Journal of Dairy Research (2013) **80** 496–502. doi:10.1017/S0022029913000484

Use of milk amyloid A in the diagnosis of subclinical mastitis in dairy ewes

Arianna Miglio[1]*, Livia Moscati[2]*, Gabriele Fruganti[1], Michela Pela[2], Eleonora Scoccia[2], Andrea Valiani[2] and Carmen Maresca[2]

[1] Department of Veterinary Clinical Science, University of Perugia, Perugia 06124, Italy
[2] Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Perugia 06124, Italy

Received 27 February 2013; accepted for publication 29 August 2013; first published online 8 October 2013

Subclinical mastitis (SM) is one of the most important diseases affecting dairy ewes worldwide, with negative impact on the animal health, farm income and public health. Animals with SM often remain untreated because the disease may not be revealed. Increase in somatic cell count (SCC) and positive bacteriology for mastitis pathogens in milk samples are indicative of SM but the evidence of only one of these alterations must suggest an uncertain SM (UM). UM is defined when positive bacteriological examination (Latent-SM) or SCC > 500 000 cells/ml (non-specific-SM) are detected in milk. Nevertheless, SCC and bacteriological examination are expensive, time consuming and are not yet in use at the farm level in dairy ewes. Recently, a sensitive acute phase protein, amyloid A, displaying multiple isoforms in plasma and different body fluids including mammary secretion (milk amyloid A-MAA), has been investigated as a marker of mastitis in cows and, in a few studies, in sheep. The aim of this trial was to compare the concentration of MAA of single udder-halves in ewes with healthy udder-halves (HU-control group) and naturally occurring subclinical mastitis, both confirmed (SM group) and uncertain (UM groups: Latent-SM and non-specific-SM), for monitoring udder health. The reliability of a specific ELISA kit for the measurement of MAA was also tested. During a 3-month trial period, 153 udder halves were assigned to the experimental groups based on their health status: 25 with SM, 40 with UM (11 with latent-SM and 29 with non-specific-SM) and 88 HU. SCC and bacteriological analysis were performed to establish the control and subclinical mastitis groups. MAA concentrations in milk samples were measured using a specific commercially milk ELISA kit. The data were submitted to statistical analysis. Significant ($P < 0{\cdot}05$) differences among the groups SM, non-specific-SM and HU were detected with the SM having the highest level and HU the lowest. MAA concentration is affected by the udder health status and is a useful indicator of subclinical mastitis and increased SCC in sheep.

Keywords: Milk amyloid A, acute phase proteins (APPs), subclinical mastitis, dairy ewes, sheep.

Mastitis is recognised as one of the most important diseases affecting dairy ewes worldwide. The high incidence and prevalence of the subclinical form of mastitis in sheep dairy flocks in Europe affects the yield and quality of the milk, with negative impact on animal health, farm income and public health (Burriel 1997; Saratsis et al. 1999; Bergonier et al. 2003; Leitner et al. 2004; Kiossis et al. 2007). The major pathogens causing subclinical mastitis (SM) in sheep are *coagulase-negative Staphylococcus* spp. (CNS), even though *Staphylococcus aureus, Streptococcus uberis* and *Entetrobacteriaceae* have been detected in milk samples from subclinical intramammary infections (Pengov 2001; Contreras et al. 2007; Vautor et al. 2009).

The scarce bibliographic data regarding SM in ewes and the proved limited use of the available tools for diagnosis of mastitis in cows continue to make SM a diagnostic problem in sheep (Bergonier et al. 2003). Sheep with clinical mastitis are easily detected by inspection and palpation of the teats and udder, and are thus treated (Mork et al. 2007). By contrast, animals with SM remain untreated because the disease may not be observed, owing to the absence of macroscopic abnormalities in the udder and milk. Therefore, the use of laboratory assays is necessary to avoid persistent udder infection and the spread of the disease in dairy flocks. Conventional methods for diagnosis of SM associate clinical evaluation of the udder with cyto-bacteriological

*For correspondence; e-mail: miglioarianna@libero.it; lmoscati@izsum.it

examination of milk (Fruganti et al. 1985; Menzies & Ramanoon, 2001; Kiossis et al. 2007). Udder half milk samples with an increase in somatic cell count (SCC) and positive bacteriology for mastitis pathogens are indicative of subclinical inflammation of the mammary gland, but the evidence of only an increased SCC or a positive bacteriology must suggest a uncertain SM (Bergonier et al. 1994, 2003; Fthenakis, 1996).

Nevertheless, SCC and bacteriological examination are expensive, time-consuming and are not yet in use at the farm level, especially in dairy ewes (McDougall et al. 2001). Furthermore, even if the SCC in milk from individual quarters is considered a standard indicator of SM in cows (Pyörälä, 2003), it may not be a specific sign of the inflammatory status of the udder-halves of ewes, owing to variability due to numerous factors other than intramammary infections such as age, breed, milking management, physiological stage of the animal (lactation stage, dry period), season, numbers of lambs born and stressors (Peris et al. 1991; Mavrogenis et al. 1995; Leitner et al. 2001; Bergonier et al. 2003). Thus there is a need to identify and evaluate other specific and rapidly assessable biomarkers that can be used to assess both SM and unfavourable changes in milk quality.

Recently, the acute phase proteins (APPs) have been well investigated as a marker of mastitis in cows (Karreman et al. 2000; Eckersall 2001; Eckersall et al. 2001; Murata et al. 2004; Nielsen et al. 2004; O'Mahony et al. 2006) but only in a very few studies in sheep (Winter et al. 2003, 2006).

Nowadays, the limited literature indicates serum amyloid A (SAA) as the most sensitive acute phase protein in ewes, whose concentration increases in the extracellular body fluid mostly in response to inflammatory and infectious conditions both acute and subclinical (Winter et al. 2003; Eckersall et al. 2007; Eckersall & Bell, 2010; Ceciliani et al. 2012). This protein is produced by the liver and, subsequently, secreted in blood serum in response to pro-inflammatory cytokines (Gronlund et al. 2003).

Nevertheless, in the case of mastitis, even if the concentration of SAA has been demonstrated to increase over 200-times during acute mammary infection (Winter et al. 2003) it does not seem to be significantly correlated with SM (Winter et al. 2003; Miglio, A et al. unpublished data). In this regard, Winter et al. (2003) showed that concentration of SAA in sheep with SM experimentally induced, reached rapidly high levels after intramammary infection; these levels were maintained for a short period of time (approximately 2–7 d) and quickly returned to physiological levels even if the ewes continued to have SM.

Recently, in ruminants has been discovered the presence of different isoforms of AA due to its extra-hepatic synthesis in various tissues by macrophages, endothelial cells, smooth-muscle cells and intestinal and mammary epithelium (McDonald et al. 2001; Larson et al. 2005). Particularly, the secretion of mammary-associated AA isoforms (milk amyloid A) has been detected in normal ovine and bovine colostrum, and in milk experimentally and naturally infected with mastitis pathogens (Winter et al. 2003, 2006; Nielsen et al. 2004; Kovacevic-Filipovic et al. 2012). In this regard, a few studies in ewe (Winter et al. 2003, Miglio, A et al. unpublished data) demonstrate the absence of a significant correlation between the concentrations of AA in serum and milk in course of SM, thus supporting the idea that AA can be synthesised locally in the inflamed ovine mammary gland and proposing milk amyloid A as a more useful diagnostic marker of mastitis in sheep.

The aim of this study was to determine the AA concentrations in milk of single udder-halves in ewes with healthy udder-halves, naturally occurring SM and uncertain SM for monitoring udder health, because of the scarce literature on this topic. Furthermore we investigated, for the first time in ewes, the reliability and the potential value of measuring milk amyloid A with a new milk ELISA kit (MAA) as a more sensitive diagnostic marker for the detection of SM in sheep flocks.

Materials and methods

Animals and milk samples

The study was carried out in 2011 at a dairy farm located in the province of Perugia, Region of Umbria (Central Italy). The farm reared 900 lactating Lacaune sheep kept in an open-sided barn. A total of 500 sheep were in lactation and the animals were milked twice a day in a milking parlour with a milking machine. The animals were fed with mixed hay supplemented with cereal grains. On 8 different days of a 3-month experimental period, 77 mid-lactating sheep (lactation months 3–7), ranging from 2 to 6 years old, were selected based on the absence of overt signs of mastitis (absence of abnormalities in the udder on inspection and palpation as well as of macroscopic changes in the mammary secretion). In addition, a complete clinical examination was performed in order to confirm the sheep were healthy. Given that the apparent healthy clinical status of every sheep was checked, a single milk sampling was performed in each udder-half by using sterile plastic vials at the beginning of the morning milking. Milk samples were refrigerated and analysed within 2 h for bacteriological examination, total microbial count (TMC) and SCC, and then immediately stored at − 30 °C until they were analysed for milk amyloid A (MAA).

Case definition (experimental groups)

Three udder-halves groups were categorised based on the single udder-half health status and the analysis of its milk sample. SCC data of each milk sample were analysed according to the cut-off value chosen at 500 000 cells/ml, consistent with the literature pertaining to mammary udder-half milk samples of adult multiparous mid-lactating sheep (Green, 1984; Bergonier et al. 1994, 2003; Fthenakis et al. 1994; Mavrogenis et al. 1995; Waage et al. 2000; Kiossis et al. 2007; Radostits et al. 2007).

https://doi.org/10.1017/S0022029913000484 Published online by Cambridge University Press

The udder-halves of all groups showed no abnormalities on inspection and palpation and their milk was not grossly altered (absence of clots in milk).

The subclinical mastitis status of an udder-half (SM group) was confirmed by positive bacteriological examination (presence of mastitis pathogens) and increase of SCC in milk (> 500 000 cells/ml).

An udder-half with uncertain subclinical mastitis (UM) was defined based on positive bacteriological examination or SCC > 500 000 cells/ml in milk. These changes in milk composition have been indicated as latent subclinical mastitis (Latent-SM group) and nonspecific subclinical mastitis (nonspecific-SM group), respectively (Fruganti et al. 1985; IDF, 1987; Albenzio et al. 2002).

According to the parameters of the SM and UM experimental groups, a healthy udder-half (HU group) was defined when it did not show overt signs of mastitis (absence of abnormalities in the udder on inspection and palpation as well as of macroscopic changes in the mammary secretion) and milk showed negative bacteriological examination and SCC ⩽ 500 000 cells/ml (control group).

Bacteriological examination and SCC

Bacteriological analysis and somatic cell count (SCC) were performed to establish the HU, UM (Latent SM and nonspecific SM) and SM groups.

Bacteriological examination of the udder-half milk samples was carried out in accordance with National Mastitis Council standards (Harmon et al. 1990; Hogan et al. 1999). For each sample, about 10 µl of milk were plated into 5% sheep blood agar and then incubated aerobically at 37 °C for up to 48 h. Colonies were isolated and identified by gross morphology and haemolysis. Appropriate biochemical tests were further performed on the colonies isolated to identify the pathogens (API Systems; bioMerieux, Marcy, France).

In each culture, the number of colony-forming units (cfu) per ml were determined for every colony type. The cut-off to categorise a milk sample as positive to mastitis pathogen was defined as growth of five or more identical colonies (⩾ 500 cfu/ml). Growth of two or more different colonies of environmental bacteria (⩾ 500 cfu/ml per type) was considered as a contaminated sample.

SCC was assessed in fresh milk by fluoro-opto-electronic cell counting (Fossomatic 5000, Foss Electric, Hillerød, Denmark).

Amyloid A in udder-half milk samples

Concentration of AA was determined in udder-half milk samples using a specific commercially available milk ELISA kit (Mast ID RANGE, Milk Amyloid A-MAA Assay kit – Tridelta Development Ltd., Wicklow, Ireland; cat. TP-807) as first described by Gerardi et al. (2009). The milk ELISA kit is a modified version of the serum ELISA kit (PHASE™ RANGE, Serum Amyloid A-SAA Assay kit – Tridelta Development Ltd., Wicklow, Ireland; cat. TP-802) used to detect amyloid A in blood serum (McDonald et al. 1991). The milk ELISA kit is based on different dilutions both of the calibration standards and of streptavidin peroxidase, according to the manufacturer. Milk samples were diluted 1 : 500 with buffer solution according to the manufacturer's instructions for sheep mammary secretion, and all the samples, including the standards, were tested in duplicate. Samples with an optical density outside the range of the standard curve were diluted further and reanalysed. Optical densities were read on an automatic plate reader (model Sunrise; Tecan, Salzberg, Austria) at 450 nm using 630 nm as reference. The limit of detection (LOD) of the ELISAs was 0·10 mg/l for milk (MAA) samples analysed with ELISA kit cat. TP-807, according to the manufacturer.

Statistical analysis

The prevalence of the SM, Non-specific SM, Latent SM and HU, and the confidence intervals (95% CI) were calculated. Mean values and SD of the MAA were calculated to describe the central location and the spread of the data of the concentration into the udder-half groups. The 95% confidence intervals of the MAA mean concentrations of experimental groups were calculated.

The normality of the distribution of the MAA data was assessed by the Shapiro-Wilk test. Data were transformed to a log scale in order to balance the distribution. MAA (mg/l of milk) was transformed as natural log (ln). One-way ANOVA was applied to compare the health status of udder halves, and $P < 0{\cdot}05$ was considered statistically significant. Bonferroni's multiple comparison test was applied for post hoc comparison.

All the statistical analyses were carried out using Stata 9.1 software.

Results

Bacteriological examination and SCC

A total of 154 udder-half milk samples were collected from the 77 Lacaune sheep considered in the trial. Because in one udder-half milk sample it was not possible to determine the SCC, 153 udder-half milk samples were examined. No contaminated sample was recorded.

The number of udder-halves with SM (presence of mastitis pathogens and SCC > 500 000 cells/ml in milk) was 25 and the overall prevalence was 16·3% (CI: 11·0–23·4%). The following pathogens were detected: *coagulase-negative Staphylococcus* spp. (*Staph. epidermidis* and *Staph. chromogenes*), *Staph. aureus, Enterococcus faecalis* and *Streptococcus uberis*.

The number of udder-halves with UM was 40 (26·1%): 11 (7·2%) with Latent-SM (presence of mastitis pathogens and SCC ⩽ 500 000 cells/ml in milk) and 29 (19%) with Non-specific-SM (absence of mastitis pathogens and SCC > 500 000 cells/ml in milk). CNS spp. (*Staph. epidermidis*

https://doi.org/10.1017/S0022029913000484 Published online by Cambridge University Press

Table 1. Measures of variability [mean, SD, SE, minimum (Min), maximum (Max)] and 95% confidence interval for MAA concentration in milk of sheep with udder-halves with subclinical mastitis (SM), uncertain subclinical mastitis (non-specific SM and latent SM) and healthy udder-halves (HU)

Milk amyloid A, μg/ml	Mean + SD, μg/ml	SE, μg/ml	Min value, μg/ml	Max value, μg/ml	95%CI, μg/ml
Healthy udder-halves (HU)	29·68 ± 27·98	2·98	0·48	132·58	23·75–35·61
Non-specific SM	77·31 ± 55·31	10·27	1·39	140·56	56·28–98·35
Latent SM	44·59 ± 42·07	12·268	7·77	137·09	16·33–72·85
Udder-halves with SM	114·37 ± 41·14	8·23	18·41	142·43	97·39–131·35

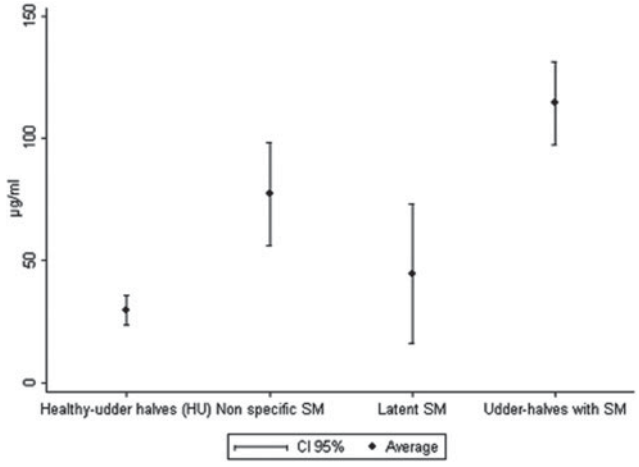

Fig. 1. Mean and 95% confidence interval for MAA concentration in milk from dairy ewes with udder-halves with subclinical mastitis (SM), uncertain subclinical mastitis (non-specific SM and latent SM) and healthy udder-halves (HU).

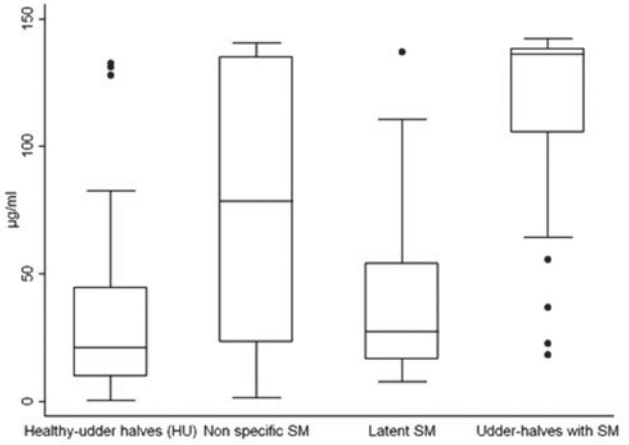

Fig. 2. Difference between concentrations of Milk Amyloid A in milk from dairy ewes with udder-halves with subclinical mastitis (SM), uncertain subclinical mastitis (non-specific SM and latent SM) and healthy udder-halves (HU). The plots show the median (line within box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and outliers (dots).

and *Staph. chromogenes*) were the only organisms isolated in the milk samples with Latent SM.

The number of healthy udder halves (HU) was 88 (57·5%).

In our investigation the ewes with SM were 22 (28·6%): 3 (3·9%) from both the udder-halves and 19 (12·4%) from one udder-half with the contralateral healthy (14 animals) or with Non-specific SM (5 animals).

The sheep with both the udder-halves healthy (HU) were 23.

The remaining animals (31) had at least one udder-half with UM (44·1%): 11 (44·1%) ewes with Latent SM (all from one udder half) and 20 sheep with Non-specific SM (18 from one udder-half and 3 from both udder-halves).

Amyloid A in udder-half milk samples

Average values ± SD, CI and median values for the MAA were calculated (Table 1, Figs. 1 & 2). MAA concentrations (average and 95% CI) were affected by the healthy udder-half status of sheep (Fig. 1).

Milk amyloid A data were not normally distributed. A log transformation was necessary in order to normalise the distribution of data before carrying out the ANOVA.

MAA data showed a detectable difference in content in the comparison between mastitic (SM group), suspected mastitic (Non-specific SM group) and healthy udder-halves (HU group).

Application of Bonferroni's test between each of all possible pairwise comparisons showed a statistically significant ($P<0·05$) effect of MAA concentrations and healthy status of the mammary gland (Table 2). Particularly, significant ($P<0·05$) differences were detected among experimental groups SM, Non-specific SM and HU with SM having the highest level and HU the lowest level. A significant difference was also detectable in the comparison between SM and Latent SM groups, but not between Latent SM and Non-specific SM, and Latent SM and HU groups.

Discussion

The prevalence of subclinical mastitis in the flock investigated was similar to those reported in the literature regarding lactating ewes from different geographical areas (Watkins et al. 1991), especially with reference to countries in the Mediterranean basin (Italy, Greece, France and Spain) (De La Cruz et al. 1994; Gonzalez-Rodriguez et al. 1995; Albenzio et al. 2002; Bergonier et al. 2003; Contreras et al. 2007; Kiossis et al. 2007).

https://doi.org/10.1017/S0022029913000484 Published online by Cambridge University Press

Table 2. Bonferroni adjustment in pairwise comparisons. Concentration of Milk Amyloid A in milk samples from udder-halves with subclinical mastitis (SM), uncertain subclinical mastitis (non-specific SM and latent SM) and healthy udder-halves (HU)

	Bonferroni's test	
Udder halves health status	*P* value	Difference, μg/ml
Udder halves with Non specific SM healthy – udder halves (HU)	0·000	47·63
Udder halves with Latent SM healthy – udder halves (HU)	1	14·91
Udder halves with Latent SM – Udder halves with Non specific SM	0·09	– 32·72
Udder halves with SM healthy – udder halves (HU)	0·000	84·69
Udder halves with SM – Udder halves with Non specific SM	0·003	37·06
Udder halves with SM – Udder halves with Latent SM	0·000	69·78

On the other hand, the high percentage of UM found in this study, in agreement with previous reports on lactating ewes (Fthenakis et al. 1994; Albenzio et al. 2002), emphasises the importance of early identification of this condition in sheep flocks, in view of the higher susceptibility to develop mammary infection in the affected udder-half.

Furthermore, it is important to notice the possible spread of the infection from the affected udder-half to the contralateral gland, as demonstrated in the 3 animals showing both the udder halves with SM. Nevertheless this phenomenon is not commonly detected as suggested by the considerable percentage (25/88; 28%) of milk samples from healthy udder-halves originating from ewes that have the contralateral infected (SM or Latent SM).

Consistent with the results of several studies (Las Heras et al. 1999; Albenzio et al. 2002; Al-Majali & Jawabreh 2003; Leitner et al. 2004; Contreras et al. 2007; Vautor et al. 2009), in this study environmental pathogens such as CNS and *Str. uberis*, as well as *Staph. aureus* and *Ent. faecalis*, were the prevalent pathogens isolated from the milk of sheep with SM. Similarly, CNS, which are mostly the cause of opportunistic mastitis and are normally found on teat skin, were the only bacteria isolated from the udder-halves with latent SM, as found previously (Fruganti et al. 1985; Radostits et al. 2007).

It should be taken into account that the occurrence of latent SM in 11 udder halves tested in our study could be related to cutaneous contamination and subsequent multiplication of bacteria in the udder, chronic CNS infection, initial or final stage of udder inflammation, mastitis with an acute phase of short duration or infection with low pathogenic bacterial strain (Fruganti et al. 1985; Saran & Leitner, 2000).

Similarly, in the 29 udder-halves with non-specific SM detected, even if the SCC was high, it may not have been possible to isolate the aetiological agent owing to a number of causes: its poor concentration or intermittent excretion, the mastitis pathogens were not detectable using the conventional bacteriological tests, the infection was supported by endotoxins, or the biomarkers of the immunity response (lysozyme, lactoferrin and neutrophil granulocytes) may have thwarted the pathogen detection or elimination (Fruganti et al. 1985; Saran & Leitner, 2000; Albenzio et al. 2002).

Sheep with both these alterations should necessarily be identified, isolated, properly treated and then re-examined to avoid the spread of the infection in the flock.

Recent reports demonstrate that AA concentrations, evaluated in ovine milk with a serum ELISA kit (mAA), reflect the severity of mastitis; the increase in mAA levels during experimentally induced *Staph. epidermidis* (Winter et al. 2003) and naturally occurring (Winter et al. 2006) SM in ewes has been demonstrated. To our knowledge, this is the first study to report the use of a more sensitive assay (milk ELISA kit TP-807, Milk Amyloid-MAA) to determine amyloid A in sheep udder-half milk samples.

The results indicate that there were different concentrations of MAA in the experimental groups, although a statistically significant difference ($P<0·05$) was observed only between udder-halves with SM, Non-specific SM and HU.

Particularly, MAA concentrations were significantly higher in the SM group (mean ± SE: 114·37 ± 8·23 μg/ml) compared with Non-specific SM (77·31 ± 10·27 μg/ml), Latent SM (44·59 ± 7·77 μg/ml) and HU (29·68 ± 2·98 μg/ml) groups.

In our study the mean MAA concentration of the udder-halves with SM was similar to what recorded by Winter et al. (2003) (171 ± 141·7 μg/ml) and Winter et al. (2006) (121 ± 25·3 μg/ml) in the same animal species and tends to be higher than those reported in affected bovine quarters (Eckersall 2001; Gronlund et al. 2003, 2005; Nielsen et al. 2004; Gerardi et al. 2009; Safi et al. 2009; Kovacevic-Filipovic et al. 2012). This finding could be explained because mastitis in dairy ewes is characterised by AA concentrations in milk that peak at least 10-fold higher than in cows in the acute form and remain at higher levels in the subclinical form (Winter et al. 2006).

Interestingly, in this trial the healthy udder-halves showed MAA concentrations higher than those found by Winter et al. (2003) (mean 1·4 μg/ml) and Winter et al. (2006) (mean 8·6 μg/ml) in ewes of the same breed. It could be due to the more sensitive assay we used to determine MAA that has a lower LOD (0·10 mg/l), which allows it to detect lower quantities of amyloid A than the serum ELISA kit TP-802 (mAA).

In our study, values of MAA identified in healthy udder-halves ranged from 23·75 to 35·61 μg/ml (CI) and were higher than the reference range (0–7·5 μg/ml) identified in healthy bovine quarter milk with the same ELISA kit by Gerardi et al. (2009) and than those found using the serum

https://doi.org/10.1017/S0022029913000484 Published online by Cambridge University Press

ELISA kit by other authors (Eckersall 2001; Gronlund et al. 2003; Nielsen et al. 2004; Gerardi et al. 2009). This suggests that normal ovine milk contains a greater amount of this APP than bovine milk.

Additionally, in our study MAA concentrations of udder-halves with SM and Non-specific SM were in the range 97·39–131·35 μg/ml (CI) and 56·28–98·35 μg/ml (CI), respectively. Surprisingly, these results show that the detection of the marker MAA with the Milk ELISA kit is also useful to differentiate the healthy udder-half from those with high SCC and to distinguish between a highly cellular milk sample with mastitis pathogen and one with high SCC but not pathogens.

Furthermore, in the present study, there was a tendency for higher MAA concentration in udder-halves with Latent SM compared with HU and a higher MAA concentration in udder halves with Non-specific SM compared with Latent SM, even though these differences were not significant ($P=1$ and $P=0·09$, respectively). The lack of significant differences among the groups Latent SM and the Non-specific SM, and between the groups Latent SM and HU may be related both to the limited number of cases of Latent SM and to the small concentration of bacteria they contain. Indeed, recently, it has been demonstrated that levels of MAA seem to be associated even with the quantity of bacterial DNA in the milk sample (Kalmus et al. 2013).

In conclusion, this study contributes to supporting the importance of the acute phase protein MAA as a sensitive diagnostic marker of SM in ewes, with particular emphasis on the use of the specific milk ELISA assay. Furthermore, we consider that the concentration of MAA can be a useful indicator of mammary gland inflammation and of unfavourable changes in milk quality in sheep, although a clear and standardised cut-off value for the healthy udder-half should be confirmed. These results encourage further study on the physiology of MAA in sheep breeds different from the Lacaune included in our trial, and which is considered one of the most resistant to mastitis (Barillet et al. 2001; Rupp et al. 2009). More studies are also needed to confirm the usefulness of MAA to detect milk samples with mastitis pathogens but not high SCC (latent SM). Although MAA determination requires an ELISA method that is carried out routinely only in reference laboratories, systematic control of this biomarker on dairy farms could reduce the laboratory costs and the time required for milk analysis. The online measurement of this protein with automated milking systems could enable early detection of mammary inflammation and infection, reducing the economic impact and improving the health and welfare of dairy ewes as well as public health. Moreover, even the determination of MAA in a given period of time could be useful in monitoring the health status of the ovine mammary gland.

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence the content of the paper.

References

Albenzio M, Taibi L, Muscio A & Sevi A 2002 Prevalence and etiology of subclinical mastitis in intensively managed flocks and related changes in the yield and quality of ewe milk. *Small Ruminant Research* **43** 219–226

Al-Majali AM & Jawabreh S 2003 Period prevalence and etiology of subclinical mastitis in Awassi sheep in southern Jordan. *Small Ruminant Research* **47** 243–248

Barillet F, Rupp R, Mignon-Gasteau S, Astruc JM & Jacquin M 2001 Genetic analysis for mastitis resistance and milk somatic cell count score in Franch Lacaune dairy sheep. *Genetic Selection Evolution* **33** 397–415

Bergonier D, Van de Wiele A, Arranz JM, Barillet F, Lagrifoull G, Condorcet D & Berthelot X 1994 Detection of subclinical mammary infections in the ewe by mean of somatic cell counts: proposal of physiological thresolds. In *Proceedings of the International Symposium of Somatic Cells and Milk of Small Ruminants*, Bella, 25–27 September 1994, Italy, EAAP Publication No 77, pp. 41–47

Bergonier D, De Cremoux R, Rupp R, Lagriffoul G & Berthelot X 2003 Mastitis of dairy small ruminants. *Veterinary Research* **34** 689–716

Burriel AR 1997 Dynamics of intramammary infection in the sheep caused by coagulase-negative staphylococci and its influence on udder tissue and milk composition. *Veterinary Record* **140** 419–423

Ceciliani F, Ceron JJ, Eckersall PD & Sauerwein H 2012 Acute phase protein in ruminants. *Journal of Proteomics* **75** 4207–4231

Contreras A, Sierra D, Sanchez A, Corrales JC, Marco JC, Paape MJ & Gonzalo C 2007 Mastitis in small ruminants. *Small Ruminant Research* **68** 145–153

De La Cruz M, Serrano E, Montoro V, Marco J, Romeo M, Baselga R, Albizu I & Amorena B 1994 Etiology and prevalence of subclinical mastitits in the Manchega sheep at mid-date lactation. *Small Ruminant Research* **14** 175–180

Eckersall PD 2001 Acute phase proteins as markers of infection and inflammation: monitoring animal health, animal welfare and food safety. *Irish Veterinary Journal* **53** 307–311

Eckersall PD & Bell R 2010 Acute phase proteins: biomarkers of infection and inflammation in veterinary medicine. *Veterinary Journal* **185** 23–27

Eckersall PD, Young FJ, McComb C, Hogarth CJ, Safi S, Weber A, McDonald T, Nolan AM & Fitzpatrick JL 2001 Acute phase proteins in serum and milk from dairy cows with clinical mastitis. *Veterinary Record* **148** 35–41

Eckersall PD, Lawson FP, Bence L, Waterston MM, Lang TL, Donachie W & Fontaine MC 2007 Acute phase protein response in an experimental model of ovine caseus lymphadenitis. *BMC Veterinary Research* **19** 35

Fruganti G, Ranucci S, Tesei B & Valente C 1985 Valutazione dello stato sanitaria della mammella di pecore durante un intero ciclo di lattazione. 'Safety evaluation of the sheep udders during an entire lactation period'. *Clinica Veterinaria* **108** 286–296

Fthenakis GC 1996 Use of somatic cell counts or of indirect tests in milk for the diagnosis of subclinical mastitis in ewes. In *Proceedings of Somatic Cells and Milk of Small Ruminants, International Symposium*, Bella, Italy. pp. 27–29 (Ed. R Rubino). The Netherlands

Fthenakis GC, Marples RR, Richardson JF & Jones JE 1994 Some properties of coagulase-negative staphylococci isolated from cases of ovine mastitis. *Epidemiology Infection* **112** 171–176

Gerardi G, Bernardini D, Elia CA, Ferrari V, Iob L & Segato S 2009 Use of Serum Amyloid A and milk amyloid A in the diagnosis of subclinical mastitis in dairy cows. *Journal of Dairy Research* **76** 411–417

Gonzalez Rodriguez MC, Gonzalo C, San Primitivo F, Carmenes P & Rodriguez MCG 1995 Relations between somatic cell count and intramammary infection of the half udder in dairy ewes. *Journal of Dairy Science* **78** 2753–2759

Green TJ 1984 Use of somatic cell counts for detection of subclinical mastitis in ewes. *Veterinary Record* **114** 43–48

Gronlund U, Hulten C, Eckersall PD, Hogarth C & Persson Waller K 2003 Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced *Staphylococcus aureus* mastitis. *Journal of Dairy Science* **70** 379–386

Gronlund U, Hallen Sandgren C & Persson Waller K 2005 Haptoglobin and serum amyloid A in milk from dairy cows with chronic sub-clinical mastitis. *Veterinary Research* **36** 191–198

Harmon RJ, Eberhart RJ, Jasper DE, Langlois BE & Wilson RA 1990 *Microbiological Procedures for the Diagnosis of Bovine Udder Infection*. Arlington VA, USA: National Mastitis Council Inc

Hogan JS, Gonzalez RN, Harmon RJ, Nickerson SC, Olivers SP, Pankey JW & Smith KL 1999 *Laboratory Handbook on Bovine Mastitis*. Madison WI, USA: National Mastitis Council Inc

Kalmus P, Simojoki H, Pyörälä S, Taponen S, Holopainen J & Orro T 2013 Milk haptoglobin, milk amiloyd A, and N-acetyl-b-d-glucosaminidase activity in bovines with naturally occurring clinical mastitis diagnosed with a quantitative PCR test. *Journal Dairy Science* **96** 3662–3670

IDF 1987 *Bovine Mastitis. Definition and Guidlines for Diagnosis*. Bull. No211.Brussels, Belgium: International Dairy Federation

Karreman HJ, Wentink GH & Wensing T 2000 Using serum amyloid A to screen dairy cows for subclinical inflammation. *Veterinary Quarterly* **22** 175–178

Kovacevic-Filipovic M, Ilic V, Vujcic Z, Dojnov B & Bozic T 2012 Serum Amyloid A isoforms in serum and milk from cows with *Staphylococcus aureus* subclinical mastitis. *Veterinary Immunology and Immunopathology* **145** 120–128

Kiossis E, Brozos CN, Petridou E & Boscos C 2007 Program for the control of subclinical mastitis in dairy Chios breed ewes during lactation. *Small Ruminant Research* **73** 194–199

Larson MA, Weber A, Weber T & McDonald T 2005 Differential expression and secretion of bovine serum amlyloid A3 (SAA3) by mammary epithelial cells stimulated with prolactin or lipopolysaccharide. *Veterinary Immunology and Immunopathology* **107** 255–264

Las Heras A, Dominguez L & Fernandez JF 1999 Prevalence and aetiology of subclinical mastitis in dairy ewes of de Madrid region. *Small Ruminant Research* **32** 21–29

Leitner G, Chaffer M, Caraso Y, Ezra E, Kababea D & Saran A 2001 Udder infection and milk somatic cell count, NAGase activity and milk composition-fat, protein and lactose-in Israeli Assaf and Awassy sheep. *Small Ruminant Research* **49** 157–164

Leitner G, Chaffer M, Shamay A, Merin U, Saran A & Silanikove N 2004 Changes in milk composition as affected by subclinical mastitis in sheep. *Journal of Dairy Science* **87** 46–52

Mavrogenis AP, Koumas A, Kakoyiannis CK & Taliotis CH 1995 Use of somatic cell counts for the detection of subclinical mastitis in sheep. *Small Ruminant Research* **17** 79–84

McDonald TL, Weber A & Smith JV 1991 A monoclonal antibody sandwich immunoassay for serum amyloid A (SAA) protein. *Journal of Immunological Methods* **144** 149–155

McDonald TL, Larson MA, Mack DR & Weber A 2001 Elevated extrahepatic expression and secretion of mammary-associated serum amyloid A3 (M-SAA3) into colostrum. *Veterinary Immunology and Immunopathology* **83** 203–211

McDougall SM, Murdough P, Pankey W, Delaney C, Barlow J & Scruton D 2001 Relationships among somatic cell count, California mastitis test, impedance and bacteriological status of milk in goats and sheep in early lactation. *Small Ruminant Research* **40** 245–254

Menzies PI & Ramanoon SZ 2001 Mastitis of sheep and goat. *Veterinary Clinics of North America: Food Animal Practice* **17** 333–358

Mork T, Waage S, Tollersrud T, Kvitle B & Sviland S 2007 Clinical mastitis in ewes; bacteriology, epidemiology and clinical features. *Acta Veterinaria Scandinavica* **49** 23

Murata H, Shimada N & Yoshioka M 2004 Current research on acute phase proteins in veterinary diagnostic: an overview. *Veterinary Journal* **168** 28–40

Nielsen BH, Jacobsen S, Anderson PH, Niewold TA & Heegaard PM 2004 Acute phase protein concentrations in serum and milk from healthy cows, cows with clinical mastitis and cows with extramammary inflammatory conditions. *Veterinary Record* **154** 361–365

O'Mahony MC, Healy AM, Harte D, Walshe KG, Torgerson PR & Doherty ML 2006 Milk Amyloid A: correlation with cellular indicis of mammary inflammation in cows with normal and raised serum amyloid A. *Research in Veterinary Science* **80** 155–161

Pengov A (2001) The role of coagulase negative Staphylococcus spp. and associated somatic cell counts in the ovine mammary gland. *Journal of Dairy Science* **84** 572–574

Peris C, Molina P, Fernandez N, Rodriguez M & Torres A 1991 Variation in somatic cell count, California mastitis test and electrical conductivity among various fractions of ewe's milk. *Journal of Dairy Science* **74** 1553–1560

Pyörälä S 2003 Indicators of inflammation in the diagnosis of mastitis. *Veterinary Research* **34** 565–578

Radostits OM, Gay CC, Hinchcliff KW & Constable PD 2007 *Veterinary Medicine. A Textbook of the Disease of Cattle, Sheep, Pigs, Goats and Horses*, 10th edition. p. 675. St Luis Mo, USA: Saunders Elsevier

Rupp R, Bergonier D, Dion S, Aurel MR, Robert-Granier C & Foucras G 2009 Response to somatic cell count-based selection for mastitis resistance in a divergent selection experiment in sheep. *Journal of Dairy Science* **92** 1203–1219

Safi S, Khoshvaghti A, Reza S, Bolourchi M & Nowrouzian I 2009 Acute phase proteins in the diagnosis of bovine subclinical mastitis. *Veterinary Clinical Pathology* **35** 1–6

Saran A & Leitner G 2000 Interaction between bacteria, immunity and therapy in the mammary gland. In *Proceedings of the International Symposium on Immunology of Ruminant Mammary Gland*. Stresa, Italy, June 11–14. pp. 290–297. (Ed. A Zecconi)

Saratsis P, Alexopoulos C, Tzora A & Fthenakis GC 1999 The effect of experimentally induced subclinical mastitis on the milk yield of dairy ewes. *Small Ruminant Research* **32** 205–209

Waage S, Skei HR, Rise J, Rogdo T, Sviland S & Odegaard SA 2000 Outcome of clinical mastitis in dairy heifers assessed by reexamination of cases one month after treatment. *Journal of Dairy Science* **83** 70–76

Watkins GH, Burriel AR & Jones JE 1991 A field investigation of subclinical mastitis in sheep in southern England. *British Veterinary Journal* **147** 413

Winter P, Fuchs K, Walseh K & Colditz IG 2003 Production of serum amyloid A in serumand milk during experimental *Stapphylococcus epidermidis mastitis* in ewes. *Veterinary Record* **152** 558–562

Winter P, Miny M, Fuchs K & Baumgartner W 2006 The potential of measuring serum amyloid A in individual ewe milk and in farm milk for monitoring udder health on sheep dairy farms. *Research in Veterinary Science* **81** 321–326

Vautor E, Cockfield J, Le Marechal C, Le Loir Y, Thiery R & Lindsay J 2009 Difference in virulence between *Staphylococcus aureus* isolates causing gangrenous mastitis versus subclinical mastitis in a dairy sheep flock. *Veterinary Research* **40** 56