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Study on the possible survival of *Staphylococcus chromogenes* through the dry period in dairy ewes

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ABSTRACT

CNS (Coagulase-Negative-Staphylococci) in dairy ewes are the most commonly isolated microorganisms during subclinical mastitis and are related to milk loss. The objective of this study was to investigate whether CNS can survive during the dry period, despite the use of drying-off treatment, and persist during the subsequent lactating period in dairy ewes. 47 primiparus dairy Chios ewes were used. All animals were sampled during the last milking before drying-off and they were then randomly assigned into one of two groups. Ewes of the treated group ($n = 21$) were administered a dry period intramammary preparation (Nafpenzal®), and ewes of the control group ($n = 26$) received no drying-off treatment. Immediately after lambing and once every month until the end of lactation, milk samples were collected for microbiological examination and for the determination of SCC. Daily milk yields were also recorded during the sampling days. Twelve out of the total 47 animals were found to carry the same microorganisms during 2 subsequent milking periods (pre and post partum). *Staphylococcus chromogenes* was the most commonly identified CNS species (7 animals). All 25 isolated *Staphylococcus chromogenes* strains were subjected to pulsed field gel electrophoreses in order to identify the presence of the same or different clones of the bacteria. Two clones were identified. Both clones were isolated before drying-off and re-isolated during the subsequent lactation period (>90% similarity). Apart from the first month of lactation, mean milk yield of treated group was higher than that of the control group, but the difference was significant only after the third month of lactation ($P < 0.05$). Results from the present study suggest that drying-off treatment is beneficial for milk production, but is not advantageous for the elimination of *Staphylococcus chromogenes* infections.

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1. Introduction

CNS (Coagulase-Negative-Staphylococci) play an important role in a ewe's udder health, especially for the establishment of subclinical mastitis (Bergonier et al., 2003; Kiossis et al., 2007; Leitner et al., 2001, 2004). Unlike cows, CNS in sheep are the most commonly isolated microorganisms during mastitis and are related to reduction of milk yield which ranges from 10% to 50% compare to healthy ewes (Bergonier and Berthelot, 2003; Contreras et al., 2007; Watson and Buswell, 1984). The prevalence of intramammary infections with CNS in ewes has been reviewed to range between 25% and 93% (Bergonier and Berthelot, 2003). In sheep, effective udder health

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management during the end of a lactation period is notable for maintaining mammary health during the subsequent lactating period (Fthenakis et al., 2012). This includes the dry period itself, and the administration of intramammary antibiotic preparations at drying-off (Chaffer et al., 2003; Gonzalo et al., 2004). The cure rate of existing infections after treatment is reported between 65 and 95% (Bergonier and Berthelot, 2003; Chaffer et al., 2003; Gonzalo et al., 2004; Hueston et al., 1989).

In dairy heifers, some CNS can survive and persist in the udder for several weeks (Aarestrup and Jensen, 1997). The necessity of treating CNS infections during the milking period is questioned based on the evidence of sufficient self cure rate (Pyörälä and Taponen, 2009; Wilson et al., 1999).

The objective of this study was to investigate whether CNS can survive the dry period, despite the use of intramammary preparations, and persist during the subsequent lactating period in dairy ewes.

2. Materials and methods

2.1. Animals and experimental design

In the present study 47 primiparus dairy Chios ewes, bred under semi-intensive conditions, were used. The animals were machine milked twice a day and during the commencement of the study they were at the end of their first lactating period. At the start of the drying-off procedure all ewes were clinically examined, with special attention given to their mammary glands. Individual milk yield during the last day of milking was <300 ml. Udder drying-off of all animals took place abruptly. After discarding the first two squirts of secretion milk samples were collected aseptically into sterile containers for microbiological examination. Another sample was then collected for the determination of somatic cell count (SCC) in a 10 ml tube containing 10 mg sodium azide. Samples were kept at 4 °C during transportation to the laboratory and the examinations were performed within 2 h of their collection. SCC determination was performed with Fossomatic® 4000 (A/S N. Foss Electric, Hillerød, Denmark). In all cases, samples were collected from both teats of each ewe. The animals were then randomly assigned into one of two groups. Ewes of the treated group ($n = 21$) were administered a dry period intramammary preparation (Nafpenzal®, Intervet International BV, Boxmeer, The Netherlands), and ewes of the control group ($n = 26$) received no antimicrobial agent. Immediately after lambing and once every month until the end of lactation, milk samples were collected for microbiological examination and for the determination of SCC, as previously described. Daily milk yields were also recorded during the sampling days.

2.2. Bacteriological examination

100 μl of every sample was spread on sheep blood agar 7% (Liofilchem s.r.l., Italy), MacConkey agar and Manitol dextrose agar (MerckK GaA, Darmstadt, Germany) incubated at 37 °C for 48 h under aerobic conditions, in order the presence of strains of *Staphylococcus* spp., *Streptococcus* spp., *Trueperella pyogenes*, *Pasteurella multocida*, *Mannheimia haemolytica*, *Pseudomonas aeruginosa*, *Escherichia coli* and/or other members of the Enterobacteriaceae family can be detected. All isolates were stored at −80 °C using 15% Brain Heart Infusion Broth (Liofilchem s.r.l. Bacteriology products, Italy) with glycerol for further molecular investigation. The vast majority of the stains were identified, according to Quinn et al. (1999), as *Staphylococcus* species, while the biochemical identification was performed using the API ID 32 STAPH (bioMιrieux, Marcy l'Etoile-France).

Pulsed-field gel electrophoresis (PFGE) was carried out as the protocol previously described for DNA macrorestriction from *Staphylococcus aureus*, with slight modifications (McDougal et al., 2003). Briefly, bacteria were grown overnight in 5 ml Brain Heart Infusion broth at 37 °C, harvested by centrifugation and washed by TE buffer (100 mM Tris-base, 100 mM EDTA, pH 8). 100 μl of cell suspension in TE containing 1 μg of lysostaphin (Sigma Aldrich, Poole, UK) were mixed with an equal volume of 1.8% low melting point agarose in TE. The cell-agarose suspension was added into a block molds and allowed to solidify at 4 °C for 15 min, 2 plugs made by each strain. Agarose plugs were placed in 3 ml EC-buffer [6 mM Tris HCl, pH 8; 1 M NaCl; 100 mM EDTA, pH 8; 0.5% Brij 58 (Sigma Aldrich, Poole, UK); 0.2% sodium desoxycolate (Difco Laboratories, Detroit, USA); 0.5% sodium N-lauroylsarkosine] and lysed overnight at 37 °C. Subsequently, the plugs were washed 4 times in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) each one for 15 min with gentle agitation at 54 °C and finally stored in fresh TE-buffer at 4 °C until further analysis. Digestion of DNA was performed with 30 U of the restriction enzyme *Sma*I (TaKaRa, Kyoto, Japan) for 4 h at 25 °C. Restriction fragments of DNA were separated by PFGE using 1% agarose gels in 0.5X Tris-borate-EDTA buffer with CHEF-DR III (Bio-Rad, Hercules, CA, USA). *Salmonella* serotype Branderup strain H9812 digested with 40 units XbaI (TaKaRa, Kyoto, Japan) was used as size standard. Electrophoresis conditions were 14 °C for 21 h, with pulse time ranging from 5 to 40 s at an angle of 120°, the voltage was 6 V/cm. Gels were stained with a solution of ethidium bromide and photographed. The band patterns were visually compared and were classified on the basis of identity of the DNA according to the previously described criteria (Tenover et al., 1995). A database containing all the SmaI PFGE patterns was created by using Bionumerics software (version 6.6 Applied Maths, Sint-Martens-Latem, Belgium) from Applied Maths (Sint-Martens-Latem, Belgium), where band patterns over the multiple gels were normalized and compared. Clustering was performed by using the Dice similarity coefficient and the unweighted pair group method with arithmetic means (UPGMA), with 1% of tolerance and 0.5% optimization. The cluster cutoff was set at 90% and the resulting clusters were designated by capital letters.

2.3. Statistical analysis

Differences between groups were tested by using the Student's *t*-test. The data related to milk yield and SCC were subjected to one-way analysis of variance (repeated measures ANOVA), in order to establish differences between monthly sampling times; differences among means in this case were tested by Duncan's test. Normality and homogeneity of variance was tested by applying Kolmogorov–Smirnov and Levene's test, respectively. Significance was taken at the level of $P < 0.05$.

3. Results

During the course of the experiment, 5 and 10 ewes allocated to the treated group and to the control group, respectively, were removed from the study due to health or general condition problems. Data from these ewes were used until removal of the study.

3.1. Genotypic characterization

Twelve out of the total 47 animals were found to carry the same species during 2 subsequent milking periods (pre and post partum). *Staphylococcus chromogenes* was the most commonly identified CNS species (7 animals) and thus selected for further investigation. In detail, 7 animals were found to carry *Staphylococcus chromogenes*, 3 *Staphylococcus aureus*, 1 *Staphylococcus warneri* and 1 *Staphylococcus lentus* (Table 1). Of the 7 animals with *Staphylococcus chromogenes* 6 belonged to the treated group and 1 to the control group. All 25 *Staphylococcus chromogenes* strains that were isolated in different milking periods were subjected to pulsed field gel electrophoreses in order to identify the presence of the same or different clones of the bacteria. Two clones were identified. Both clones were isolated before drying-off and re-isolated during the subsequent lactation period (clones >90% similarity, Dice coefficient UPGMA). All strains showed similar resistance to the same antibiotics (Fig. 1).

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