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Clinical evaluation of the use of enrofloxacin against *Staphylococcus aureus* clinical mastitis in sheep

A.R Attili[a], S. Preziuso[a], V. Ngu Ngwa[a,b], A. Cantalamessa[a], M. Moriconi[a], Vincenzo Cuteri[a,*]

[a] School of Biosciences and Veterinary Medicine, University of Camerino, Via Circonvallazione, 93/95, Matelica (MC) 62024, Italy
[b] School of Veterinary Medicine and Sciences, University of Ngaoundere, P.O. Box 454, Ngaoundere, Cameroon

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ABSTRACT

The aims of this work were to evaluate the potential role of enrofloxacin in controlling the severity of the clinical mastitis in sheep caused by *Staphylococcus aureus*; to improve cure rates and to minimize the related effects of the disease on the mammary glands. This study was conducted in commercial dairy flocks where there was on going intensive monitoring of subclinical mastitis by Somatic Cell Count (SCC) and bacteriology. Two groups of animals were selected from these flocks. Group A ($n = 34$ animals) and Group B ($n = 39$ animals) were treated with 2.5 mg/kg bw and 5 mg/kg bw, respectively of enrofloxacin (Baytril® 5% injectable solution, Bayer, Italy) for three consecutive days (2 doses per day). The effectiveness of the enrofloxacin in curing the *S. aureus*-induced clinical mastitis was monitored through SCC, rectal temperature, and by systemic and local mammary gland reactions from the 1st to the 14th day post treatment. The presence of *S. aureus* in milk samples was confirmed by bacteriological examination and PCR before and after treatment. Bacteriological cure was 39% in Group A and 82% in Group B. Both doses significantly reduced SCC ($P < 0.001$), while the reduction in Group B was also significantly higher than Group A. Mean rectal temperature as well as local mammary gland and systemic reactions, also decreased significantly in both groups ($P < 0.001$). In conclusion, both enrofloxacin concentrations provide bacteriological cure but the higher concentration resulted in greater reduction of clinical mastitis in sheep caused by *S. aureus*.

1. Introduction

Mastitis is considered as one of the most important diseases affecting domestic animals and is caused by several etiologic agents. The disease is economically relevant for ovine dairy farmers due to the premature culling of ewes and reduced performance of lambs. Although subclinical mastitis occurs worldwide, its economical importance is especially significant in the Mediterranean area, which could boasts of the highest concentration of dairy sheep producers in Europe (Mavrogianni et al., 2011; Gelasakis et al., 2015). Several pathogens can cause mastitis, but *Staphylococcus spp.* is the most frequently diagnosed causal microorganism of intra-mammary infection (IMI) in goats and sheep (Menzies and Ramanoon, 2001; Gonzalo et al., 2004). IMI caused by *Staphylococcus aureus* (*S. aureus*) warrants special attention because this bacterium is responsible for both clinical and subclinical acute mastitis. Various isolates of *S. aureus* secrete toxins as well as thermostable enterotoxins that contribute to the pathogenesis of mastitis. Thus, a main priority should be the implementation of programs to eradicate *S. aureus* from ovine dairy herds. The pervasiveness and aetiology of clinical and subclinical mastitis in dairy ewes show that *S. aureus* prevalence ranges from 20 to 60% of the isolated bacteria (Bergonier et al., 2003). In contrast to bovine and goat mastitis, little information is available about the drugs used for the clinical mastitis caused by *S. aureus* in sheep. Lactoferrin, penicillin and flunixin-meglumine are known to be effective in goat mastitis caused by *S. aureus* (Mavrogianni et al., 2004; Lacasse et al., 2007). Antibiotics like oxytetracycline and penicillin-benzathine also act as good antimicrobial agents against sheep mastitis, but recent studies suggest that staphylococci are becoming highly resistant to oxytetracycline in cattle (Kumar et al., 2011; Preziuso et al., 2013), and to tetracycline in small ruminants (Chirles et al., 2012).

Enrofloxacin is a synthetic bactericidal agent in the fluoroquinolone group developed specifically for veterinary use and

* Corresponding author.
E-mail addresses: annarita.attili@unicam.it (A.R Attili), silvia.preziuso@unicam.it (S. Preziuso), ngwavictor@yahoo.com (V. Ngu Ngwa), andrea.cantalamessa@unicam.it (A. Cantalamessa), martina.moriconi86@gmail.com (M. Moriconi), vincenzo.cuteri@unicam.it (V. Cuteri).

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registered in several countries including Europe and the USA. The main characteristics of this antimicrobial agent are low host toxicity, high bioavailability, excellent tissue penetration and long serum half-life (Brown, 1996; Lizondo et al., 1997). The pharmacokinetic studies on the use of enrofloxacin in lactating sheep show that it acts as a good antimicrobial agent when administered through intravenous, intramammary or subcutaneous routes (Haritova et al., 2003; Rahal et al., 2006; Cadenas et al., 2012). Furthermore, it is highly efficacious against most Gram-negative and some Gram-positive bacteria, some rickettsial and mycoplasmas organisms, and has been observed to be active even at lower concentrations compared to other classes of antimicrobial agents (Vancutsem et al., 1990; Langston et al., 1996). Therefore, this has necessitated the need to carry out further studies on the biological activity of enrofloxacin especially against sheep mastitis.

Thus, this study aims at evaluating the role of a commercial available antibiotic, enrofloxacin, in controlling clinical mastitis caused by *S. aureus* and also, at determining the most appropriate dosage regime in sheep.

2. Materials and methods

2.1. Animals, experimental design and milk samples

A stratified random sampling was carried out in two ovine flocks with a history of clinical mastitis caused by *S. aureus*, and known to be officially free of brucellosis. The flocks were located in Central Italy (43°16′N – 13°00′E) and consisted of a total of 2600 winter-lambing Sarda ewes grazing on upland pastures at an average altitude of 1150 FASL.

Two groups of animals (one per flock) were selected and named as Group A ($n = 36$) and Group B ($n = 39$). The animals selected and enrolled in each group were all female sheep aged more than one year, at the end of lactation, and showing clinical symptoms of mastitis such as acute inflammation of the udder, and, bacteriologically, *S. aureus* positive. The ewes should also answered at the following criteria:

2.1.1. Inclusion criteria

- Lactating sheep.
- Sheep with clinical mastitis, either acute or hyperacute, affecting at least one udder quarter.
- Sheep with body temperature higher than 39,6 °C.
- Sheep whose symptoms have been present for no more than 12 h.
- Sheep with systemic signs of disease: decreased appetite and/or lethargy and/or other general signs of disease.
- Sheep with clinical symptoms of mastitis lasting no longer than 12 h at the inclusion visit.

2.1.2. Exclusion criteria

- Sheep with concomitant medical, infectious or surgical illnesses;
- Sheep receivingsystemic or intramammary treatments, either anti-infective or anti-inflammatory, within the previous ten days.
- Sheep with apparent teat lesion.
- Sheep withmilk production lower than 0.5 L before the onset of the inclusion clinical signs.

Group A and Group B animals were treated with 2.5 mg/kg bw and 5 mg/kg bw of enrofloxacin antibiotic (Baytril®—5% injectable solution, Bayer, Italy) respectively, for three consecutive days (2 doses per day). The clinical symptoms of mastitis were monitored from the first day (D1) of treatment until day 14 (D14). Rectal temperature was measured at days D1, D2, D2 + 12 h, D3 + 12 h, D7, D14. Somatic Cell Count (SCC) was performed at D1 and D14. Local mammary gland reaction and systemic reaction (attitude and appetite) were also monitored at D1, D2, D3, D5 and D14, and a score was given for the local mammary gland reaction (0 = normal; 1 = hot and pain; 2 = severe inflammation; and 3 = cyanotic) and for the systemic reaction (good, sufficient, moderate and severe).

Milk samples were collected from the affected udder during the morning or evening milking period (D1, D7 and D14). The first squirts of milk were discarded and approximately 50 mL of milk were taken in sterile vials for the bacteriological and SCC tests. The samples were collected aseptically, in accordance with International Dairy Federation guidelines, identified with appropriate label, and transported to the laboratory at 4 °C within 3 hours and immediately analysed.

Somatic Cell Count was performed using a Fossomatic 5000 instrument (Foss Electric, Hillerod, Denmark), based on flow cytometry, within 5 h post collection, after heat treatment of the milk samples at 40 °C for 15 min (Gonzalo et al., 1994; Green, 1984). The mean value result was normalized and used for statistical analysis.

2.2. Bacteriological examination and bacterial identification

The collected milk samples, were cultured on different agar media, namely Columbia Agar supplemented with 5% sheep blood (with and without Streptococcus Selective Supplement), Baird-Parker medium and MacConkey Agar (Oxoid, Milan, Italy).

The organisms were considered based on colony morphology, Gram staining results (Gram-positive cocci in cluster), characteristic haemolytic patterns, and biochemical tests (catalase-positive and coagulase-positive) (Balows et al., 1991), and identified as *S. aureus* using API-Staph gallery (BioMérieux, France). Finally, the *S. aureus* strains were confirmed by PCR (Baron et al., 2004).

Briefly, since a biochemical similarity exists among *S. aureus* and other coagulase-positive staphylococci, a molecular identification of *S. aureus* was performed by amplifying the *nuc* gene by species-specific oligonucleotide primers (*SAnuc*-F: 5′-TGCTATGATTGTGGTAGCCATC-3′ and *SAnuc*-R: 5′-TCTCTAGCAAGTCCCTTTTCCA-3′). In addition, primers targeting a highly conserved region of 16S rDNA (16S-F: 5′-GGACGGGTGAGTAACACGTGG-3′ and 16S-R: 5′-TCCCGTAGGAGTCTGGACCGT-3′) were used as an internal control in a multiplex PCR (Baron et al., 2004). To prepare the DNA, three colonies of freshly sub-cultured strains were suspended in 50 μl lysozyme 5U/μl (Sigma, Germany). Then the tube was vortexed at 1000 rpm for 1 min. After incubation for 1 h at 37 °C, 0.75 μl proteinase K 20 μg/μl (Sigma, Germany) was added and the suspension was re-incubated for 1 h at 56 °C. Finally, the proteinase K was inactivated by boiling the mixture for 10 min and then cooling it in ice for 2 min. After centrifugation at 8000 rpm for 10 min, the resulting supernatant was used for PCR.

The PCR reaction mixture (25 μl) contained 10 pmol of the *SAnuc* primers and 5 pmol of the 16S primers, 2X Taq PCR mastermix (Qiagen GmbH, Germany), 1.9 μl of MgCl2 25 mM (Sigma, Germany), 3 μl of the template DNA and water. The mixture then underwent thermal cycling using a Hybaid, PCR Express, California. It was brought to 95 °C for 4 min, underwent 35 cycles (95 °C for 1 min, at 55 °C for 1 min and at 72 °C for 1 min) and then a final extension at 72 °C for 7 min. Electrophoresis of 10 μl of the reaction products was conducted in a 2.0% agarose gel (Qbiogene, Germany) with Tris-acetate electrophoresis buffer (TAE, 4.0 mmol/l Tris–HCl 1 mmol/l EDTA, pH 8.0). The PCR products of the *S. aureus nuc* gene were 420 bp and the internal control was 252 bp.

2.3. Antibiotic sensitivity test

The in vitro antimicrobial sensitivity of each strain was tested using the disk diffusion method (Bauer et al., 1959). Bacterial