Original Article

Subclinical bovine mastitis associated with *Staphylococcus* spp. in eleven Uruguayan dairy farms

Rosario de los Santos[1], Álvaro González-Revello[1], Lucía Majul[1], Ana Umpiérrez[2], Ariel Aldrovandi[3], Andrés Gil[4], Darío Hirigoyen[1,5], Pablo Zunino[2]

[1] *Departamento de Ciencia y Tecnología de la Leche, Facultad de Veterinaria, Universidad de la República (UdelaR), Montevideo, Uruguay*
[2] *Departamento de Microbiología, Instituto de Investigaciones Biológicas Clemente Estable (IIBCE), Montevideo, Uruguay*
[3] *Departamento de Calidad Alimentaria, Facultad de Veterinaria, Universidad de la República (UdelaR), Montevideo, Uruguay*
[4] *Departamento de Bioestadística e Informática, Facultad de Veterinaria, Universidad de la República (UdelaR), Montevideo, Uruguay*
[5] *Instituto Nacional de Investigación Agropecuaria (INIA), La Estanzuela, Colonia, Uruguay*

Abstract

Introduction: Bovine mastitis is the most common disease affecting the dairy industry, with staphylococci being considered as one of the most significant and prevalent causes. This study aimed to assess the presence of staphylococcal subclinical mastitis (SCM) in Uruguayan dairy farms and to identify *Staphylococcus aureus* (SA) and non-aureus staphylococci (NAS) in milking cows. In addition, the antibiotic susceptibility of isolated staphylococci was evaluated.

Methodology: We tested 546 apparently healthy milking cows from 11 farms for detecting SCM using the California Mastitis Test (CMT). The cows were not treated with antibiotics. CMT-positive samples were cultured, and colonies compatible with *Staphylococcus* spp. were further identified through molecular techniques. The susceptibility of the *Staphylococcus* spp. isolates against thirteen antibiotics was determined using the disk diffusion method.

Results: Subclinical staphylococcal mastitis was present in almost all (82%) farms. SA (n = 39) was more common than NAS (n = 9) in the 48 samples tested. Isolates exhibited resistance to one, two, and even three different antibiotics. Resistance to penicillin was the most frequent among SA (23/39) and NAS (4/9). No staphylococci isolates exhibited resistance to cefoxitin, vancomycin, trimethoprim-sulfamethoxazole, erythromycin, or clindamycin.

Conclusions: Staphylococcal SCM is one of the most common diseases in Uruguayan dairy farms. SA was the prevalent pathogen, however SA and NAS mastitis coexisted in many farms. NAS were identified and its distribution was similar to other countries. Penicillin had the highest and most frequent percentage of resistance.

Introduction

Bovine mastitis is one of the most common and expensive diseases affecting the dairy industry worldwide and the most important cause associated with poor milk quality [1]. Mastitis is caused by microorganisms, typically bacteria, which enter the bovine mammary gland through the teat canal, establishing an intramammary infection (IMI) and resulting in an inflammatory reaction [2]. Mastitis can be present in a clinical or a subclinical form. Clinical mastitis (CM) can be detected through physical examination. Subclinical mastitis (SCM) is more difficult to identify and requires additional tools.

Furthermore, SCM is detectable through different tests [3]. The California Mastitis Test (CMT) is a simple, cheap, and rapid screening test for mastitis based on the amount of cellular nuclear protein present in the milk sample. Since inflammatory cells are the predominant cell type present in mastitic milk, the CMT reflects the somatic cell count (SCC) level quite accurately and is a reliable indicator to assess the severity of infection. The test is appropriate for cow-side evaluation of udder health and the procedure can be taught quickly to producers and the milking staff [4].

Multiple players have a role in the development and outcome of mastitis, including bacteria, farmers, and

hosts. When the balance tilts in favor of the pathogen, mastitis occurs [5].

The staphylococci group is considered one of the most isolated, significant, and prevalent bacteria that cause bovine mastitis [6]. *Staphylococcus aureus* (SA) is a contagious udder pathogen that, if appropriate control measures are not introduced, spreads during milking from infected quarters to healthy ones [7-9]. It has been traditionally classified as a major mastitis pathogen that can cause CM but often also causes SCM, which remains persistent and increases milk SCC.

Other staphylococci, such as non-aureus staphylococci (NAS), have been traditionally regarded as opportunistic pathogens of minor importance, causing mild symptoms and usually SCM associated with only a moderate increase of SCC [10]. However, recent studies propose that infections by NAS may cause more severe harm than previously thought [11]. When studying cows with mastitis caused by *Staphylococcus chromogenes*, authors have found similar SCC compared to cows with SA mastitis [12]. Among NAS, differences in niche adaptation, behavior, epidemiology, ecology, the effect on udder health, virulence factors, and antimicrobial susceptibility have been reported [13,14].

Identification of NAS species has become relevant to design appropriate control strategies in dairy herds where mastitis is a problem, and different patterns of NAS prevalence have been found in herds [13,15]. However, Uruguayan studies have usually described NAS as a relatively harmless bacterial group without a species identification. Some NAS species recovered from intramammary infection (IMI) are specifically adapted to the udder, whereas other species are environmental opportunists that only sporadically cause infections [15,16]. NAS mastitis-associated pathogens have become more predominant than SA in several countries including Uruguay, especially in farms that have successfully controlled SA mastitis using the ten-point strategy [16-18].

Mastitis is still the most commonly treated disease of dairy cows. Data from the US Department of Agriculture (USDA) revealed that among the farms that treated cows with any antibiotic, 85.4% were used for mastitis [19]. *Staphylococcus* spp., are the leading pathogens of bovine mastitis, and when SA is prevalent, mastitis is much more difficult to control with antibiotics than NAS.

Antibiotic therapy of mastitis involves the detection of the infected quarter, the causative pathogen and characteristics of the animal, the udder pathology, and its clinical history [20]. Several antimicrobial treatment regimens are available, with different antimicrobial compounds, routes of administration, probability of cure, and costs [21, 22].

The aim of this study was to assess the presence, causative agents and prevalence of staphylococcal SCM in eleven dairy farms of Uruguay and to determine antibiotic resistance of the isolated staphylococci.

Methodology

Milk farms and cows' inclusion criteria

Eleven commercial dairy herds located in the Uruguayan provinces of San José, Canelones, and Colonia, with a production system mostly based on grazing, were selected to participate in this study. Their accessibility, size and bulk milk tank quality variables, bacterial count (BC) and bulk tank milk somatic cell count (BTSCC) were considered for the selection of herds.

For animal selection, apparently healthy milking cows (not on current or previous antibiotic treatment in the last five days) were evaluated to determine the presence of SCM.

General features of the farms

Eleven farms were included in this study. All farms milked Holstein cows. Herd sizes ranged from 25 to 390 milking cows. Three main groups of farms according to the number of cows in milking (0-50, 51-100, and more than 100), were considered. Five of them had less than 50 milking cows, another 5 farms had from 51 to 100 milking cows, and one had more than 100 milking cows.

Milk sampling and SCM diagnosis

All apparently healthy milking cows were tested to evaluate the presence of SCM using CMT. If any grade of CMT was detected, the quarter was sampled following the National Mastitis Council recommendations and sent to the laboratory for further SCC analysis [4], and milk pathogen isolation and identification [23]. This decision was made considering that a cow quarter SCC of 200,000 cells/mL or more (and for heifers 150,000 cell/mL or more) would indicate disease. For the trace level of CMT, we considered a cell count range between 100,000 and 300,000 cells/mL [24].

Isolation and identification of staphylococci

A set of rapid and simultaneous tests that allowed us to have a positive predictive value above 95% for SA were employed for the phenotypic identification [25].

Once the milk samples arrived at the laboratory, they were inoculated onto blood agar plates (5% ovine blood) and Baird-Parker Agar (BPA) plates. All plates were incubated aerobically at 37 °C for 48 h before being examined for the presence of staphylococcal-like colonies [26]. The isolates that grew were preliminarily evaluated by Gram staining and catalase production. All catalase-positive, Gram-positive, coccus-shaped bacterial isolates were tentatively identified as *Staphylococcus* spp. and tested for coagulase production using rabbit plasma. Additional selective media were used to improve positive predictive value, including Mannitol-Salt Agar and, deoxyribonuclease (DNase) test. Voges-Proskauer test was performed to differentiate SA from the other coagulase-positive staphylococci [25,27].

Catalase-positive isolates were examined for hemolysis on blood agar plates. Their presence was recorded as a predictor factor for staphylococci identification. Colony morphology was also observed. Isolates that exhibited positive Gram stain colonies of irregular appearance, grayish tan, no hemolysis or produced a narrow (< 2 mm) diffuse zone of complete hemolysis at 24 h or diffuse zone of incomplete hemolysis and had a negative coagulase reaction, were at first classified as NAS [28]. Coagulase activity was determined in tubes (tube coagulase test, TC) and examined after 4 and 24 h of incubation [29]. DNase test was carried out using commercially available DNase-Agar, prepared in Petri dishes, inoculated with the isolates (spots), and incubated at 37 °C for 24 h. Those isolates identified as positive after this test, showing a clear zone around the bacterial growth after incubation (DNase activity), were considered positive.

Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA), Baird Parker Agar (BPA), Mannitol Salt Agar, DNAse medium, and Voges-Proskauer Broth (VP) were from HIMEDIA (Mumbai, India. Sheep Blood was supplied from Biokey SRL (Montevideo, Uruguay. SA reference strains ATCC 29213, 25923 and 6538 were used as controls [25,30,31].

Molecular identification was made by partial amplification and sequencing of the 16S rDNA using 27F and 1492R universal primers [32]. Genomic DNA was extracted from overnight bacterial cultures using a commercial kit (Gen Elute Bacterial Genomic; Sigma, St. Louis, MO, USA).

Polymerase Chain Reaction (PCR) used for molecular identification was carried out in a final volume of 25 μL containing 1x PCR buffer, 1.5 mM MgCl$_2$, 0.2 mM dNTP Mix, 0.5 μM of each primer, 1 μL of DNA and 1U Taq DNA polymerase (Invitrogen Life Technologies, São Paulo, Brazil). Amplification consisted of an initial denaturation step at 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min. The PCR reactions were performed in a Sensoquest Labcycler Thermocycler (Sensoquest, Gottingen, Germany).

PCR products were examined using agarose (1%) gel electrophoresis and visualized using GelRed (Biotium, Fremont, CA, USA) in a UV light transilluminator (Labnet International, Inc., Edison, NJ, USA). Amplicons were sequenced at Macrogen Inc. (Seoul, South Korea). Obtained sequences were analyzed using BLASTn to compare with data available in the GenBank database (NCBI) [33].

To estimate the prevalence of staphylococcal SCM we considered quarters of milking cows that were diagnosed with SCM, and we divided them by the total number of cows or quarters sampled.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the isolates was determined using the disk diffusion method according to the guidelines of Clinical Laboratory Standards Institute [34]. The antimicrobials used were clindamycin (DA2, 2 μg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 μg), enrofloxacin (ENR, 5 μg), erythromycin (E15, 15 μg), ciprofloxacin (CIP5, 5 μg), cefoxitin (FOX30, 30 μg), rifampicin (RD5, 5 μg), amoxicillin-clavulanic acid (AMC 30, 20/10 μg), oxytetracycline (OT30, 30 μg), penicillin (P10, 10 μg), gentamicin (CN10, 10 μg), chloramphenicol (C30, 30 μg), and vancomycin (VA30, 30 μg). The SA strain ATCC 29213 was used in each assay as a quality-control strain. Resistance to methicillin was indirectly tested through cefoxitin. Also, cefoxitin is an alternative marker for the presence of *mecA* since it is a more powerful inducer of the MecA regulatory system than penicillin and therefore improves the expression of this gene and consequently also improves the detection of resistance to methicillin [35]. E-test methods were used for clindamycin and vancomycin for those staphylococci that were apparently resistant when tested with Kirby and Bauer assay. The E-test manufactured by bioMérieux (Lyon, France), was conducted according to the manufacturer's instructions. All organisms were tested using Mueller-Hinton agar (supplemented with 5% defibrinated sheep blood for staphylococci). Minimum inhibitory concentrations (MICs) were analyzed using the CLSI criteria [34].

Data Analysis

Data were analyzed using Exploratory Data Analysis and presented in a descriptive way.

Results

Subclinical mastitis prevalence in cows and quarters after CMT and SCC tests were sequentially performed

A total of 546 apparently healthy milking cows and their functional quarters (*n* = 2184) were tested to evaluate the presence of SCM (Table 1).

A total of 257 cows (11.2%; CI 95: 9.96-12.61) were positive according to the CMT test (traces 1, 2 or 3). These were classified provisionally as affected quarters because not all grade traces were considered positive for SCM. The SCC test performed after CMT provided the final accurate diagnosis with the differentiation between healthy and ill cows. The prevalence of SCM per quarter was highly variable increasing as farms were smaller.

The group of sampled cows included 70% healthy animals. With regards to CMT, the level traces plus level 1 were more frequent than level 2 plus level 3. In other words, herds had mostly healthy individuals, and, in the group of affected cows, the inflammation rate of the mammary gland was mild or moderate and the highest level of CMT was the least frequent. Also, 17 milk samples of different quarters apparently affected with SCM, according to CMT analysis, exhibited a normal SCC and were discarded (not shown). The number of cows that were positive for SCM (at least one quarter affected) were 163, and this represented 29.9% (CI 95: 26.2-33.8) (Table 1).

According to the survey performed on the farm's owners before sampling, 18.2% of the farms exhibited historical records of SCC lower than 200,000 cells/mL, while the remaining 81.8% showed a cell count that ranged from 200,000 to 400,000 cells/mL.

Mastitis ranking varied among the reasons for cows culling during lactation. In 45.5% of the farms, it was the first reason, in 18.2% the second, in 9.1% the third, in 18.2% the fourth and in 9.1% this aspect remained unknown.
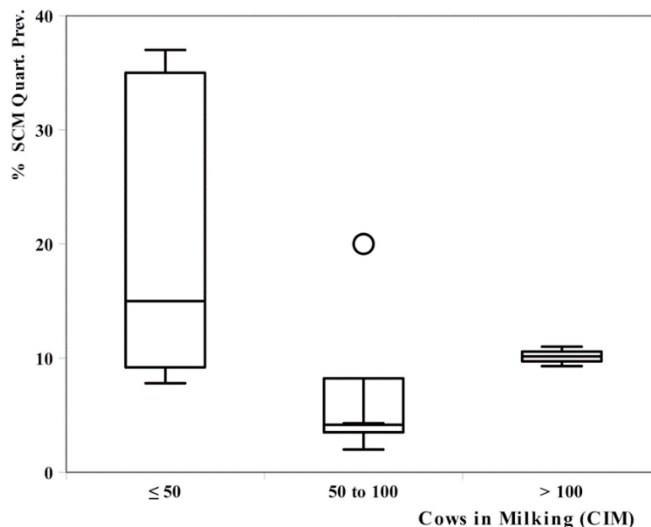
In 63.6% of the dairy farms, the prevalence of mastitis did not change compared to the previous year,

Table 1. Proportion estimated (prevalence) of cows and quarters with SCM.

	SCM	Proportion (95% CI)
Cows[1]	No	0.7015 (0.6616 - 0.7385)
	Yes	0.2985 (0.2615 - 0.3384)
Quarters[2]	No	0.8878 (0.8739 - 0.9004)
	Yes	0.1122 (0.0996 - 0.1261)

[1]: Number of cows studied: 546; [2]: Number of quarters studied: 2184 (Results are shown as the proportion of total sampled quarters).

Figure 1. Subclinical mastitis prevalence per quarter (SCMP/qtr.) grouped per size of farm as cows in milking.

in 27.3% of farms the prevalence decreased, and in 9.1% the prevalence increased. As seen in Figure 1, the prevalence of mastitis was more variable in farms with a smaller number of cows. Because of that, it was not possible to determine a significant trend between these two variables.

Prevalence of staphylococci and distribution among farms

After performing CMT, 257 positive milk samples were submitted for SCC test. Seventeen exhibited normal counts, so they were discarded from the trial and the remaining 240 samples were plated. Ninety-eight did not result in any bacterial growth, and another 2 were contaminated. Finally, bacterial growth was seen after culturing the remaining 140 samples. Ninety-two of them were bacteria other than staphylococci (bacilli and other cocci). The most frequently isolated pathogen was SA (80.9%, 39 isolates), followed by *S. chromogenes* (4 isolates), *S. haemolyticus* (4 isolates) and *S. warneri* (1 isolate). Although SA was the most frequently isolated *Staphylococcus* species, it was not present in 2 out of the 11 farms. The staphylococci distribution among farms is illustrated in Figure 2.

Antibiotic resistance

Thirty out of 48 staphylococci isolates were resistant to at least one antibiotic, whereas 18 showed no resistance (Table 2). Resistance to penicillin was most frequently detected with 23/39 (59%) resistant SA, 2/4 (50%) resistant *S. haemolyticus,* 1/4 (25%) resistant *S. chromogenes* and, 1/1 (100%) resistant *S. warneri*. None of the staphylococci isolates exhibited

resistance to cefoxitin, vancomycin, trimethoprim-sulfamethoxazole, erythromycin, or clindamycin. Only SA showed resistance to ciprofloxacin, rifampin, amoxicillin-clavulanic acid, gentamicin, and chloramphenicol (Table 2). Meanwhile, *S. chromogenes* showed phenotypic resistance to penicillin and enrofloxacin, and *S. haemolyticus* expressed resistance to penicillin and oxytetracycline. Finally, *S. warneri* was resistant to only penicillin. Overall, 18 were resistant to a single compound, 8 to two, and 4 to three different compounds (Table 2).

Discussion

Bovine mastitis is considered to be the commonest treated disease of dairy cows worldwide and staphylococci are the most widespread pathogens related to this pathology [1]. Besides this, SA has been described as the most prevalent pathogen causing CM and SCM in Uruguay and worldwide [1,36]. However, it has been reported that once SA is controlled, NAS becomes an important emergent cause of bovine mastitis instead [37].

The aim of this study was to assess the presence and prevalence of staphylococcal SCM in 11 dairy farms in Uruguay and to determine the antibiotic resistance patterns of the isolated staphylococci.

Three main categories of farms were studied. Five farms had less than 50 milking cows, 5 farms had from 51 to 100 milking cows, and 1 had more than 100 milking cows. Considering the proportion of healthy and mastitic cows, and, within the mastitic group, the degrees of CMT exhibited and the quarters SCC, the results were similar to a previous study by our group [38]. The SCC observed in most farms is compatible with the SCM persistent infection with staphylococci present in almost all the farms. After bacterial

Figure 2. Staphylococci distribution among farms.
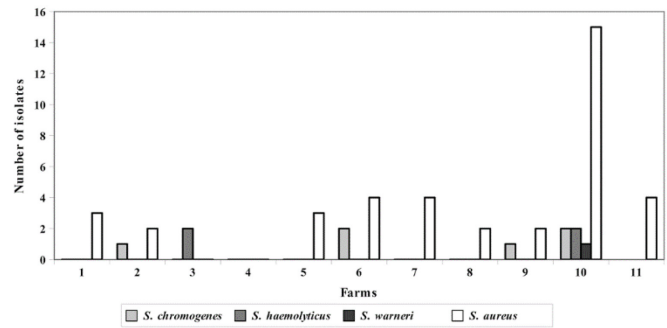
identification, SA was the most frequently isolated staphylococci (about 85%), while NAS isolation rate was about 15%. These results are different from previous work by our group, where NAS was the most isolated bacterial group in two Uruguayan farms [38], but similar to that reported by Persson *et al.* [39], who observed that the most common isolates in Sweden were SA (54% of total staphylococci, 19% of total isolates), followed by NAS (46% of total staphylococci, 16% of total isolates). One explanation for the differences in NAS prevalence between studies may be due to the udder microbiota, which has been proposed to be herd-specific [40].

Accurate species identification in bovine mastitis is essential for studying NAS epidemiology, since NAS show a diverse behavior according to species [41,42]. *S. chromogenes* and *S. haemolyticus* were the predominant species with a relative frequency of 8.5% of total staphylococci. Similar results were reported by DeVisscher *et al.* [40], who found that *S. chromogenes* was the most frequently NAS isolated species, although at a higher frequency than our study, from infected quarters. Differences in percentages are possibly due to

Table 2. Staphylococcus species and antibiotic resistance.

Antimicrobial	S. aureus	S. chromogenes	S. haemolyticus	S. warneri	Resistant isolates
DA2μ	2	0	0	0	0
SxT	0	0	0	0	0
ENR	1	1	0	0	2
E15	0	0	0	0	0
CIP5	1	0	0	0	1
FOX30	0	0	0	0	0
RD5	1	0	0	0	1
AMC30	3	0	0	0	3
OT30	3	0	1	0	4
P10	23	1	2	1	27
CN10	1	0	0	0	1
C30	1	0	0	0	1
VA30	0	0	0	0	0
					32*

There were some isolates resistant to more than one antibiotic. Drug code: DA2μ: clindamycin; SXT*: sulfamethoxazole + trimethoprim; ENR*: enrofloxacin; E15*: erythromycin; CIP5*: ciprofloxacin; FOX30*: cefoxitin; RD5*: rifampicin; AMC30*: amoxicillin + clavulanic acid; OT30*: oxytetracycline; P10*: penicillin; CN10*: gentamicin; C30*: chloramphenicol; VA30*: vancomycin.

the number of sampled dairy farms. In Argentina, a country in our region where dairy activity is relevant, Raspanti *et al.* [43] isolated NAS from bovine SCM and determined that *S. chromogenes* and *S. haemolyticus* were the predominant species. The intramammary infection (IMI) prevalence of *S. chromogenes* differed among parities in that study. In our work, cows affected with NAS had an average age of 2.5 years, and were in their first lactation, while 75% of them were in the late lactation, the remaining 25% were in the first third.

Antibiotic resistance was evaluated, and isolates were mainly susceptible to the tested antibiotics. Resistance to penicillin was the most frequently detected one in all our isolated staphylococci (59%). The occurrence of this antibiotic resistance was previously reported in SA associated to SCM (36%) and NAS (33.3%) by Giannecchini *et al.* in Uruguay in 2014 [44]. Penicillin is the antibiotic most frequently used in Argentina for bovine mastitis treatment, and Raspanti *et al.* found over 50% of resistant NAS [43]. Similarly, Persson *et al*. reported that in Sweden, 4% of the SA isolates and 35% of the NAS isolates were resistant to penicillin, whereas resistance to other antimicrobials was uncommon [39]. Antimicrobial resistance, particularly in the NAS group is increasing worldwide, although reports are scarce [43]. The efficiency of antibiotic treatment is associated with a rational use of antibiotics. In 2017, our group detected that the annual proportion of staphylococci from SCM isolates, resistant to penicillin significantly decreased from 28.7% in 2008 to 6.5% in 2015 [45]. The average number in the 8-year period showed 19% of SA resistant to penicillin and an overall trend of risk reduction for this resistance over the years ($p < 0.05$) [45]. Similar trends were found in the same conditions in New Zealand by McDougall *et al.* [46]. These authors determined that 25% of SA associated to SCM were resistant to penicillin. We estimate that when the antibiotics use is satisfactory, and the antibiotic susceptibility is checked before dosage together with good biosecurity practices, resistance can decrease with time. Concerning the other tested antibiotics, we did not find any resistance to clindamycin or erythromycin either in SA or in NAS, which had been previously determined in Uruguayan dairy farms and among livestock environments [44,47].

Conclusions

Staphylococcal mastitis remains an important issue in farms of all sizes leading to the culling of cows in many cases. In this work, *S. aureus* and NAS *S. chromogenes*, *S. warneri*, and *S. haemolyticus* were

identified as the causative agents, and their distribution was similar to that in other countries. We do not know how each species affect the udder and milking yields in our farm work conditions because the species diagnosis is usually performed in a very limited number of cows. This can contribute to the persistence of NAS because control strategies may vary according to different species. As far as we are aware, this is the first study in our country that includes NAS identification by molecular methods in a mastitis study. SCM was identified on almost all the farms. Penicillin had the highest and most frequent percentage of resistance among staphylococci. However, different isolates of SA also exhibited resistance to ciprofloxacin, rifampin, amoxicillin-clavulanic acid, gentamicin, and chloramphenicol. NAS was resistant to enrofloxacin, oxytetracycline, and penicillin. Since SA is the most isolated pathogen, control measures at milking and prescribing antibiotics must be reformulated. We recommend rotating the antibiotic and isolating the pathogen to test its antibiotic resistance in all farm sizes, to obtain a better response to therapy with routine drugs.

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Corresponding author

Rosario de los Santos, DMTV, MSc.
Profesor Asistente, Departamento de Ciencia y Tecnología de la Leche, Facultad de Veterinaria, UdelaR
Lasplaces 1550, Montevideo, Uruguay
Tel: 598-26222411
Fax: 598-26222411
Email: rosario.rdelos@gmail.com

Conflict of interests: No conflict of interests is declared.