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Prevalence and effect of intramammary infection due to coagulase-negative staphylococcal (CNS) species in somatic cell counts in milk from Holstein dairy cows in Boyacá, Colombia

R. J. Andrade-Becerra^{1*}, L. E. Tarazona-Manrique², J. C. Vargas-Abella³

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ABSTRACT

Mastitis is one of the most important illnesses in specialized dairy herds worldwide due to the effects on production and animal health. The types caused by CNS has a special importance in a production where the main pathogens are controlled. The objective of the present work is to determine the prevalence of CNS in a dairy herd in Boyacá and also quantify the effects of every species of CNS in SCC. 40 cows were selected and sampled during 6 months, CMT was performed, and results from 1 to trace were sampled. The routine bacteriological test was also performed for CNS identification, and the isolating of CNS was performed through *rpoB* gene identification and through the type of strain using the pulse gel electrophoresis procedure. Out of 960 samples, 619 were positive for CNS growth. The most prevalent species were *Staphylococcus epidermidis*, *S. chromogenes*, *S. sciuri*, *S. simulans*, *S. haemolyticus* and *S. capitis*. The results that were found here are similar to the results observed in different parts of the world, which confirms that they are pathogens that must be constantly evaluated because they can go unnoticed in routine controls, especially in those farms where major pathogens are not a serious problem. The results determined in this study demonstrate that CNS generates a slight increase in somatic cells.

Keywords: Mastitis, California mastitis test, coagulase-negative staphylococcus, somatic cell count.

Prevalencia y efecto de la infección intramamaria debida a especies de estafilococos coagulasa negativo (ECN) en el conteo de células somáticas en leche de vacas Holstein en Boyacá, Colombia

RESUMEN

La mastitis es una de las enfermedades más importantes en los rebaños lecheros especializados alrededor de todo el mundo debido a los efectos sobre la producción y la salud animal. Los tipos ocasionados por estafilococos coagulasa negativo (ECN) tienen una importancia especial en una producción en la que los principales patógenos están

¹ Gipatracol research group, laboratory of milk quality analysis and mastitis control. School of veterinary medicine and zootechnics. Universidad Pedagógica y Tecnológica de Colombia, sede Tunja (Colombia). roy.andrade@uptc.edu.co

² Gipatracol research group, laboratory of milk quality analysis and mastitis control. School of veterinary medicine and zootechnics. Universidad Pedagógica y Tecnológica de Colombia, sede Tunja (Colombia).

³ Gipatracol research group, Laboratory of milk quality analysis and mastitis control. School of veterinary medicine and zootechnics. Universidad Pedagógica y Tecnológica de Colombia, sede Tunja (Colombia).

controlados. El objetivo del presente trabajo es determinar la prevalencia del ECN en un hato lechero en Boyacá y cuantificar los efectos de cada especie de ECN en el conteo de células somáticas (CCS). Se seleccionaron 40 vacas y se tomaron muestras durante 6 meses, se realizó californina mastitis test (CMT) y se tomaron muestras de los resultados desde 1 hasta donde hubo trazas. También se realizó la prueba bacteriológica de rutina para la identificación del ECN y el aislamiento del ECN se realizó mediante la identificación del gen *rpoB* y del tipo de cepa, usando el procedimiento de electroforesis en gel de pulso. De 960 muestras, 619 fueron positivas para el crecimiento del ECN. Las especies más prevalentes fueron *Staphylococcus epidermidis*, *S. chromogenes*, *S. sciuri*, *S. simulans*, *S. haemolyticus* y *S. capitis*. Los resultados encontrados aquí son similares a resultados en diferentes partes del mundo, lo que confirma que son patógenos que deben ser evaluados constantemente porque pueden pasar desapercibidos en los controles de rutina, especialmente en aquellas fincas donde los patógenos mayores no son un problema grave. Los resultados determinados en este estudio demuestran que el SNC genera un ligero aumento de células somáticas.

Palabras clave: mastitis, prueba de mastitis California, estafilococos coagulasa-negativos, conteo de células somáticas.

INTRODUCTION

Mastitis caused by intramammary infection (IMI) is one of the most important illnesses in specialized dairy herds worldwide due to the effects on mammary tissue, the decrease of milk production, the poor quality of raw milk, the increase of culling rate of animals, and the additional expenses for treatments and milk that is lost because of the antibiotic treatment (Taponen *et al.* 2016; Krishnamoorthy *et al.* 2016). Globally, the focus of control programs is for major pathogens like *Staphylococcus aureus* and *Streptococcus agalactiae*, but it is important to analyze also minor pathogens like coagulase-negative *Staphylococcus* species, which are one of the most prevalent groups that cause subclinical mastitis in dairy cows (Vanderhaeghen *et al.* 2014; Tarazona *et al.* 2019).

Staphylococci are a diverse group of gram-positive cocci, which can infect the mammary gland. For intramammary infection diagnosis, *Staphylococci* are classified according to its ability to coagulate

plasma in coagulase-positive or coagulase-negative, the microbiological test used to make this determination is the rabbit plasma coagulase test (Andrade *et al.* 2012). Coagulase-negative *staphylococcus* (CNS) can be found in teat canals, teat skin and teats ducts. They are classified as minor pathogens causing mastitis, and they rarely differ when IMI is diagnosed, it is also possible that they can cause food-borne disease due to pathogenic factors (De Freitas *et al.* 2013; Levinson *et al.* 2016; Dufour *et al.* 2012).

The CNS regularly isolated in subclinical mastitis are *Staphylococcus epidermidis*, *S. chromogenes*, *S. sciuri*, *S. simulans*, *S. haemolyticus*, *S. hominis*, *S. xylosum*, *S. equorum*, *S. capitis* and *S. auricularis* (Andrade *et al.* 2012; Andrade *et al.* 2018). These bacteria are sometimes referred to environmental staphs, and they are the most frequent organisms isolated from milk samples obtained in herds that have controlled the major pathogens (Krishnamoorthy *et al.* 2016; Dufour *et al.* 2012). They

are also related with a mild increase of somatic cells count (SCC) in bulk milk in a herd, and with an increase in IL-6 in milk and serum, in concentrations up to 20 times more than in clinically healthy cows (Thorgberg *et al.* 2009; Bochniarz *et al.* 2017), and in acute-phase inter-alpha-trypsin inhibitor heavy chain 4. However, there is no consensus regarding the effects of IMI with NEC on milk production, and its physicochemical characteristics (Tomazi *et al.* 2015).

Some cows with IMI caused by CNS species like *S. chromogenes*, *S. simulans*, and *S. xylosus* had a SCC similar to that observed in IMI caused by *S. aureus*, others like *S. auricularis* had minimal impact in SCC (Tomazi *et al.* 2015; Supré *et al.* 2011). In Colombia, the studies focused on the previous topic are few (Andrade *et al.* 2014; Tarazona *et al.* 2019). Therefore, due to the absence of data related with the prevalence of CNS in Colombia and due to the variability in SCC in IMI caused by CNS, the objective of this study was to determine the prevalence of CNS in a dairy herd in Boyacá and also quantify the effects of every species of CNS in the SCC.

MATERIALS AND METHODS

Population and sampling

The research was carried out in the municipality of Paipa. A longitudinal, prospective, descriptive study was developed. 1 herd was included in the study, this one had a total of 72 animals in production, of which 40 Holstein cows between 2 and 4 calvings, and between 60 and 90 days in milk (DIM), were selected. These cows corresponded to 55% of the animal presents in the herd. The average bulk tank milk somatic cell count (SCC) in

the last month was 232.000 cells/ml, and 845 l of milk was produced per day on average. They grazed on kikuyu (*Cenchrus clandestinus*), and they received water *ad libitum*. The milking system was mechanic with 1 operator.

This research was approved by the Research Ethics Committee of the Universidad Pedagógica y Tecnológica de Colombia (UPTC), sede Tunja (Approval letter, 22 April 2019).

Sampling and transport

Monthly, milk samples were taken from every teat, between July and December 2019. The samples were taken following the instructions from the National Mastitis Council (2017). All mammary glands were evaluated and no one showed apparent sings like pain or inflammation such as reddish tones in the mammary gland, tenderness, adhesions, and hard areas. Later California mastitis tests (CMT) were performed in all teats. The CMT results were scored and interpreted as either (Godden *et al.* 2017) 0 (negative or no thickening of mixture), trace (slight thickening), 1 (+, or low thickening but no tendency to form a gel) 2 (++ , or immediate thickening, with a slight gel formation), or 3 (+++, or gel formed, and the surface of the mixture becomes elevated) inflammatory response based on the viscosity of the gel formed by mixing reagent with milk (Tarazona *et al.* 2019; Mendoza *et al.* 2017; Tolosa *et al.* 2013).

Milk samples were taken from every teat that showed 0, trace, and 1 results. They were stored in sterile plastic bags, the procedure described by the National Mastitis Council (NMC) was followed (NMC, 2004). The collected samples were kept and transported under refrigeration until they arrived at the laboratory for analysis

(Tarazona *et al.* 2019). All samples were taken by a veterinarian and transported under refrigeration to the milk quality analysis and mastitis control laboratory (MQAMC). They also were transported to the Veterinary Microbiology Laboratory (VML) of the Universidad Pedagógica y Tecnológica de Colombia in Tunja.

Bacteriological tests and species identification

A routine diagnosis was carried out, also the isolation of non-common microorganisms following the methodology proposed in the Laboratory Handbook on Bovine Mastitis, by the NMC (NMC, 2017). 1 ml of milk sample were inoculated on sheep blood agar. After that, the samples were incubated during 24 hours in an aerobic medium at 37°C, gram staining (positive), hemolysis pattern, catalase reaction (positive), and coagulase tests (negative) (Soler *et al.* 2019; Thorgberg *et al.* 2009). Milk samples that showed growth of 3 or more species of pathogens were considered contaminated, and they were excluded (Dufour *et al.* 2012; Koop *et al.* 2012).

Genetic characterization of CNS

The identification of each CNS species was carried out in a private external laboratory through the identification of the *rpoB* gene, through real-time PCR. However, it was not possible to access the primer sequences.

Colony Forming Units and SCC

SCC was performed using Fossomatic 4000™ equipment from the FOSS Company®, and Colony Forming Units/ml (CFU/ml) was performed using a Bacto-Scan™ FC+ (Vásquez *et al.* 2012).

Definition of positive case

A positive case (for every mammary quarter) was considered infected by CNS when growth of > 500 Colony Forming Units/ml (CFU/ml) of a particular organism and only one colony type on the plate were isolated (Dieser *et al.* 2014; Raspanti *et al.* 2016).

Statistical analysis

All the data were grouped in an Excel document for windows 2010. Later, they were

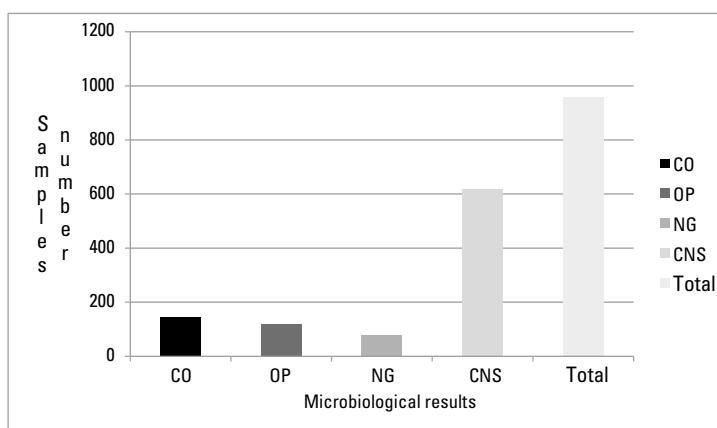


FIGURE 1. Results of microbiological characterization of milk samples. CO: Contaminated; OP: Others Pathogens; NG: No growth; CNS: Coagulase Negative Staphylococcus

processed through the statistical program Statgraphics centurion XVI®. Average Geometric measurement SCC was calculated using monthly dates for every infected teat. The correlation between CNS species causing a positive case and average geometric count SCC were compared using variance analysis of Kruskal-Wallis (Anova) with comparison for peers post-hoc ($P < 0,05$)

RESULTS

Microbiological characterization

Due to the fact that each teat was evaluated separately, a total of 960 samples of milk were taken. The results of the

microbiological analyses were: 1) 144 contaminated samples (15%); 2) 120 samples with growing of others type of pathogens principally *Streptococcus* spp. and *Corynebacterium* spp. (12,5%) and 3) 77 samples without growing (8%) (figure 1).

The 619 remaining samples (64,47%) were positive for almost 1 strain of CNS. 11 species of CNS were identified (table 1). The duration of infection ranged between 1 and 6 months. Species like *S. epidermidis* and *S. chromogenes* were found in several samples in 1 month with high numbers of colonies, while species like *S. hyicus*, *S. auricularis* and *S. equorum* were found in samples in all 6 months with few colonies (table 1).

TABLE 1. Percentage of isolations for every species of CNS and geometric mean of SCC. The letters indicate membership in a statistical group, different letters indicate the statistical difference between groups

Species of CNS	Number of isolations	Percentage of isolation (%)	Geometric mean Of SCC $\times 10^3$ cell/mL*	p < 0,05
<i>S. epidermidis</i>	87	14,05	250	a
<i>S. chromogenes</i>	115	18,57	200	ab
<i>S. sciuri</i>	65	10,5	240	a
<i>S. simulans</i>	76	12,27	220	a
<i>S. haemolyticus</i>	58	9,36	230	a
<i>S. capitis</i>	70	11,3	190	ab
<i>S. hominis</i>	34	5,49	36	d
<i>S. xylosus</i>	23	3,71	80	c
<i>S. equorum</i>	8	1,29	40	d
<i>S. auricularis</i>	32	5,16	15	e
<i>S. hyicus</i>	36	5,81	12	e
Mixed CNS	15	2,42	87	c
Total	619	100		

Statistical differences ($p < 0,05$) were found in the median geometric mean of SCC between species of CNS. The major results of SCC were found for six species of CNS, which were *S. epidermidis*, *S. chromogenes*, *S. simulans*, *S. sciuri*, *S. haemolyticus* and *S. capitis*. For the remaining CNS species, values less than 100×10^3 cell/ml were found, and the mixed infection (*S. auricularis* and *S. equorum*) had a result of 87×10^3 cell/ml (table 1).

DISCUSSION

Many factors are associated with an increase of SCC. Even in the absence of bacterial growth, this increase leads usually to traumatic damages (lacerations, cuts, injuries), that get worst due to chemical irritants or external conditions, causing that the teats or the udder, in general, get affected, and increasing the somatic cell count. This could explain the absence of growth of bacterium colonies in the 77 samples (Andrade *et al.* 2018; Tarazona *et al.* 2019).

CNS are minor pathogens that caused mastitis. They are important in the dairy industry worldwide, however, their importance had not been previously evaluated in Colombia. In this study a total of 619 samples (64,47%) were positive to almost 1 CNS species by cultured as well as by pulse gel electrophoresis. These results were similar to those reported by (Hosseinzadeh & Dastmalchi 2014), who determined in Iran that 68,35% of isolations in mastitis milk samples corresponded to CNS; nonetheless, these results were higher compared to the results of Taponen *et al.* (2016), who in Finnish farms determined IMI due to CNS in the 13,35% of the samples.

Studies in CNS causing mastitis in dairy cows show a wide variation in the

prevalence of distinct species. Hosseinzadeh & Dastmalchi (2014) showed that the CNS species more prevalent in their study were *S. haemolyticus* (40,7%) and *S. chromogenes* (15,7%), results that were higher than the one reported here. However, Bochniarz *et al.* (2017) reported that *S. xylosum*, *S. chromogenes*, *S. haemolyticus*, *S. simulans* and *S. sciuri* were the most prevalent species in subclinical mastitis milk samples, all these species were found in the present study. Another study in the United States carried out by Gillespie *et al.* (2009) showed that *S. chromogenes* and *S. epidermidis* were the most prevalent species in dairy herds.

Jenkins *et al.* (2019) in several dairy herds in the United States showed that the most prevalent species of CNS present in that country were *S. chromogenes* followed by *S. haemolyticus*, *S. simulans*, *S. epidermidis*, *S. hominis*, *S. auricularis*, *S. sciuri*, *S. devriesei*, *S. capitis*, *S. cohnii* and *S. warneri*, results that were similar to those reported here.

In 89 Canadian herds, Fry *et al.* (2014) reported that 46% of the samples were positive for one of the 20 species of CNS. These results are lower compared to the results showed here. From those isolations, *S. chromogenes*, *S. simulans*, *S. haemolyticus*, *S. epidermidis* and *S. xylosum* were the most prevalent, results that were similar to those reported here. This similarity showed that CNS had parallel behavior in Colombia compared to North American countries.

However, results by De Freitas *et al.* (2013) in Brazil determined that *S. hyicus*, *S. warneri* and *S. epidermidis* were the most prevalent species in dairy herds in São Paulo, results that were different from those described here, principally related to *S. warneri*. Similarly, Costa *et al.* (2008) studied IMI caused by CNS in Brazilian

herds located in the states of São Paulo and Pernambuco. They reported that *S. warneri*, *S. hyicus*, *S. chromogenes*, and *S. epidermidis* were the most prevalent.

In Argentina, Raspanti *et al.* (2016) determined the prevalence of CNS in dairy herds, showing that *S. chromogenes*, *S. haemolyticus*, *S. warneri*, *S. xylosus*, *S. simulans* and *S. epidermidis* were the principal pathogens present in Argentinian dairy cows, results similar to those found here.

All these studies showed that regardless of the country the species that can infect the mammary gland were similar, and that this similarity can be due to similar management practices between herds evaluated. Also, in several countries management control can be used to combat the pathogens to achieve similar results.

Various factors are associated with pathogenicity, sustainability and spreading of CNS in cows. Factors such as hemolytic and proteolytic activity, higher tolerance to post milking teat disinfection, and the ability to evade immune mechanisms might provide colonization advantages for some species like *S. chromogenes* and *S. haemolyticus* in the mammary gland (Bochniarz & Wawron 2012; Hosseinzadeh & Dastmalchi 2014; Piessens *et al.* 2012; Quirk *et al.* 2012).

With respect to SCC, the statistical analyses showed that the species *S. epidermidis*, *S. chromogenes*, *S. sciuri*, *S. simulans* and *S. haemolyticus* have not statistically differences between the average geometric count of SCC determined for each species, which means that the increases in SCC are similar when any of these pathogen are present in mammary glands. However, not all species generated a similar SCC average (table 1).

The SCC results found here for *S. epidermidis*, *S. chromogenes*, *S. sciuri*, *S.*

simulans and *S. haemolyticus* were higher than the obtained by Thorgberg *et al.* (2009), who determined values of SCC for milk samples of persistent or non persistent subclinical mastitis with the same pathogens, 223×10^3 cells/ml and 189×10^3 cells/ml, respectively. But our results were lower compared to the results of Dufour *et al.* (2012), who found for *S. chromogenes* a SCC of 277×10^3 cells/ml; for *S. haemolyticus*, 662×10^3 cells/ml; and for *S. simulans*, 525×10^3 cells/ml. These differences may be due principally to factors associated with cow immunity, and the quantity of CFU chosen to determine a positive case (1000 CFU/mL), while in this study it was 500 CFU/mL.

It has been shown that *S. epidermidis*, *S. chromogenes* and *S. simulans* generally induced a mild to strong inflammatory reaction as measured by CMT (CMT scores 3-5), whereas persistent IMI with others CNS species, including *S. xylosus* and *S. haemolyticus*, mostly had a low CMT score (1-2) (Vanderhaeghen *et al.* 2014). Our findings were different to those proposed by Vanderhaeghen *et al.* (2014) due to the pathogens mentioned above in our study, which show CMT scores between trace and 1, and these were related to those mentioned by Taponen *et al.* (2006), who said that these pathogens can cause subclinical and clinical mastitis in similar proportions.

Sampimom *et al.* (2009) found lower results with respect to SCC related with *S. chromogenes*, *S. epidermidis* and *S. capitis* of 192, 132, and 187×10^3 cells/ml, respectively. However, for *S. xylosus* the results were 400×10^3 cells/ml, values that were higher compared to our results. These results were similar to those mentioned by Supré *et al.* (2011), who mentioned that *S. simulans* and *S. xylosus* can had a

substantial effect on milk SCC, an effect comparable to that of *S. aureus*.

In their study, Fry *et al.* (2014) obtained similar results related to the principal pathogenic species (*S. chromogenes*, *S. simulans*, *S. epidermidis*, *S. xylosus*, *S. capitis* and *S. haemolyticus*). They did not find statistical differences between SCC in milk samples of dairy herds in the USA, and they mentioned that these pathogens are primarily of environmental origin, and therefore these species may be acting as opportunistic mastitis pathogens.

An increased prevalence of CNS IMI is associated with several cow and herd level factors, but not with any specific milking or housing system (Taponen *et al.* 2016). It has been demonstrated that the principally source of CNS was the environment (Piessens *et al.* 2011), Nagase *et al.* (2002) demonstrated that CNS like *S. warneri*, *S. hominis* and *S. capitis* can be found in dairymen, which means that these can be a potential source for colonization of these species in bovine udder tissue, as these species are commonly found living on the skin and the mucous membranes of humans. Also, Thorgberg *et al.* (2009), who used the same technique as in the present study, found *S. epidermidis* in milk, and the milker's skin, which indicated that this pathogen may be transmitted from the milkers to cows.

CONCLUSIONS

There were determined 11 species of CNS in IMI in cows from Boyacá, Colombia, and the most prevalent species were *S. chromogenes*, *S. simulans*, *S. epidermidis*, *S. xylosus*, *S. capitis* and *S. haemolyticus*. All of this species have an SCC lower than 250×10^3 cells/ml. The results found here are similar to results in different parts

of the world, which confirms that they are pathogens that must be constantly evaluated because they can go unnoticed in routine controls, especially in those farms where major pathogens are not a serious problem. The results determined in this study demonstrate that the CNS generates a slight increase in somatic cells.

Conflict of Interest

The authors declare no conflicts of interest.

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