http://dx.doi.org/10.1590/1678-4162-12271

Arq. Bras. Med. Vet. Zootec., v.74, n.4, p.563-575, 2022

Biofilm production by *Staphylococcus* spp. isolated from bovine mastitis in dairy herds in state of Acre, Brazil and its implications

[*Produção de biofilme por* Staphylococcus *spp. isolados de mastite bovina, em rebanhos leiteiros do estado do Acre, Brasil, e suas implicações*]

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ABSTRACT

This study aimed to identify the *Staphylococcus* species responsible for bovine mastitis in dairy herds in northern Brazil, to investigate the capacity of biofilm production, and to analyze the association of biofilm production with multiresistance and intensity of California Mastitis Tests (CMT) reactions that can make treatment more difficult and cause misdiagnoses, respectively. Milk samples were collected from 23 dairy farms located in five municipalities in the state of Acre. A total of 339 crossbred cows were tested by CMT, with 109 animals (229 udder ceilings) reacting to the test. After bacterial isolation in blood agar, the catalase-positive and gram-positive cocci were submitted for identification by MALDI-TOF MS. Of 103 strains identified as staphylococci, *Staphylococcus chromogenes* (58.3%) and *Staphylococcus aureus* (19.4%) were the most prevalent species. Biofilm production was quantitatively evaluated using a microplate adherence test. Among the *Staphylococcus* strains, 71.8% were biofilm producers. Most strains of *S. chromogenes* (68.3%) had the capacity to produce biofilms, ranging from weak (43.3%), moderate (13.3%), and strong (11.7%) producers. Among *S. aureus* strains, 50% were non-biofilm producers, and none were strong producers. Our data showed an association between biofilm production capacity and multidrug resistance. In addition, there was a reduction in the response to the CMT test, which can mask the diagnosis.

Keywords: biofilm, bovine mastitis, *Staphylococcus chromogenes*, *Staphylococcus aureus*, multidrug resistance

RESUMO

O presente estudo teve como objetivos identificar as espécies de estafilococos envolvidas com mastite em rebanhos leiteiros no norte do Brasil, investigar a capacidade de produção de biofilme e analisar a associação da produção de biofilme com multirresistência e intensidade de reações de CMT que podem dificultar o tratamento e causar erros de diagnósticos, respectivamente. Amostras de leite foram coletadas em 23 propriedades leiteiras, situadas em cinco municípios do estado do Acre. Um total de 339 vacas mestiças foram testadas pelo CMT, sendo detectados 109 animais (229 tetos) reagentes ao teste. Após isolamento em ágar sangue, os cocos Gram positivos produtores de catalase foram submetidos à identificação por MALDI TOF MS. Um total de 103 estirpes foram identificadas como estafilococos, sendo mais prevalentes as espécies Staphylococcus chromogenes *(58,3%) e* S. aureus *(19,4%). A produção de biofilme foi avaliada quantitativamente pelo teste de aderência em microplaca. Dentre todas as amostras de estafilococos, 71,8% foram produtoras de biofilme. A maioria das amostras de* S. chromogenes *(68,3%) apresentou capacidade de produzir biofilme, variando de fraco (43,3%), moderado (13,3%) a forte (11,7%) produtor. Entre as amostras de* S. aureus*, 50% não foram produtoras e nenhuma foi produtora forte. Essa capacidade de produção de biofilme mostrou-se associada à multirresistência a antimicrobianos, além de diminuir a intensidade no teste CMT, podendo mascarar o diagnóstico.*

Palavras-chave: biofilme, mastite bovina, Staphylococcus chromogenes, Staphylococcus aureus, *multirresistência*

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Submitted: December 20, 2020 Accepted: January 12, 2022.

INTRODUCTION

Bovine mastitis is an important disease in dairy herds worldwide, causing losses in milk production (Langoni *et al*., 2011; Hoogeveen *et al.*, 2011). *Staphylococcus* spp. stand out as an etiologic agent of this pathology, and the *Staphylococcus aureus* species is observed in most cases (Pyörälä and Taponen, 2009; De Vliegher *et al*., 2012). Although considered less pathogenic, intramammary infections caused by other species of the genus have also been associated with considerable losses in milk production and tend to persist throughout the production period (Fry *et al*., 2014b).

The role of various virulence factors has been studied for a long time to better understand the ability of these bacteria to persist and spread in the host, as well as in the expression of severe disease and the interference of antimicrobial therapy (Girardini, 2013; Marques *et al*., 2013). A variety of bacteria have the capacity to produce biofilms, which consist of an agglomeration of bacterial cells embedded in a heterogeneous extracellular matrix. Bacteria in biofilms have specific physiological characteristics, are protected from the action of macrophages, and are more resistant to various antimicrobials (Marques *et al*., 2013).

Strains multiplied in the form of biofilms differ *in vivo* in their ability to spread in herds, cause a decrease in immune response, and cause clinical mastitis or persistent infections, in addition to losses in milk production (Israel *et al*., 2018). Regarding the biofilm production of *Staphylococcus* spp. isolated from bovine mastitis, studies have usually focused on the species *S. aureus* (Melchior *et al.*, 2011; Girardini, 2013). The analysis of species other than *S. aureus* isolated from mastitis is important for understanding the disease and identifying potential targets for new therapies.

In our previous study, *Staphylococcus chromogenes* were identified as a mastitis agent among *Staphylococcus* species in herds investigated in the state of Acre, in the northern region of Brazil (Israel *et al*., 2018). Investigations on the prevalence and characterization of the etiologic agents of bovine mastitis have been mainly performed in South and Southeast regions. Although *S. chromogenes* is a known agent of mastitis, this species is still poorly studied.

Therefore, this study aimed to identify the *Staphylococcus* species responsible for bovine mastitis in a greater number of herds in the state of Acre, northern Brazil, to investigate the capacity of biofilm production, and to analyze the association of biofilm production with multiresistance and intensity of CMT reactions that can make treatment more difficult and cause misdiagnoses, respectively.

MATERIAL AND METHODS

The study was approved by the Ethics Committee on the Use of Animals in Education Hand Research (CEUA) of the Federal University of Acre (UFAC), under identification number 61/2015.

Milk samples were collected from 23 dairy farms located in the state of Acre, northern Brazil, from November 2015 to September 2018. The farms were selected by non-probabilistic sampling due to ease of access through the roads and availability of the producers.

Milk collection was performed aseptically from individual mammary quarters with clinical signs and altered milk or CMT positivity. The CMT results for each mammary quarter were classified according to the intensity of the reaction: negative (0), mild (+), moderate (++), and intense (+++) (Fonseca and Santos, 2000).

Milk samples were subjected to bacterial culture on blood agar (Laboclin, Pinhais, PR, Brazil) for 24 h at 37ºC (NMC, 2004). Positive-catalase and Gram-positive isolates were identified by the MALDI-TOF technique (Matrix Associated Laser Desorption-Ionization - Time of Flight - Mass Spectrometry), on the MALDI Biotypes 3.1 equipment (Bruker Daltonik, Bremen, Germany) (Pasternak, 2012).

The determination of antimicrobial resistance was determined in a previous study by the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2018). The following antimicrobials were tested: penicillin G, cefoxitin, ceftiofur, enrofloxacin, ciprofloxacin, gentamicin, streptomycin, clindamycin, tetracycline, erythromycin,

rifampicin, chloramphenicol, and linezolid. Cefoxitin disks were used to detect methicillin-resistant *Staphylococcus* spp. (MRS). Isolates resistant to three or more antimicrobial classes and MRS are considered multidrug resistant (MDR) (Magiorakos *et al*., 2012). In this study, these data were used to analyze the association between biofilm production and MDR.

Biofilm production was quantitatively evaluated using the microplate adherence test, with modifications (Christensen *et al*., 1985; Cucarella *et al*., 2001). The bacteria were inoculated at a concentration of 1 CFU/mL in tryptic soy medium (TSB; Kasvi, Curitiba, PR, Brazil) and incubated at 37ºC for 24h. Subsequently, the cells in suspension were inoculated in sterile polystyrene microplates with 96 wells, diluted in TSB (1:40), and incubated for another 24h without shaking. After incubation, the wells were washed twice with 200 µL of sterile saline solution, dried in an oven at 60ºC and left until the plate was dried for 30 min. An aliquot of 200µL of 1% crystal violet was added and plates were stained for 15 min. The wells were then washed three times with distilled water and dried at room temperature. The absorbance was determined at 490 nm using an ELISA reader (Polaris, Celer Biotecnologia, Belo Horizonte, MG, Brazil). Uninoculated wells containing only TSB served as negative controls. The readings were performed in triplicate, and the optical density (OD) values were taken as the average of the readings. The strains were classified into four categories according to the average OD related to the results obtained for the negative control (ODNC) (Peña and Uffo, 2013). The categories were based on the following criteria: non-adherent (NA), OD ≤ ODNC, weakly adherent (+) when ODNC<OD≤2×ODNC, moderately adherent (++), when 2 × ODNC < OD ≤ 4 × ODNC or strongly adherent (+++), when 4 × ODNC < OD.

The results of the experiments were analyzed using descriptive statistics based on the distribution of the relative and absolute frequencies. The results were analyzed using Fisher's exact test to investigate the association between biofilm production and multidrug resistance and the G test to investigate the association between biofilm production and the CMT test (Zar, 2010). A 95% confidence interval (CI) was calculated.

RESULTS AND DISCUSSION

Of 339 cows, 109 (32.2%) animals distributed in 21 herds were diagnosed with mastitis, with most cases being subclinical (n: 105; 96.3%). A total of 1340 mammary quarters were examined, of which 17.1% (n = 229) had an inflammatory process: 1.2% (n = 16) with clinical process and 15.9% (n = 213) with subclinical.

In southern Brazil, Kozerski *et al*. (2014) observed a bovine mastitis rate (33%) like that observed in our study. A lower frequency (20.2%) was reported by another study that investigated herds in the northern region of the country, with 4.6% being clinical mastitis and 15.6% subclinical (Oliveira *et al*., 2011). Considering mammary quarters, the clinical mastitis rates vary in different studies performed in Brazil. In the southern region, similar rates were found by Ribeiro *et al*. (2006) (1.2%) and Oliveira *et al*. (2011) (1.3%). In contrast, Pinheiro *et al*. (2009) and Martins *et al*. (2010) detected higher rates of 7.0% (southeastern region) and 5.8% (Midwest region), respectively. According to Fonseca and Santos (2000), the internationally accepted level per herd for clinical mastitis is less than 1.0% and that for subclinical mastitis is less than 15%. Therefore, the prevalence of mastitis observed in our study was higher than the rates considered tolerable for this disease.

Staphylococcus spp. (n = 103) were isolated from 56.9% (n = 62/109) of the cows and 38.9% (n = 89/229) of the mammary quarters with mastitis. From some mammary quarters, more than one bacterial colony with different phenotypic characteristics was identified by MALDI-TOF. Among the 103 *Staphylococcus* isolates, the following species were identified: *S. chromogenes* (n = 60; 58.2%), *S. aureus* (n = 20; 19.4%), *S. hyicus* (n = 8; 7.8%), *S. saprophyticus* (n = 6; 5.8%), *S. haemolyticus* (n = 4; 3.9%), *S. epidermidis* (n = 3; 2.9%), *S. kloosii* (n = 1; 1%), and *S. xylosus* (n = 1; 1%). *S. chromogenes* was the prevalent species and was detected in most of the farms (n = 18/23) included in the study, followed by *S. aureus* (n = 7/23). The distribution of species involved in cases of bovine mastitis and the number of affected cows, as well as the number of mammary quarters, in the different properties investigated are shown in Table 1.

Table 1. Distribution of *Staphylococcus* species isolated from bovine mastitis among the different dairy farms

Farm	Number of the cows (teats) with *Staphylococcus*				
	S. chromogenes (n: 60)*	*S. aureus* (n: 20)*	*S. hyicus* (n: 8)*	*S. saprophyticus* (n: 6)*	Others (n: 9)*
A	1 (1)	1 (1)	-	-	-
B	1 (1)	-	-	-	-
C	3 (4)	-	-	1 (1)	1 (2)[a]
E	3 (5[+])	-	-	1 (1)	-
F	4 (7)	-	-	2 (3[+])	-
G	2 (3)	-	-	-	-
H	3 (3[+])	-	2 (3)	-	2 (2)[b, c]
I	2 (3)	-	1 (1)	-	4 (4)[d]
J	8 (10[++])	-	1 (1[+])	-	1 (1)[a]
K	4 (7)	2 (2)	1 (2)	-	-
L	-	4 (5[+])	-	-	-
M	1 (1)	1 (4)	-	-	-
N	2 (2)	-	-	-	-
O	1 (1)	-	-	-	-
Q	2 (3)	1 (1[+])	-	-	-
R	1 (1)	-	-	-	-
S	1 (1)	3 (4)	-	-	-
U	1 (1)	-	-	-	-
W	1 (2)	1 (1)	-	-	-

*Number of isolates, [a]*S. epidermidis*, [b]*S. kloosii*, [c]*S. xylosus*, [d]*S. haemolyticus.*
[+] Isolated from the same species on the same teats, but phenotypically distinct

S. aureus has been the most prevalent species of the genus in cases of bovine mastitis in most studies (Rabello, 2007; Kozerski *et al.*, 2014; Duarte *et al.*, 2017). Kozerski *et al*. (2014) found 49.1% of *S. aureus* in southern Brazil. Marques *et al*. (2013) isolated *Staphylococcus* spp. in southeastern Brazil and found only 15.2% of *S. aureus* isolates.

In our study, *S. chromogenes* was the most frequently isolated species and was broadly distributed in the farms investigated. However, the *S. chromogenes* frequency rate was not higher than the *S. aureus* frequency rate in herds where both pathogens were the cause of mastitis.

Originally, *S. chromogenes* was considered a subspecies of *S. hyicus*, but it was later classified as a separate species (Hajek *et al*., 1986). It has been reported as coagulase-negative staphylococci (CoNS) species most frequently associated with mastitis in dairy cattle in several studies and is associated with persistent infections (Tomazi *et al*., 2014; Srednik *et al.*, 2015). According to Zhang and Maddox (2000), *S. chromogenes* can cause bovine mammary infections more severely than other CoNS. In the study by Myllys *et al*. (1994), there was no significant difference in the parameters of inflammation between infections caused by *S. aureus* and *S. chromogenes*.

Studies have demonstrated the spread of *S. chromogenes* in cattle herds from different geographical areas, including Brazil (Tremblay *et al.*, 2013; Fry *et al.*, 2014b; Tomazi *et al.*, 2014; Lange and Brito, 2015; Srednik *et al*., 2015). Nevertheless, other CoNS species have a higher frequency than *S. chromogenes* in both clinical and subclinical mastitis (Bochniarz *et al*., 2014). In our study, *S. chromogenes* was prevalent only in the subclinical form of the disease. The increased frequency of this species may also have been observed due to the use of new diagnostic techniques such as MALDI-TOF. The use of new bacterial identification tools can increase the knowledge about the etiology and prognosis of bovine mastitis.

Among *S. chromogenes* isolates, 36.7% (n=22/60) showed the ability to coagulate rabbit plasma in the tube coagulase test. Reagent and non-reagent strains in the coagulase test were recovered simultaneously from some teats. Of

the coagulase-positive strains, 72.7% (n=16/22) were biofilm producers. Among coagulase-negative strains, 65.8% (n = 25/38) had the ability to produce biofilms. There was no association between coagulase-positive strains and biofilm production (p>0.7).

Although it is generally coagulase-negative, coagulase-positive *S. chromogenes* strains have been previously reported in other studies (Lange *et al.*, 2011; Santos *et al.*, 2016). In these strains, the plasma clotting activity may be caused by protease interference, but further studies are necessary to explain this. Coagulase-positive *S. chromogenes* strains can be misdiagnosed as *S. aureus* or other coagulase-positive staphylococci (CoPS). *S. chromogenes* are phylogenetically more like CoPS and coagulase-variable staphylococci than CoNS (Santos *et al*., 2016). In the *S. chromogenes* genome, an open reading frame with 41% identity with the coagulase gene of CoPS *Staphylococcus pseudintermedius* (Fry *et al.*, 2014a). These data reinforce the requirement for more discriminatory and accessible methodologies for different laboratories. MALDI-TOF reliably identifies several species of *Staphylococcus*, but its access is restricted to laboratories that have the equipment. Identification by MALDI-TOF of several species of *Staphylococcus* is reliable (Carpaij *et al*., 2011; Legarraga *et al.*, 2013). In the study by Tomazi *et al*. (2014), all *S. chromogenes* isolates were identified using this technique.

Most of the *Staphylococcus* spp. (n=74; 71.8%) isolates were classified as biofilm producers, with 47.6% (n=49/103) weak, 12.6% (n=13/103) moderate, and 11.7% (n=12/103) strong. Non-producing isolates were observed among the *S. chromogenes* (n=19/60; 31.7%) and *S. aureus* (n = 10/20; 50%) isolates. Except for *S. aureus*, most of the isolates from all species were classified as weak producers. Strong production was observed among *S. chromogenes*, *S. hyicus*, *S. saprophyticus*, and *S. kloosii* isolates (Table 2).

Table 2. Biofilm-producing ability and levels among *Staphylococcus* spp. isolates recovered from bovine mastitis in the dairy herds

Species	Number of strains (%) with biofilm-producing ability			
	Unable (0)	Weak (+)	Moderate (++)	Strong (+++)
S. chromogenes (n: 60)	19 (31.7)	26 (43.3)	8 (13.3)	7 (11.7)
S. aureus (n: 20)	10 (50.0)*	9 (45.0)	1 (5.0)	0
S. hyicus (n: 8)	0	5 (62.5)	1 (12.5)	2 (25.0)
S. saprophyticus (n: 6)	0	3 (50.0)	1 (16.7)	2 (33.3)
S. haemolyticus (n: 4)	0	4 (100)	0	0
S. epidermidis (n: 3)	0	2 (66.7)	1 (33.3)	0
S. kloosii (n: 1)	0	0	0	1 (100)
S. xylosus (n: 1)	0	0	1 (100)	0
Total	29 (28.2)	49 (47.6)	13 (12.6)	12 (11.7)

Biofilm production levels – (0): unable; (+): weak; (++): moderate; (+++): strong producer.
*p< 0.05

Both biofilm-producing and non-producing *Staphylococcus* spp. strains were isolated from the nine farms. Biofilm-producing strains were prevalent on properties, except for two farms (N and Q) (Figure 1). Considering only *S. chromogenes*, biofilm-producing strains were more distributed (n = 12/18) and prevalent (n = 5/8) than non-producing strains (Figure 2.A). Biofilm-producing *S. aureus* strains (n = 10/20; 50%) were less frequent than strains of other species (n = 64/83; 77%; p = 0.037) (Table 2). This may explain the lower spread of this species. However, biofilm-producing *S. aureus* strains were prevalent in relation to non-producing strains in only one (farm M) of the herds where this pathogen caused mastitis (Figure 2.B).

Both *S. chromogenes* and *S. aureus* were isolated from animals on the six farms. Although *S. aureus* is considered the most prevalent cause of bovine mastitis, in our study, *S. chromogenes* was found in higher proportion (Figure 3).

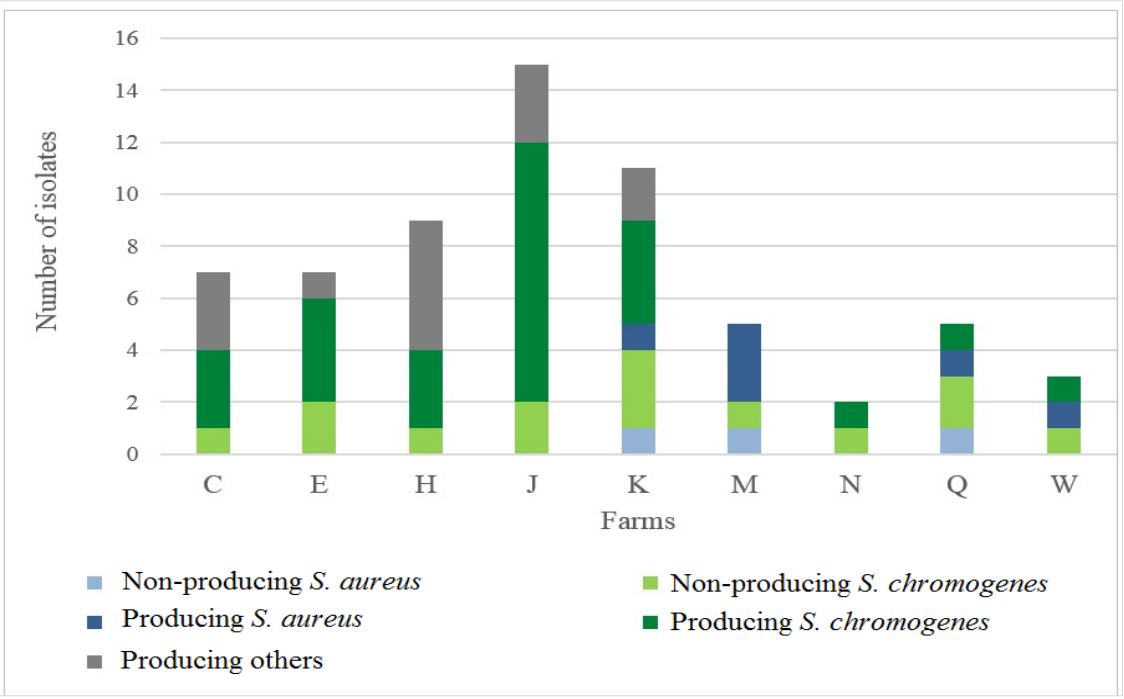

Figure 1. Distribution of *Staphylococcus* spp. strains with different biofilm production levels on farms where both non-producers and producers were isolated.

S. chromogenes and *S. aureus* strains with different biofilm production levels were distributed in different herds and within the same herd (Figure 2).

Bochniarz *et al*. (2014) evaluated the biofilm production of CoNS strains isolated from cows with mastitis and found 38.5% of the *S. chromogenes* strains with this capacity. In another study conducted by this group in 2016, the ability to produce biofilms was confirmed in 63.2% of the *S. chromogenes* strains. Furthermore, biofilm-producing *S. aureus* strains were not predominant in relation to *S. chromogenes* strains, independent of the production capacity, in most herds where these two species were responsible for mastitis (Figure 3). Biofilm production *in vitro* may not reflect what occurs *in vivo* because of potential environmental effects on genetic regulation pathways (Marques *et al*., 2013).

The isolation rates of biofilm-producing *Staphylococcus* spp. have varied in different studies evaluating strains recovered from bovine mastitis. Marques *et al*. (2013) and Noel *et al*. (2016) observed that 76.8% and 74.4% of the strains were biofilm producers, respectively. Higher frequencies (96.7%) were observed by Srednik *et al*. (2015) (85.1%) and Tremblay *et al*. (2013) using a similar test method. On the other hand, Simojoki *et al*. (2012) observed that most strains were non-biofilm producers (68.7%). *S. chromogens* was the species with the lowest number of strains capable of producing biofilms. In the study by Tremblay *et al*. (2013), biofilm-producing *S. xylosus* strains were the most frequent, followed by *S. haemolyticus*, while *S. epidermidis* strains were the least frequent. The observed differences can be explained by the distribution of CoNS species in different countries. In addition, intra-species variation is not uncommon and has been observed for *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, and *S. hominis* (Tremblay *et al*., 2013; Oliveira *et al*., 2015). Ajitkumar *et al*. (2013) identified three distinct genotypes among *S. chromogenes* strains, which may explain the variation in biofilm production in this species.

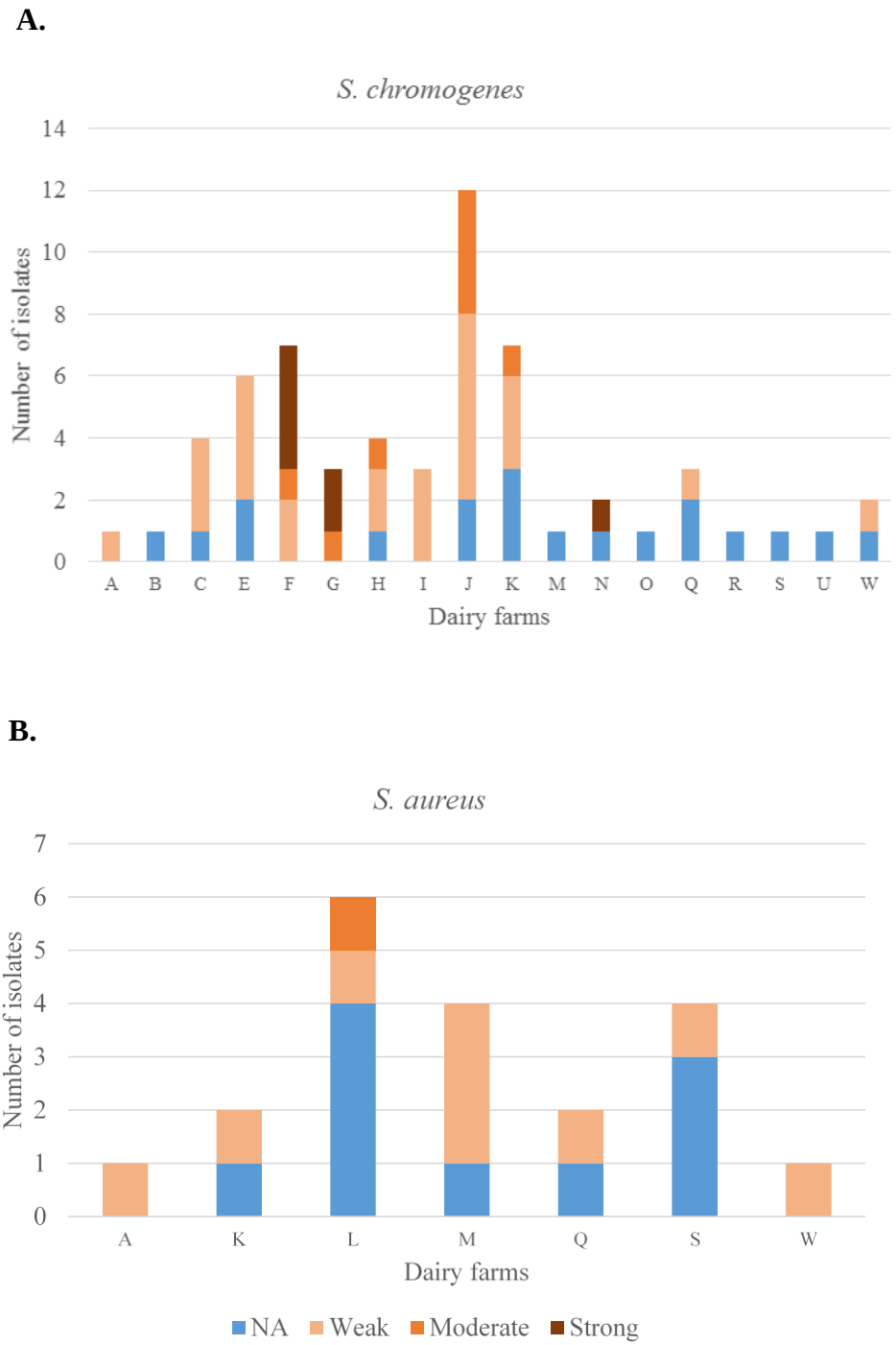

Figure 2. Distribution of *S. chromogenes* (A) and *S. aureus* (B) isolates that not producing and producing biofilm with different degrees in dairy farms. NA: non-adherent (non-producer).

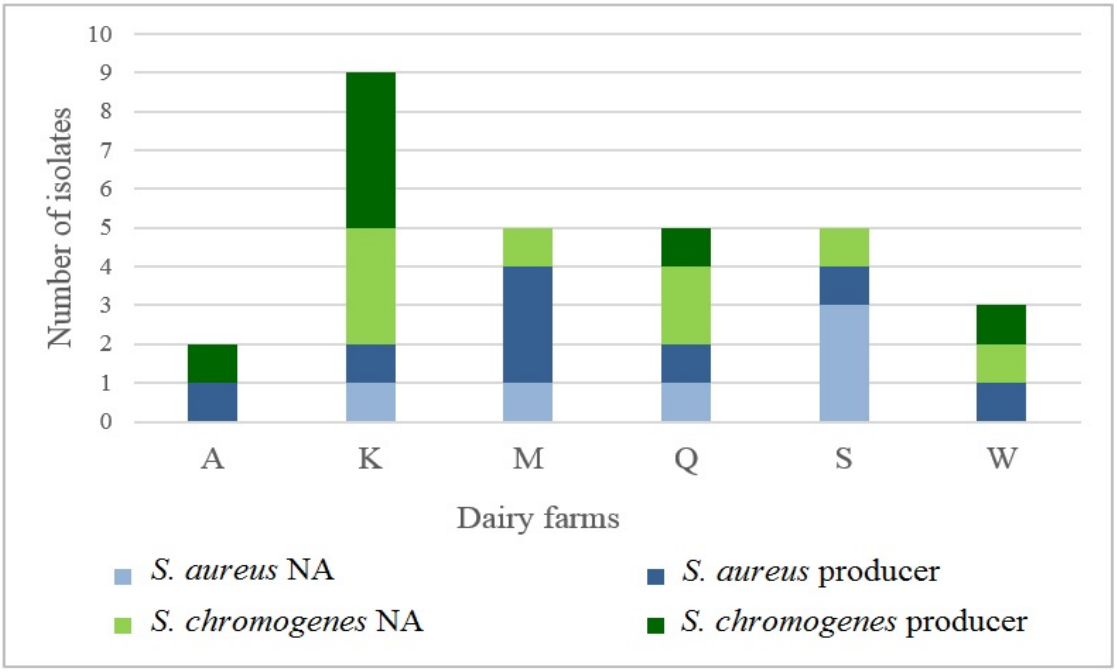

Figure 3. Distribution of biofilm non-producing and producing *S. chromogenes* and *S. aureus* isolates among dairy farms where both species caused mastitis. NA: non-adherent (non-producer).

The antimicrobial resistance profiles of *Staphylococcus* isolates were determined in a previous study by this group. MDR strains (n = 19/103; 18.4%) were isolated from intramammary infections in different farms. Of these strains, 79% (n = 15/19) were MRS. Most MDR strains, including MRS, were of the species *S. chromogenes* (n = 12/19, 63.2%) (Table 3). Among the *S. chromogenes* strains, 20% (n = 12/60) were MDR with resistance ranging from four to eight antimicrobial classes. The occurrence of MDR strains isolated from mastitis is worrying because it can have an impact on public health and veterinary medicine.

The association between biofilm production (different levels) and multidrug resistance was tested using Fisher's test (Table 4). Fisher's test shows where the biofilm intensities have significant differences (between the observed and the expected), in this case it was "NA" and "+". Thus, it is understood that when all strains of *Staphylococcus* spp. are evaluated, in "NA" strains there is no multidrug resistance. When the formation of biofilm was weak "+", the strains tended not to be multidrug resistant, whereas strains with moderate "++" and intense "+++" biofilm formation were shown to be equal in regard to the chances of finding MDR strains or non MDR strains. Among the *S. aureus* strains, only one was multidrug resistant, and the one with the highest intensity of biofilm production "++". These results are interesting to demonstrate, even with a small percentage of MDR strains (n = 19/103; 18.4%), they were concentrated in strains with moderate to intense biofilm formation capacity.

Bacterial biofilms are one of the factors that contribute to the failure of bovine mammary gland infections (Chagas, 2015). An association was observed between biofilm production levels and the presence or absence of multidrug resistance among *S. chromogenes* strains (Table 5). This species was the most prevalent mastitis agent and represented the majority of MDR strains (n = 12/19; 63.2%). The p-value in the table indicates the significance of the Fisher's exact test. Assuming equality in the expected values of 'no' and 'yes' multiresistance in each biofilm-producing level, it is seen that the differences was significant in only "NA". In biofilm-producing *S. chromogenes* isolates, regardless of the production level, there is a probability of finding MDR and non-MDR strains, suggesting that biofilm-producing strains have greater resistance to antimicrobials than non-biofilm producers. These two virulence

factors are related, both of which can contribute to treatment failure and facilitate their dissemination. The clonal relationship of these strains may explain the association observed in our study. However, molecular methodologies must be used to answer this question.

Table 3. Distribution of multidrug resistant (MDR) and methicillin-resistant *Staphylococcus* spp. (MRS) strains isolated from mastitis among dairy herds

Farm	Year	Species	
		MDR (n: 19)	MRS (n:12)
C	2016	*S. chromogenes* (1) *S. epidermidis* (1)	*S. chromogenes* (1) *S. epidermidis* (1)
E	2018	*S. chromogenes* (2)	*S. chromogenes* (2)
F	2018	*S. chromogenes* (2) *S. saprophyticus* (1)	*S. chromogenes* (1) *S. saprophyticus* (1)
G	2018	*S. chromogenes* (1)	-
H	2018	*S. chromogenes* (1) *S. hyicus* (1) *S. xylosus* (1)	*S. hyicus* (1) *S. xylosus* (1)
I	2018	*S. chromogenes* (1) *S. haemolyticus* (1)	*S. chromogenes* (1) *S. haemolyticus* (1)
J	2018	*S. chromogenes* (4) *S. epidermidis* (1)	*S. chromogenes* (4) *S. epidermidis* (1)
L	2018	*S. aureus* (1)	-

MDR: multidrug resistant (resistant to $\geq$ 3 antimicrobial classes and MRS); MRS: Methicillin- resistant *Staphylococcus.*

Table 4. Relationship between biofilm production levels by *Staphylococcus* spp. and multidrug resistance

Biofilm	Multiresistance		P (Fisher test)
	No	Yes	
NA	29	0	0.0000
+	39	11	0.0064
++	6	6	1.0000
+++	10	2	0.1900

NA: nonadherence (non-producer); + (weak); ++ (moderate); +++ (strong).

Table 5. Relationship between biofilm production levels by *S. chromogenes* and multidrug resistance

Biofilm	Multiresistance		P (Fisher test)
	No	Yes	
NA	19	0	0.001
+	19	7	0.153
++	5	3	1.000
+++	5	2	1.000

NA: non-adherence (non-producer); + (weak); ++ (moderate); +++ (strong).

It is important to note that all MDR strains were biofilm producers to some degree. The concentration of antimicrobials required to eliminate biofilm-producing bacteria can be up to 1000 times greater than that needed to eliminate the same species in a planktonic state (Van Praagh *et al*., 2011; Hook *et al*., 2012).

The resistance mechanisms of biofilms are not yet fully understood and are usually multifactorial. It is important to test the antibiofilm activity of antimicrobials that are potentially useful for treating infections associated with biofilms. According to Stypulkowski (2014), standardized assays and parameters such as minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), usually determined with cells in suspension, are inadequate to assess antimicrobial activity against sessile cells in biofilms. The ideal in situations where there is the possibility of biofilm formation is the determination of the concentration of antimicrobial necessary to inhibit the growth of cells in the biofilm (MBIC - Minimum Biofilm Inhibitory Concentration), therefore, it is of great importance to develop studies which evaluate the activity of antimicrobials against *Staphylococcus* spp. surrounded by biofilms in search of new alternatives to treat these infections.

Table 6 shows the CMT reaction intensity of cows infected with distinct *Staphylococcus* species. Most strains were isolated from cows that exhibited mild reactions during the test. CMT is one of the most used tests for the diagnosis of subclinical mastitis. It is an indirect indicator of somatic cell counts (mainly leukocytes) in milk. The positive reaction intensity varies among mild (+), moderate (++), and intense (+++), according to the inflammatory reaction (Fonseca and Santos, 2000).

The mechanism by which the host interacts with the polysaccharides of the bacterial biofilm remains poorly understood. Among the few studies, it appears to be highly complex (Watters *et al*., 2016). Several studies have shown that certain bacterial exopolysaccharides can inhibit the general immune response to biofilms (Raffatellu *et al*., 2005; Bylund *et al*., 2006; Murofushi *et al*., 2015). In addition to the inhibition of innate immune cells, such as neutrophils and macrophages, certain biofilm exopolysaccharides have been shown to inhibit complement activation (Pier *et al*., 2001). In summary, exopolysaccharides associated with biofilms may play a vital role in bacterial evasion. This fact may explain the results observed in our study in relation to biofilm-producing levels by *S. chromogenes* and the CMT reaction intensity (Table 7).

The same approach was used with data referring to *S. chromogenes*. In this case, the association between CMT reaction intensity and biofilm production level was significant (G test; $p < 0.05$). (Table 7). The difference in these results can be explained by the fact that some milk samples from mammary quarters with determined CMT reaction had different Staphylococcus species with different levels of biofilm production (103 Staphylococcus strains in 89 mammary quarters); therefore, analyses of all Staphylococcus species would not be of interest. In addition, the association of Staphylococcus strains (except *S. chromogenes*) is difficult because the number of these species is low.

Table 6. Degree of CMT reaction between *Staphylococcus* spp. isolated from teats with bovine mastitis in dairy herds

Species	CMT		
	+	++	+++
S. chromogenes (n:60)	35 (58.3%)	10 (16.7%)	15 (25.0%)
S. aureus (n:20)	12 (60.0%)	7 (35.0%)	1 (5.0%)
S. hycus (n:8)	3 (37.5%)	2 (25.0%)	3 (37.5%)
S. saprophyticus (n:6)	2 (33.3%)	3 (50.0%)	1 (16.7%)
S. haemolyticus (n:4)	2 (50.0%)	1 (25.0%)	1 (25.0%)
S. epidermidis (n:3)	3 (100%)	0	0
S. kloosii (n:1)	0	1 (100%)	0
S. xylosus (n:1)	0	0	1 (100%)
Total (n:103)	57 (55.3%)	24 (23.3%)	22 (21.4%)

CMT reaction intensity: + (mild), ++ (moderate), +++ (intense).

Table 7. Relationship between the intensity of *S. chromogenes* biofilm-producing levels and CMT reaction intensity.

Biofilm	CMT			Total
	+	++	+++	
NA	11	4	4	19
+	15	1	10	26
++	3	4	1	8
+++	6	1	0	7
Total	35	10	15	60

Teste-$G_{0,05;6}$ = 14.74; P = 0.02; CMT reaction intensity: + (mild); ++ (moderate); +++ (intense). NA: non-adherence (non-producer); + (weak); ++ (moderate); +++ (strong).

The data in Table 7 show that there is an inverse relationship between the intensity of the CMT reaction and the level of biofilm production. This result is an important finding, suggesting that biofilm-producing strains may interfere with CMT results. In comparison to planktonic growth, biofilms secrete less pro-inflammatory factors, protect against phagocytes, and attract myeloid-derived suppressor cells (MDSCs) (Otto, 2018).

CONCLUSIONS

S. chromogenes was the species most frequently isolated among the farms investigated, demonstrating its importance as an etiological agent of bovine mastitis. Most *Staphylococcus* spp. strains were biofilm-producing at different levels. An inverse relationship between the intensity of biofilm-producing *S. chromogenes* and CMT reaction intensity was found. Furthermore, the MDR strains exhibited moderate to intense biofilm-forming capacity. Infections caused by strains with these two characteristics have a higher risk of treatment failure. The large number of *S. chromogenes* strains able to produce biofilms isolated from bovine mastitis can lead to a change in the prevalence of etiological agents. The significant number of *S. chromogenes* strains with important virulence factors, such as biofilm production and multidrug resistance associated with a lower reaction in the CMT test is an important finding as it suggests that biofilm-producing strains make treatment difficult and may mask field diagnosis, demonstrating that these characteristics help this species to stand out and spread to a greater degree through herds.

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