https://doi.org/10.22319/rmcp.v12i4.5645

Article

Relationship between antibiotic resistance and biofilm production of *Staphylococcus aureus* isolates from bovine mastitis

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Abstract:

The objective was to analyze the relationship between the antibiotic-resistance profile and the biofilm formation of *S. aureus* isolates from bovine mastitis. Thirty (30) isolates of *S.*

aureus from cases of subclinical mastitis in dairy farms in semi-intensive production and backyard production systems, located in the states of Guanajuato and Michoacán, Mexico, were analyzed. An antibiogram was performed by the Kirbi-Bauer disc-diffusion method. Biofilm formation was determined by the violet crystal staining method. For the evaluation of antibiotic resistance genes and biofilm formation, genomic DNA was obtained from a colony for the identification of the genes: *blaZ*, *mecA*, *tetK*, *tetM*, *gyrA* and *gyrB*, and *icaA* and *icaD*. The results showed that 100 % of the isolates were resistant to penicillin and dicloxacillin, followed by cefotaxime (86.6 %), ampicillin and cephalotin (83.3 %) and ceftazidime (80.0 %), while a 36.6 % resistance to oxacillin was observed. It was identified that all isolates of *S. aureus* had the ability to form biofilm with a range between 20 to 98 %. It was also observed that isolates with a high multi-resistance presented a greater formation of biofilm, establishing a significant positive correlation. In conclusion, *S. aureus* isolates from bovine mastitis presented high levels of antibiotic resistance; as well as an important biofilm-forming capacity, demonstrating the existence of a positive correlation between these two factors.

Key words: Antibiotics, Mastitis, DNA, Biofilm.

Received: 20/03/2020

Accepted: 12/03/2021

Introduction

The processing of bovine milk is a sector of utmost importance in the livestock industry, in Mexico, a production of more than 12 million tons was estimated in 2019[(1)], which places it within the top ten milk-producing countries worldwide[(1)]. One of the main goals of a dairy farm should be to have an efficient milk production and that it is healthy and free of contaminants, so it is essential that the mammary gland is healthy[(2)]. In this sense, mastitis is the most common and costly disease in dairy cattle, since it affects the welfare of the cow and causes economic problems due to losses in production, decrease in quality and quantity of milk, premature elimination of the cow, cost of veterinary treatment and the discarding of milk due to antibiotic contamination[(3,4)].

Staphylococcus aureus is a ubiquitous pathogen that causes a variety of infections in humans and animals and is one of the main causative agents of bovine mastitis[(5,6)]. This Gram-positive bacterium produces chronic, persistent and recurrent infections, since it is able to overcome all the barriers of the host defense system, because it has a wide spectrum of virulence factors such as the production of enzymes, antigens, adhesins and toxins, among others[(7)]. These

virulence factors eventually confer on the bacterium multi-resistance to antibiotics and the formation of biofilms[8]. The biofilm is a consortium of microorganisms that is embedded within a polymer matrix, consisting mainly of exopolysaccharides, proteins and nucleic acids, which allows the bacterium to adhere to a biotic or abiotic surface[9]. The formation of biofilm is a life strategy for most bacteria, since it provides them with stability, performs catalytic functions, increases the chances of transfer of genetic material and resistance to antibiotics, participates in cellular communication processes and offers protection to survive adverse and variable environmental conditions; which contributes to its successful colonization in the host[10]. Multi-resistance to antibiotics and the formation of biofilms are characteristics of virulence that are related to each other in an important way. In this sense, it is known that the biofilm formed by *S. aureus* significantly increases antibiotic resistance by inhibiting the penetration of the antimicrobial, which results in an increasingly serious situation in the therapeutic combat of this microorganism[11].

Currently, this research group has a collection of isolates of *S. aureus* from bovine mastitis that were collected in the states of Guanajuato and Michoacán, Mexico, which have presented very high levels of multi-resistance to antibiotics (70 to 100 %)[12], which is consistent with the low efficiency in the therapies used in the production units of the region. Unfortunately, in Mexico so far, the ability of these bacteria that cause bovine mastitis to form biofilms, and its possible relationship with antibiotic resistance levels, have not been evaluated. Based on the above, the objective of this work was to analyze whether there is a correlation between the antibiotic-resistance profile and the biofilm formation of *S. aureus* isolates from bovine mastitis.

Material and methods

Isolation of *S. aureus*

Thirty (30) isolates of *S. aureus* from cases of subclinical mastitis of cows located in dairy farms located in localities in the states of Guanajuato and Michoacán, Mexico, which use semi-intensive production and backyard production systems, were analyzed. The sampling, isolation and characterization of the isolates was already reported by Varela *et al* [12] in 2018.

Antibiotic multi-resistance profile

An antibiogram was performed by the Kirbi-Bauer disc-diffusion method[13], using Biorad® sensidiscs with the following antibiotics and concentrations: penicillin (PE) 6 µg, oxacillin (Oxa) 6 µg, dicloxacillin (DC) 30 µg, pefloxacin (PEF) 5 µg, cefuroxime (CXM) 30 µg, gentamycin (GE) 120 µg, cefotaxime (CTX) 30 µg, sulfamethoxazole + trimethoprim (SXT) 1.25 and 23.75 µg, tetracycline (TE) 30 µg, ampicillin (AM) 10 µg, erythromycin (E) 15 µg,

ceftazidime (CAZ) 30 µg and cephalotin (CF) 30 µg. The results are reported as sensitive, intermediate and resistant based on the parameters established in Performance Standards for Antimicrobial Susceptibility Testing, 2019[14]. Once the resistance profile was established, the 30 isolates were classified as described below: Group 1: High resistance (resistant to 11-13 antibiotics), Group 2: Medium resistance (9-10 antibiotics), Group 3: Low resistance (4-8 antibiotics).

Biofilm formation

To measure the biofilm-forming capacity of *S. aureus* isolates, the violet crystal staining protocol[11] was used, as described below: the bacterial isolate was cultured in LB medium and incubated 24 h at 37 ºC. The tests were performed on sterile plates of 96 wells and placed ≈ 1 x 10^6 CFUs in a final volume of 100 µL in each well. Isolates were incubated for 48 h at 37 °C.

Once the incubation time had elapsed, the supernatant was discarded, the wells were washed with 100 µL of PBS solution (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO_4) and the wells were dried. Subsequently, 100 µL of violet crystal solution (0.5 % weight/volume) was added to each well and it was left to stand for 15 min. The dye was then removed, and it was washed twice with 100 µL of PBS. Then, 125 µL of 95 % ethanol (volume/volume) was added and it was vigorously resuspended to dissolve the dye. The absorbance reading was taken at 495 nm in an ELIREAD microelisa analyzer (Kontrolab®, Guidonia, Italy). Once the data were obtained, the percentage of biofilm formation was plotted using as 100 % the registered absorbance of the certified strain of *S. aureus* (ATCC 27543). Three independent experiments with three repetitions were conducted.

Analysis of resistance genes and biofilm formation

To evaluate the presence of genes related to antibiotic resistance and biofilm formation, it was carried out from genomic DNA, which was obtained by chopping a bacterial colony from a fresh culture plate to later place it in the PCR reaction mixture. The oligonucleotides used in this study are shown in Table 1. The reaction was performed in a final volume of 20 µL containing 0.4 µM oligonucleotides, 200 µM deoxynucleotides triphosphates (Invitrogen, Carlsbad, California, United States), 2 mM magnesium chloride (Invitrogen, Invitrogen, Carlsbad, California, United States) and 1 U of Taq polymerase (Invitrogen, Invitrogen, Carlsbad, California, United States). The amplification conditions were as follows: initial denaturation temperature at 95 °C for 10 min, followed by 30 denaturing amplification cycles for 10 min at 94 °C, alignment for 1 min at the specific temperature of oligonucleotides (Table 1), polymerization for 30 sec at 72 °C, and a final extension cycle for 7 min at 72 °C. Amplicons (5 µL) were analyzed by 1 % agarose gel electrophoresis (weight/volume) and

stained with ethidium bromide. It was considered positive for the gene, the presence of an amplification band corresponding to the size of the expected product.

Table 1: Oligonucleotides (OLIG) used

OLIG	Sequence	AT (°C)	SEP (bp)	Reference
blaZ	5´-TAAGAGATTTGCCTATGCTT-3´ 5´-TTAAAGTCTTACCGAAAGCAG-3´	49	377	Yang *et al*., 2016[29]
mecA	5´-GTAGAAATGACTGAACGTCCGATGA-3´ 5´-CCAATTCCACATTGTTTCGGTCTAA-3´	62	310	Elhassan *et al*., 2015[27]
tetK	5´-GTAGCGACAATAGGTAATAGT-3´ 5´-GTAGTGACAATAAACCTCCTA-3´	49	360	Yang *et al*., 2016[29]
tetM	5´-AGTGGAGCGATTACAGAA-3´ 5´-CATATGTCCTGGCGTGTCTA-3´	49	158	Yang *et al*., 2016[29]
gyrA	5´-AATGAACAAGGTATGACACC-3´ 5´-ACGCGCTTCAGTATAACGC-3´	49	222	Hashem *et a*l., 2013[28]
gyrB	5´-CAGCGTTAGATGTAGCAAGC-3´ 5´-CCGATTCCTGTACCAAATGC-3´	49	250	Hashem *et al*., 2013[28]
icaA	5'-CCTAACTAACGAAAGGTAG-3´ 5'-AAGATATAGCGATAAGTGC-3'	50	1315	Dhanawade, 2010[49]
icaD	5'-AAACGTAAGAGAGGTGG-3' 5'-GGCAATATGATCAAGATAC-3'	50	381	Dhanawade, 2010[49]
nuc	5´-GACTATTATTGGTTGATCCACCTG-3´ 5´-GCCTTGACGAACTAAAGCTTCG-3´	54	218	Brakstad *et al*., 2002[50]

AT= alignment temperature; SEP= size of the expected product.

To analyze the genetic basis of bacterial resistance mechanisms, the presence of the *blaZ* and *mecA* genes for beta-lactam antibiotics was analyzed[15,16]; *tetK* and, *tetM* for tetracyclines[16] and *gyrA* and *gyrB* for quinolones[17].

Statistical analysis

Three independent experiments were carried out in which the absorbance produced by the staining of the formed biofilm was measured. The experiments were done in triplicate. The difference between the highest absorbance minus the largest amount of biofilm was obtained, this difference is defined in this study as absorbance.

The 30 isolates of *S. aureus* were classified into three groups according to the level of antibiotic resistance: high, medium and low, of 10 isolates in each and were subsequently

evaluated according to their absorbance. The results of the positive controls for each resistance level of each experiment were included in the analyses.

The normality of the dependent variable absorbance was evaluated using the Chi-square goodness of fit test, resulting in normal (P>0.05). Data were analyzed with an analysis of variance (ANOVA) with a factorial design with a completely randomized arrangement. The model used is shown below:

Yijk=μ + EXi + GRj + EXixGRj + eijk, where:

Yijk= is the k-th observation of absorbance, of the i-th experiment and the j-th degree of resistance,
μ=general mean as a constant parameter,
EXi= i-th experiment, j=1, 2 and 3,
GRj= j-th degree of resistance, i= 1, 2 and 3,
GRixEXj= interaction between the i-th degree of resistance and the j-th experiment,
eijk= experimental error.

Additionally, the Spearman rank correlation between degree of resistance and absorbance was estimated.

Results and discussion

Antibiotic-resistance profile of *S. aureus* isolates from bovine mastitis

The results show that 100 % of the isolates are resistant to penicillin and dicloxacillin, in addition, 86.6, 83.3 and 80.0 % show resistance to ampicillin, cephalotin and ceftazidime, respectively; it was also observed that 36.6 % of the isolates are resistance to oxacillin. Overall, all isolates analyzed were resistant to at least 33.3 % of the antibiotics tested (Table 2).

Table 2: Resistance profile of *S. aureus* isolates

Isolate		Antibiotic												
		PE	OXA	DC	PEF	CXM	GE	CTX	SXT	TE	AM	E	CAZ	CF
Group 1	1													
	2													
	3													
	4													
	5													
	6													
	7													
	8													
	9													
	10													
Resistant isolates, %		100	90	100	100	100	70	100	80	90	100	70	90	100
Group 2	1													
	2													
	3													
	4													
	5													
	6													
	7													
	8													
	9													
	10													
Resistant isolates, %		100	70	100	20	90	20	80	20	50	90	60	80	90
Group 3	1													
	2													
	3													
	4													
	5													
	6													
	7													
	8													
	9													
	10													
Resistant isolates, %		100	30	100	30	40	0	80	0	30	60	0	70	60
Total, %		100.0	36.6	100.0	50.0	76.6	70.0	86.6	33.3	56.6	83.3	43.3	80.0	83.3

Color code: black= isolates resistant; gray= isolates intermediate resistant; white= isolates sensitive.

Antibiotic resistance is a phenomenon that continues to increase and that significantly affects the health sector, both in human medicine and veterinary medicine, since it hinders the proper management of infectious diseases. Such is the case of bovine mastitis; *S. aureus*, as one of the main bacteria isolated from bovine mastitis, has high resistance rates. In recent years, globally, the selection of bacterial resistance mechanisms continues to increase[18]. In this sense, in several countries where the resistance profile of *S. aureus* from bovine mastitis was analyzed, percentages of resistance to penicillin close to 100 %[19-23] were found, which coincides with the results reported here. Regarding the resistance presented to oxacillin, contrasting results were found, since studies carried out in countries such as India and China showed resistance levels from 48 to 84 %[21,24]; however, in Germany, Japan and Colombia, the levels of resistance to oxacillin are minimal (2-7 %)[21,23,25], while these results show intermediate levels of resistance (33.3 %). In Mexico, studies reveal high levels of bacterial resistance to penicillin, amoxicillin and dicloxacillin (100 %)[26,27], which is consistent with what was reported in this study. Likewise, there is a significant increase in resistance to cephalotin, for example, in 2008 it was reported that 30 % of the strains of *S. aureus* studied had resistance[27]; however, in this work resistance of up to 83 % was found. These variations may be due to the possible genetic variability of the isolates, climatological differences, as well as geographical discrepancies among other factors[28]. Based on these differences, the need to carry out works such as the one presented here is highlighted, to define the virulence characteristics of *S. aureus* isolated from particular regions; to generate the necessary information that allows the implementation of more efficient treatments for subclinical bovine mastitis.

The presence of the *blaZ* gene was observed in 100 % of the isolates analyzed, while only 36.6 % were positive for the *mecA* gene (Table 3). These results are consistent with what was previously reported, where the presence of these genes was shown in all the isolates analyzed[19,29]. The presence of the *tetK*, *tetM*, *gyrA* and *gyrB* genes was expressed in a descending way according to the groups analyzed, which is consistent with the phenotype found in the antibiograms. This coherence between the phenotype and the genotype of bacterial resistance has already been demonstrated before[29,30], so that the resistance observed can be attributed to the presence and eventual expression of the genes analyzed[31].

Table 3: Analysis of resistance genes and biofilm formation

Isolates		Resistance genes						Biofilm	
		blaZ	***mecA***	***tetK***	***tetM***	***gyrA***	***gyrB***	***icaA***	***icaD***
Group 1	1	+	+	+	+	+	-	+	+
	2	+	+	+	-	+	+	+	+
	3	+	+	-	+	+	-	+	+
	4	+	+	-	-	+	-	+	+
	5	+	-	+	+	+	+	-	+
	6	+	+	-	-	+	+	+	+
	7	+	+	-	+	+	+	+	-
	8	+	-	+	+	+	+	+	+
	9	+	+	-	+	+	+	+	+
	10	+	+	-	+	+	+	+	+
Presence of the gene %		100	80	40	70	100	70	90	90
Group 2	1	+	+	+	-	-	+	+	+
	2	+	-	+	-	-	-	+	+
	3	+	-	-	+	-	+	+	-
	4	+	-	+	-	-	-	+	+
	5	+	+	+	+	+	-	+	-
	6	+	+	+	+	-	-	+	+
	7	+	-	+	-	-	-	+	+
	8	+	-	+	-	-	-	-	+
	9	+	+	+	-	+	+	-	+
	10	+	+	-	+	+	+	+	+
Presence of the gene %		100	50	80	40	30	40	80	80
Group 3	1	+	-	+	-	-	-	+	+
	2	+	-	-	+	-	-	+	-
	3	+	-	+	+	-	-	+	+
	4	+	-	+	+	-	-	+	+
	5	+	+	+	+	-	+	+	+
	6	+	-	-	+	-	-	+	+
	7	+	-	-	-	-	-	-	+
	8	+	-	-	-	+	+	+	+
	9	+	-	-	-	-	+	+	+
	10	+	-	-	-	+	+	+	+
Presence of the gene %		100	10	40	50	20	40	90	90
Total, %		100.0	46.6	53.3	53.3	50.0	50.0	86.6	86.6

Biofilm formation of *S. aureus* isolated from bovine mastitis

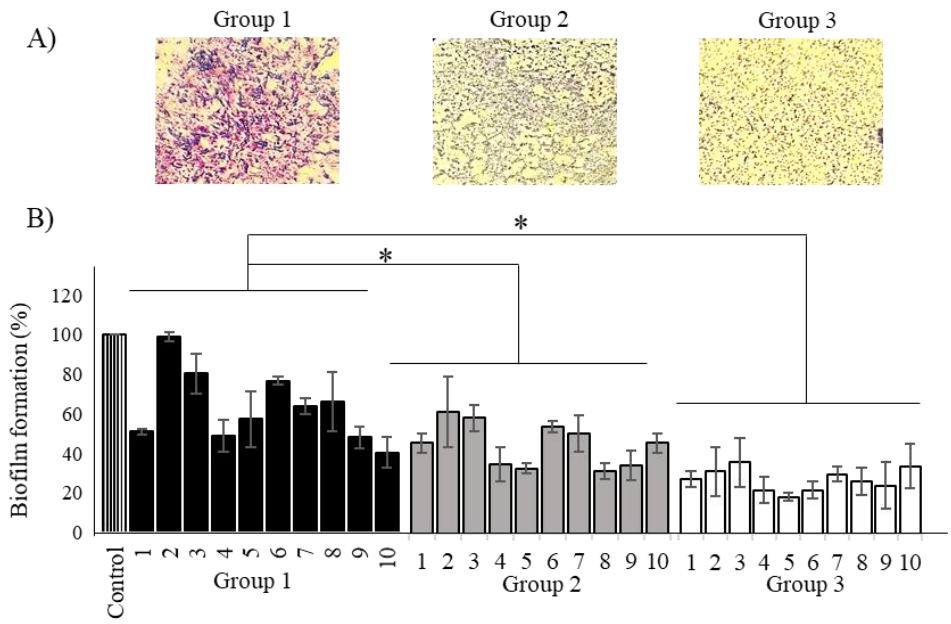
The analysis of biofilm formation showed that all isolates had the ability to form biofilm with a range from 20 to 98 %. Several studies around the world report that the strains of *S. aureus* analyzed have the capacity of biofilm formation (90 to 99 %)[32,33], which coincides with the present study.

Because *S. aureus* has high levels of antibiotic resistance, it is necessary to analyze the virulence factors and characteristics of this bacterium in order to design more efficient control strategies. In this sense, it has already been reported that *S. aureus* has the ability to form biofilm[34], which may be related to the low effectiveness that conventionally used drugs have[35].

To establish the possible relationship between resistance and biofilm formation in the 30 isolates from bovine mastitis, the following strategy was followed. First, the results of biofilm formation were analyzed according to the resistance level of the isolates (Figure 1); for which, these were ordered into three groups as described in the materials and methods section. In the analysis of variance of this study, only the effect of degree of resistance was significant ($P<0.01$), finding that the mean absorbance levels of the isolates were 1.34 (63.17 %), 0.77 (38.78 %) and 0.66 (26.28 %) for groups 1, 2 and 3, respectively. In the comparison of means, groups 1-3 were different ($P<0.05$) (Figure 1B). The estimated correlation between the degree of resistance and biofilm formation was positive (0.50) and significant ($P<0.01$) (Figure 2); in addition, these differences were analyzed microscopically, where the formation of biofilm in the isolates of group 1 were observed to be significantly increased compared to the other two groups (Figure 1A). Because the formation of biofilm by *S. aureus* induces the development of chronic and recalcitrant infections[32], the need to analyze how this virulence characteristic is related to other mechanisms, such as antibiotic resistance, stands out. With reference to the above, this relationship has been studied in both Gram-negative[36-39] and Gram-positive[40,41] bacteria; however, there are discrepancies in defining whether the correlation that occurs is positive[42,43,44] or negative[41].

In this study, it was observed that isolates with a high multi-resistance present a greater formation of biofilm; establishing a positive correlation, which is consistent with most of the works reported in this regard[42,43,44]. Finally, the presence of the *icaA* and *icaD* genes was analyzed, which are directly related to the formation of biofilm[45]. Interestingly, it was found that there is a high frequency of both genes in the isolates (86.6 %), regardless of the degree of resistance they present (Table 3). Several authors agree with this result, since they report a frequency close to 100 % of one or both genes in biofilm-forming strains of *S. aureus*[46,47,48].

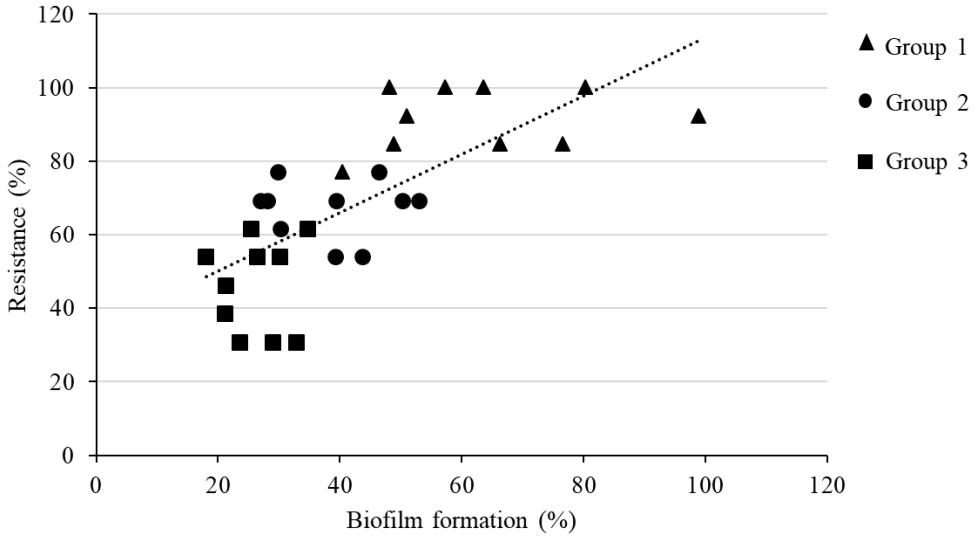
Figure 1: Biofilm formation by *S. aureus* isolates. The violet crystal staining protocol was used to measure the formation of biofilm.



A) Representative images of the biofilm staining in the three groups (Group 1: High resistance, from 11 to 13 antibiotics; Group 2: Medium resistance, from 9 to 10 antibiotics and Group 3: Low resistance, from 4-8 antibiotics), visualized with light-field microscopy.

B) The graph represents the percentage of biofilm formation of the three groups of isolates. The certified strain of *S. aureus* ATCC 27543 was used as a positive control, whose absorbance value was standardized as 100 %. The average of three independent experiments in triplicate ± their standard deviation is presented. (*) represents a statistically significant difference between groups ($P \leq 0.001$).

Figure 2: Relationship between the percentage of resistance and biofilm formation (%) of *S. aureus* isolates



Conclusions and implications

S. aureus isolates from bovine mastitis from the states of Guanajuato and Michoacán, Mexico, have high levels of antibiotic resistance; as well as an important biofilm-forming capacity. In addition, in the present work, the existence of a positive relationship between these two factors was demonstrated. These virulence characteristics may be directly associated with the low rate of clinical efficacy of treatments conventionally used on dairy farms. The variability of the results recorded in this study and other reports in various parts of the world highlight the need to conduct research on the virulence characteristics of microorganisms located in a specific geographical location and thereby establish management strategies for bovine mastitis in a comprehensive and efficient manner.

Acknowledgements

This research was funded by the University of Guanajuato through the 2018 Call (DAIP-CIIC-077/2018) and by the Secretariat of Innovation, Science and Higher Education (MA-CFINN1042).

Literature cited:

1. SIAP. Servicio de Información Agroalimentaria y Pesquera. Boletín de Leche. México. 2018.

2. Smith PB. Medicina interna de grandes animales. Serrales, DC (trad.). 4ta ed. Barcelona, España: Elsevier; 2010.

3. Taponen S, Liski E, Heikkilä AM, Pyörälä S. Factors associated with intramammary infection in dairy cows caused by coagulase-negative *Staphylococci*, *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Corynebacterium bovis*, or *Escherichia coli*. J Dairy Sci 2017;100(1):493–503.

4. Martínez-Sigales JM. Patología y clínica bovina recopilación de clases y relatos de la experiencia práctica de un veterinario de campo. Intermedica. Buenos Aires Argentina. 2016.

5. Gomes F, Henriques M. Control of bovine mastitis: Old and recent therapeutic approaches. Curr Microbiol 2016;72:377–382.

6. Monistero V, Graber HU, Pollera C, Cremonesi P, Castiglioni B, Bottini E, Ceballos-Marquez A. *et al*. *Staphylococcus aureus* isolates from bovine mastitis in eight countries: Genotypes, detection of genes encoding different toxins and other virulence genes. Toxins 2018;10:1-22.

7. Dorneles EMS, Fonseca MDAM, Abreu JAP, Lage AP, Brito MAVP, Pereira CR, Brandão HM, Guimarães AS, Heinemann MB. Genetic diversity and antimicrobial resistance in *Staphylococcus aureus* and coagulase-negative *Staphylococcus* isolates from bovine mastitis in Minas Gerais, Brazil. Microbiologyopen 2019;8:e736.

8. Thiran E, Di Ciccio PA, Graber HU, Zanardi E, Ianieri A, Hummerjohann J. Biofilm formation of *Staphylococcus aureus* dairy isolates representing different genotypes. J Dairy Sci 2018;101:1000–1012.

9. Raza A, Muhammad G, Sharif S, Atta A. Biofilm producing *Staphylococcus aureus* and bovine mastitis: A review. Mol Microbiol Res 2013;3(1):1-8.

10. Lister JL, Horswill AR. *Staphylococcus aureus* biofilms: recent developments in biofilm dispersal. Front Cell Infect Microbiol 2014;4:178:1-9.

11. Chen Y, Liu T, Wang K, Hou Ch, Cai S, Huang Y, Du Z, *et al*. Baicalein Inhibits *Staphylococcus aureus* biofilm formation and the quorum sensing system *in vitro.* Plos ONE 2016;11(4):e0153468.

12. Varela-Ortiz DF, Barboza-Corona JE, González-Marrero J, León-Galván MF, Valencia-Posadas M, Lechuga-Arana AA, Sánchez-Felipe CG. Antibiotic susceptibility of *Staphylococcus aureus* isolated from subclinical bovine mastitis cases and *in vitro* efficacy of bacteriophage. Vet Res Commun 2018;42:243–250.

13. Hudzicki J. Kirby-Bauer Disk diffusion susceptibility test protocol. Am Soc Microbiology. 2016.

14. CLSI. Performance standards for antimicrobial susceptibility testing. 20th ed. CLSI supplement M100 Wayne, PA: Clinical and Laboratory Standards Institute; 2019.

15. Yang Y, Jiang X, Chai B, Ma L, Li B, Zhang A, Cole JR, Tiedje JM, Zhang T. ARGs-OAP: online analysis pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured ARG-database. Bioinformatics 2016;32(15):2346-2351.

16. Elhassan MM, Ozbak HA, Hemeg HA, Elmekki MA, Ahmed LM. Absence of the *mecA* gene in methicillin resistant *Staphylococcus aureus* isolated from different clinical specimens in Shendi City, Sudan. Bio Med Res Int 2015;ID895860:1-5.

17. Hashem RA, Yassin AS, Zedan HH, Amin MA. Fluoroquinolone resistant mechanisms in methicillin-resistant *Staphylococcus aureus* clinical isolates in Cairo, Egypt J Infect Dev Ctries 2013;7(11):796-803.

18. Chokshi A, Sifri Z, Cennimo D, Horng H. Global contributors to antibiotic resistance. J Glob Infect Dis 2019;11(1):36–42.

19. Yang F, Zhang S, Shang X, Li H, Zhang H, Cui D, Wang X, *et al*. Short communication: Detection and molecular characterization of methicillin-resistant *Staphylococcus aureus* isolated from subclinical bovine mastitis cases in China. J Dairy Sci 2020;103:840–845.

20. Li T, Lu H, Wang X, Gao Q, Dai Y, Shang J, Li M. Molecular characteristics of *Staphylococcus aureus* causing bovine mastitis between 2014 and 2015. Front Cell Infect Microbiol 2017;7(127):1-10.

21. Cheng J, Qu W, Barkema HW, Nobrega DB, Gao J, Liu G, De Buck J, *et al*. Antimicrobial resistance profiles of 5 common bovine mastitis pathogens in large Chinese dairy herds. J Dairy Sci 2019;102:1–11.

22. Kadlec K, Entorf M, Peters T. Occurrence and characteristics of livestock-associated methicillin-resistant *Staphylococcus aureus* in quarter milk samples from dairy cows in Germany. Front Microbiol 2019;10:1295.

23. Thongratsakul S, Usui M, Higuchi H, Takahashi T, Sato T, Poolkhet Ch, Tamura Y. Prevalence and characterization of *Staphylococcus aureus* isolated in raw milk from cows in Hokkaido, Japan. Trop Anim Healt Prod 2020;52(4):1631-1637.

24. Mistry H, Sharma P, Mahato S, Saravanan R, Kumar PA, Bhandari V. Prevalence and characterization of oxacillin susceptible mecA-positive clinical isolates of *Staphylococcus aureus* causing bovine mastitis in India. Plos ONE 2016;11(9):e0162256.

25. Jiménez-Velásquez SC, Torres-Higuera LD, Parra-Arango GL, Rodríguez-Bautista JL, García-Castro FE, Patiño-Burbano RE. Perfil de resistencia antimicrobiana en aislamientos de *Staphylococcus spp*. obtenidos de leche bovina en Colombia. Rev Argent Microbiol 2020;52(2):121-130.

26. León-Galván MF, Barboza-Corona JE, Lechuga-Arana AA, Valencia-Posadas M, Aguayo DD, Cedillo-Pelaez C, Martínez-Ortega EA, Gutierrez-Chavez AJ. Molecular detection and sensitivity to antibiotics and bacteriocins of pathogens isolated from bovine mastitis in family dairy herds of Central Mexico. Bio Med Res Int 2015;2015:615153 1-9.

27. Ochoa-Zarzosa A, Loeza-Lara PD, Torres-Rodríguez F, Loeza-Ángeles H, Mascot-Chiquito N, Sánchez-Baca S, López-Meza JE. Antimicrobial susceptibility and invasive ability of *Staphylococcus aureus* isolates from mastitis from dairy backyard systems. Antonie van Leeuwenhoek 2008;94:199–206.

28. Karzis J, Petzer IM, Donkin EF, Naidoo V, Etter EMC. Climatic and regional antibiotic resistance patterns of *Staphylococcus aureus* in South African dairy herds. Onderstepoort J Vet Res 2019;86(1):e1-e9.

29. Yang F, Wang Q, Wang XR, Wang L, Li XP, Luo JY, Zhang SD, *et al*. Genetic characterization of antimicrobial resistance in *Staphylococcus aureus* isolated from bovine mastitis cases in Northwest China. J Integ Agr 2016;15(12):2842–2847.

30. Artursson K, Söderlund R, Liu L, Monecke S, Schelin J. Genotyping of *Staphylococcus aureus* in bovine mastitis and correlation to phenotypic characteristics. Vet Microbiol 2016;193:156-61.

31. Foster TJ. Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. FEMS Microbiology Reviews 2017;41:430–449.

32. Avila-Novoa MG, Iñíguez-Moreno M, Solís-Velázquez OA, González-Gómez JP, Guerrero-Medina PJ, Gutiérrez-Lomelí M. Biofilm formation by *Staphylococcus aureus* isolated from food contact surfaces in the dairy industry of Jalisco, Mexico. Hindawi J Food Qual 2018;ID1746139:1-8.

33. Piechota M, Kot B, Frankowska-Maciejewska A, Gruhewska A, Wofniak-Kosek A. Biofilm formation by methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* strains from hospitalized patients in Poland. BioMed Res Int 2018; ID4657396:1-7.

34. Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME. *Staphylococcus aureus* biofilms properties, regulation and roles in human disease. Virulence 2011;2(5):445-459.

35. Moormeier DE, Bayles KW. *Staphylococcus aureus* biofilm: A complex developmental organism. Mol Microbiol 2017;104(3):365–376.

36. Dumaru R, Baral R, Shrestha LB. Study of biofilm formation and antibiotic resistance pattern of gram-negative Bacilli among the clinical isolates at BPKIHS, Dharan. BMC Res Notes 2019;12(1):38.

37. Qi L, Li H, Zhang Ch, Liang B, Li J, Wang Li, Du X, *et al*. Relationship between antibiotic resistance, biofilm formation, and biofilm-specific resistance in *Acinetobacter baumannii*. Front Microbiol 2016;7:483.

38. Bawankar SK. Correlation of antibiotic resistance and biofilm formation amongst uropathogenic *E. coli.* Indian J Appl Res 2018;8:4.

39. Cepas V, López Y, Muñoz E, Rolo D, Ardanuy C, Martí S, Xercavins M, *et al*. Relationship between biofilm formation and antimicrobial resistance in Gram-negative bacteria. Microb Drug Resist 2019;25:1.

40. Vitale M, Galluzzo P, Bu PB, Carlino E, Spezia O, Alduina R. Comparison of antibiotic resistance profile and biofilm production of *Staphylococcus aureus* isolates derived from human specimens and animal-derived samples. Antibiotics 2019;8:97.

41. Zhang Y, Xu D, Shi L, Cai R, Li Ch, Yan H. Association between *agr* type, virulence factors, biofilm formation and antibiotic resistance of *Staphylococcus aureus* isolates from pork production. Front Microbiol 2018;9:1876.

42. Tahmasebi H, Dehbashi S, Jahantigh M, Arabestani MR. Relationship between biofilm gene expression with antimicrobial resistance pattern and clinical specimen type based on sequence types (STs) of methicillin-resistant *S. aureus.* Mol Biol Rep 2020;47(2):1309-1320.

43. Lin Q, Sun H, Yao K, Cai J, Ren Y, Chi Y. The prevalence, antibiotic resistance, and biofilm formation of *Staphylococcus aureus* in bulk Ready-to-eat foods. Biomolecules 2019;9:524.

44. Kwon AS, Park GC, Ryuc SY, Limd DH, Lime DY, Choi CL, Park Y, Lima Y. Higher biofilm formation in multidrug-resistant clinical isolates of *Staphylococcus aureus*. Int J Antimicrob Agents 2008;32:68–72.

45. O'Gara JP. *ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus.* FEMS Microbiol Lett 2007;270:179–188.

46. Castelani L, Pilon LE, Martins T, Rodrigues-Pozzi C, Rodriguez-Pozzi Arcaro J. Investigation of biofilm production and *icaA* and *icaD* genes in *Staphylococcus aureus* isolated from heifers and cows with mastitis. Anim Sci J 2015;86(3):340-4.

47. Gad GFM, El-Feky MH, El-Rehewy MS, Hassan MA, Abolella H, El-Baky RMA. Detection of *icaA, icaD* genes and biofilm production by *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from urinary tract catheterized patients. J Infect Dev Ctries 2009;3(5):342-351.

48. Kivanc SA, Arik G, Akova-Budak B, Kivanc M. Biofilm forming capacity and antibiotic susceptibility of *Staphylococcus* spp. with the icaA/icaD/bap genotype isolated from ocular surface of patients with diabetes. Malawi Med J 2018;30 (4):243-249.

49. Dhanawade NB, Kalorey DR, Srinivasan R, Barbuddhe SB, Kurkure NV. Detection of intercellular adhesion genes and biofilm production in *Staphylococcus aureus* isolated from bovine subclinical mastitis. Vet Res Commun 2010;34:81–89.

50. Brakstad OG, Aasbakk K, Maeland JA. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. J Clin Microbiol 1992;30:1654–1660.