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Article

Genetic diversity and virulence factors of *Staphylococcus aureus* strains isolated from bovine udder skin

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Abstract:

Staphylococcus aureus is a pathogen recognized as a cause of mastitis in cattle worldwide, so the objective of this work was to determine the presence of *Staphylococcus aureus* in the teat skin of the bovine udder and relate it to the presence of mastitis, as well as to determine

the virulence factors and genetic diversity of the strains. Samples of 250 milking cows were taken in three farms, in two seasons of the year, dry and rainy. In addition, the California test was performed. *Staphylococcus aureus* was isolated in salt agar and mannitol and biochemically identified and confirmed with amplification of the *femA* gene. For the identification of virulence factors, the genes *hlB*, *mec*, *saK*, *pvL*, *tsst-1*, *seA*, *seB*, *seC*, *seD* and *seE* were used by end-point PCR. For the typing of *S. aureus*, amplification and restriction of the *coag* gene was performed. The frequency of *S. aureus* was 13.4 %. No statistical relationship between the presence of *S. aureus* in the bovine udder skin and the development of subclinical mastitis was found. The most frequent enterotoxin gene in the strains was enterotoxin A. Although the percentage of typing is low, it was possible to identify two restrictotypes that group strains isolated from different cows, which shows the infectious and contagious capacity of the microorganism.

Key words: Bovine udder, *Staphylococcus aureus*, Genetic diversity, Mastitis.

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Introduction

Bovine mastitis (MB) is the inflammation of the mammary gland caused in most cases by a microorganism, which invades the udder through the teat canal[1,2]. This disease is classified, according to the clinical manifestations it presents, into clinical mastitis (CM) and subclinical bovine mastitis (SCM), the latter being the most common clinical entity[3]. Although there are numerous bacterial genera that cause mastitis, only a small number of species are prevalent and constitute a real public health problem[2].

Staphylococcus aureus is recognized as a pathogen worldwide for causing mastitis[4], being responsible for approximately one third of cases of clinical and subclinical mastitis in cattle[5,6], being considered a problem in the livestock industry due to its pathogenicity, persistence in the environment, ease of contagion between cow and cow during the milking process[2,7], due to the low rates of resolution of the disease with current treatments[8], leading to chronic infections, which persist between periods of lactation, with intermittent clinical episodes occurring from the elevation of temperature, degrees of anorexia, the appearance of clots in milk[1], which increases the risks of slaughter of the animal, work, treatment and replacement costs, veterinary visits, incorporation of protocols to avoid risk with investment

in infrastructure as well as the implementation of diagnostic methods more frequently[9]. However, mastitis not only has an impact on the producer's economy, but also becomes a public health problem by being a potential source of zoonotic transmission, since *S. aureus* has been described as capable of causing disease in humans[10,11]. Bovine mastitis has relevance in the context of food poisoning in humans. Ingestion of products contaminated with staphylococcal enterotoxins results in poisoning characterized by violent vomiting and diarrhea[12]. Among the factors related to an infection by *S. aureus* in the mammary gland is hygiene both in milking and in the udder of the animal[13,14].

In recent years, it has been suggested that the colonization of the udder skin could be a predominant factor in the development of mastitis by dragging the microorganism during milking towards the animal's teat; however, several studies differ in relation to this assertion. Cases of *S. aureus* mastitis have been described as being caused by strains highly adapted to the mammary gland and that they are different from skin isolates[2,15]. While other studies suggest that most *S. aureus* isolated from the skin and teat canal as well as extra mammary sites such as vagina, nostrils and skin of the jaws are genetically the same as those found in mammary glands or milk[16-18]. Therefore, the objective of this study was to determine the frequency of *S. aureus* in the skin of the teats of udders of cows from three dairy farms located in the south of the state of Guerrero, Mexico, the genetic diversity and virulence factors of the strains of *S. aureus*, as well as the possible relationship with bovine mastitis.

Material and methods

Farms and sampling

Three farms were included in the study. Based on the permission of the owners and the size of the farm. A sample of the teat skin was taken from the four quarters of the 250 milking cows, in two different seasons of the year: rains and dryness, having a total of 500 samples. The samples were taken with a cotton swab, which was slid on each edge of the teat and around it, covering an area of 2 cm. The California test was then performed, considering the interpretation of the test described above. All farms sell raw milk directly to consumers and one of them is characterized for being a site for the production of cheeses made with raw milk. On this farm, raw milk is collected during the morning and directly processed in a small artisanal cheese production plant.

Isolates

Samples taken with swabs from the teat skin were cultured in salt agar and mannitol. The isolates were presumptively identified as *S. aureus* according to the following scheme: mannitol positive, catalase positive, Gram positive cocci and coagulase positive in 6 h. The isolates were preserved at -20 °C in BHI (Brain Heart Infusion) broth with 15 % (V / V) of glycerol.

Molecular identification of *S. aureus*

The strains were cultured in brain-heart infusion broth and incubated at 37 °C. The control strains used in this study were *S. aureus* ATCC29231 (*sea*), *S. aureus* ATCC14458 (*seb*), *S. aureus* ATCC19095 (*sec*), *S. aureus* ATCC13563 (*sed*), *S. aureus* ATCC27664 (*see*) and *S. aureus* ATCC25923 (*femA*, *coag*, *hlb*, *sak*).

The total DNA was obtained from 1 ml from an 18 h culture of all bacterial strains including ATCC strains. The cells were sedimented from the centrifugation of cultures at 10,000 rpm for 10 min and suspended in 300 µl of lysis buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0, lysozyme 1 mg/ml) and incubated at 37 °C for half an hour or until viscosity is observed. DNA from all preparations was subsequently extracted with phenol chloroform and precipitated with ethanol. DNA samples were diluted in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0)[19].

An end-point PCR of the *femA* gene was performed on the strains for molecular confirmation of *S. aureus* with the oligonucleotides described in Table 1. The final PCR reaction mixture contained 0.2 mM of each dNTP, 3 mM of $MgCl_2$, 0.2 mM of oligonucleotides, 1 U taq DNA polymerase (Amplicon, Denmark) 5 µl of 10X buffer and 100 ng of DNA as a template.

Table 1: Oligonucleotides used for molecular identification, detection of enterotoxin genes, and molecular typing

Gene (virulence factor)	Sequence (5'- 3')	APS	Ref.
femA	femaF- AAAAAAGCACATAACAAGCG femaR- GATAAAGAAGAAAACCAGCAG	130	(20)
coa (coagulase)	coaF- CGAGACCAAGATTCAACAAG coaR- AAAGAAAACCACTCACATCA	600-900	(22)
seA (enterotoxin A)	seaF- TGCAGGGAACAGCTTTAGGC seaR- GTGTACCACCCGCACATTGA	250	(20)
seB (enterotoxin B)	sebF- ATTCTATTAAGGACACTAAGTTAGGG	400	

	sebR- ATCCCGTTTCATAAGGCGAGT		
seC (enterotoxin C)	secF- GTAAAGTTACAGGTGGCAAAACTTG secR- CATATCATACCAAAAAGTATTGCCGT	297	
seD (enterotoxin D)	sedF- GAATTAAGTAGTACCGCGCTAAATAATATG sedR- GCTGTATTTTTCCTCCGAGAGT	492	
seE (enterotoxin E)	seeF- CAAAGAAATGCTTTAAGCAATCTTAGGC seeR- CACCTTACCGCCCAAAGCTG	480	
hlB (hemolysin β)	hlbF- GTGCACTTACTGACAATAGTGC hlbR- GTTGATGAGTAGCTACCTTCAGT	300	
saK (staphylokinase)	sakF- ATCCCGTTTCATAAGGCGAGT sakR- CACCTTACCGCCCAAAGCTG	260	In this study
mecA (Methicillin resistance)	mecaF- TCCAGATTACAACTTCACCAGG mecaR- CCACTTCATATCTTGTAACG	180	(21)
tsst-1 (toxic shock syndrome toxin)	tsstF- CATCTACAAACGATAATATAAAGG tsstR- CATTGTTATTTTCCAATAACCACCCG	476	In this study

APS= amplified product size (pb); Ref.= reference.

Identification of coding genes for virulence factors

The detection of the genes *hlB*, *mec*, *saK*, *pvL*, *tsst-1*, *seA*, *seB*, *seC*, *seD* and *seE* encoding β-hemolysin, methicillin resistance region, staphylokinase, Panton Valentine toxin, toxic shock syndrome toxin and enterotoxins respectively, was based end-point PCR with the oligonucleotides described in Table 1. The final PCR reaction mixture contained: 0.2 mM of each dNTP, 3 mM $MgCl_2$, 0.2 µM of oligonucleotides, 1 U of Taq DNA polymerase (Ampliqon®, DEN), 1X Buffer and 100 ng of DNA as a template. The reaction mixtures were subjected to the following amplification conditions: initial denaturation for 5 min, at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 52 °C, 30 sec at 72 °C; and a final extension for 5 min at 72 °C for *mec*, *hlB*, *pvL*, *tsst-1*, *seA* and *seE*. Initial denaturation for 5 min at 94 °C; 30 cycles of 30 sec at 94 °C, 45 sec at 52 °C, 45 sec at 72 °C; and a final extension for 5 min at 72 °C for *saK*, *seB*, *seC* and *seD*[20,21].

The electrophoresis of the PCR products was performed in 2 % agarose gels at 80 V for 60 min. The gels were stained with Midori green (Nippon Genetics®, GER) and visualized with LED light (Nippon Genetics®, GER) at 470 nm.

Phenotypic test to show the expression of the *hlB* gene

To demonstrate the expression of the β-hemolysin, the strains were cultured by cross-streak in 5 % ram blood agar, being incubated at 37 °C in CO_2 tension for 24 h. Strains that had a halo of transparency in the perimeter of the colonies are considered β-hemolytic (*hlB*+). Strains that presented α and γ-hemolysis are considered *hlB*$^-$.

Molecular typing of *S. aureus*

In the strains molecularly confirmed as *S. aureus*, the *coag* gene was amplified by end-point PCR with the oligonucleotides described in Table 1 and with the following final mixture for each PCR reaction: 0.2 mM of each dNTP, 3 mM $MgCl_2$, 0.2 µM oligonucleotides, 1 U of Taq DNA polymerase (Ampliqon®, DEN), 1X Buffer and 100 ng of DNA as a template. The PCR protocol begins with initial denaturation for 5 min, at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 52 °C, 60 sec at 72 °C; and a final extension for 5 min at 72 °C[22]. PCR products were digested for 2 h at 37 °C with 10 U of the restriction enzyme *AluI* (Thermo Scientific®, USA) according to the protocol recommended by the manufacturer. Restriction fragments were detected by electrophoresis in 2 % agarose gels at 70 V for 60 min. The gels were stained with Midori green (Nippon Genetics®, GER) and visualized with LED light (Nippon Genetics®, GER).

Statistical analysis

The STATA V. 12 statistical package (STATA®, USA) was used to calculate simple frequencies and the Chi-square statistical test was used for possible relationships between the presence of *S. aureus* in the bovine udder skin and the development of subclinical mastitis, values of $P = <0.05$ are considered as statistically significant.

Results and discussion

In this study, of the total number of cows analyzed in the two periods, a frequency of subclinical mastitis of 6.6 % (33/500) and clinical mastitis of 0.8 % (4/500) was determined. The frequency of subclinical mastitis per dairy farm was higher in farm A (12 %) in relation to B (4 %) and C (1 %) (P=0.001) (Table 2). These differences in frequency may be related to the implementation or compliance with well-characterized strategies for the control of intra mammary infections or known as the 5-point plan, which includes the milking sequence, use of gloves, change of paper or cloth towel between the udder quarters, pre-seal and post-seal of the teat [23]. In addition to other factors such as the presence of infections and their time of evolution[24], the nature of the infectious agent, the parity and the state of lactation of the animal, as well as the nutrition and the environment where it is[25]. In this last point, it is

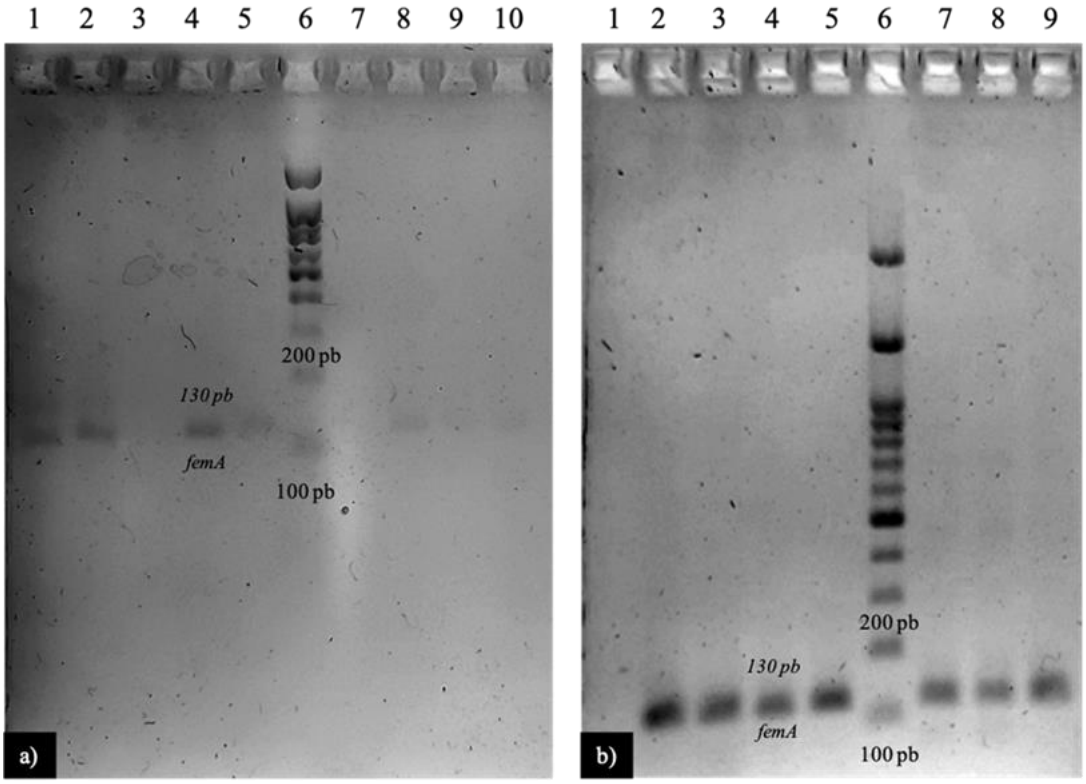
known that the rainy season (summer) is an environmental risk factor for the development of mastitis, being more frequent the appearance of cases during this season in relation to others such as winter[26], which was observed in our study, in which, during the rainy period, the frequency of subclinical mastitis was higher (10.8 %) in relation to the dry season (2.4 %) (*P*=0.001). Several authors agree on this statement, considering that humidity may have an important role; however, they also conclude that this period could coincide with the onset of lactation or the end of gestation, which should generally be considered for cows of first parity during the season with the most desirable climate, in this case summer, during which the availability of grass and therefore of feed is greater[27,28].

Table 2: Frequency of subclinical and clinical mastitis in relation to dairy farms, season and presence of *S. aureus* in the teat of cows' udders

Characteristic	Total	California Test				*p*
		Negative	Traces	Subclinical mastitis	Clinical mastitis	
Dairy farm						
A	200	8 (4)	164 (82)	24 (12)	4 (2)	
B	200	4 (2)	188 (94)	8 (4)	0	0.001
C	100	0	99 (99)	1 (1)	0	
Season						
Rainy	250	11 (4.4)	208 (83.2)	27 (10.8)	4 (1.6)	
Dry	250	1 (0.4)	242 (97.8)	6 (2.4)	0	0.001
S. aureus in udder						
Positive	67	0	66 (98.5)	1 (1.5)	0	0.106
Negative	433	12 (2.7)	385 (88.9)	32 (7.5)	4 (0.9)	

Cases of clinical mastitis only occurred in dairy farm A (2 %) and in the rainy season (1.6 %) (*P*=0.001). In the case of the isolation of the microorganism of interest from the teat skin of the udder of the cows analyzed, the frequency of *S. aureus* from the identification of the *femA* gene was 13.4 % (67/500) (Figure 1, Table 2). Other markers are used to determine the species of *S. aureus* such as the thermonuclease gene (*nuc*)[29] and the region of the 16 rRNA gene[30]; however, the *nuc* gene can be found in other *Staphylococcus* species coagulase-positive (*S. hyicus*, *S. delphini*, *S. intermedius*, *S. pseudointermedius*, *S. schleiferi*) and negative (*S. capitis*, *S. caprae*, *S. epidermidis*, *S. warneri*, *S. simulans*, *S. carnosus*, *S. kloosii*, *S. saprophyticus*)[31], so it was decided to work with this gene, which is related to the synthesis of peptidoglycan and has a high identification power for *S. aureus* resistant to methicillin[32].

Figure 1: Electrophoresis of *femA* gene amplification of *S. aureus* strains of bovine udder skin



A) 1, S522 (PL); 2, S528 (PL), 3; S519 (PL); 4, S529 (PL); 5, S521 (CL); 6, MPM; 7, negative control; 8, positive control (*S. aureus* ATCC25923); 9, S517 (PL); 10, S643 (CS).
B) 1, negative control; 2, S522 (PL); 3, S528 (PL); 4, S529 (PL); 5, S521 (CL); 6, MPM (100 PB); 7, S517 (PL); 8, S643 (CS); 9, S650 (CS).
A= barn a; B= barn B; L= rainy; S= dry; MPM= Molecular weight marker of 100 pb.
En **b**, las cepas débilmente positivas y positivas se volvieron a repetir y se modificaron las condiciones de electroforesis

S. aureus was isolated from most quarters that only had traces or low number of somatic cells, and isolated only from a cow with subclinical mastitis. Determining that there is no relationship between *S. aureus* isolates from the teat skin of cows' udder with the development of mastitis symptoms (P=0.106). Several factors could explain the absence of this relationship, from the immune status of the animal, the inoculum of the microorganism and the genetic characteristics of the strain. Even in recent years, the role of the microbiota of cow's udder has been described as an important factor in the development of mastitis, with the presence of other microorganisms that could act as antagonists of pathogens restricting their multiplication or establishment of the infection; for example, *S. chromogenes* has been described as producing bacteriocins capable of inhibiting the growth of most intramammary pathogens[33,34]. Considering that factors such as parity and the state of immunosuppression generated during gestation, lactation, metabolic profile and genetic load of the animal could

positively or negatively impact the composition of the udder microbiota and therefore the susceptibility of mastitis[35].

Although no relationship was found with the cases of subclinical mastitis in the study, other relationships were sought, such as the distribution of *S. aureus* in the three dairy farms (A, B and C), which was 14, 11.5 and 16 % respectively, with no statistically significant differences observed (*P*=0.531) (Table 3). These results could be explained by the ubiquity of the microorganism, the infectious and contagious capacity, as well as its persistence in the dairy environment[2]. In this study, the most frequently observed differences in seasonality for *S. aureus* were in the dry season (23.6 %, 29/250) in relation to the rainy season (3.2 %, 8/250), they could be explained by the accelerated growth of microorganisms associated with the temperature and humidity of the season[36], as well as an altered metabolic state, including some type of immunocompromise due to food restriction[35], which favors the colonization of the microorganisms.

Table 3: Presence of *S. aureus* in the teat of cows' udders in relation to dairy farms and season

Characteristics	Total	*S. aureus*		*P*
		Positive	Negative	
Dairy farm				
A	200	28 (14.0)	172 (86.0)	
B	200	23 (11.5)	177 (88.5)	0.531
C	100	16 (16.0)	84 (84.0)	
Season				
Rainy	250	8 (3.20)	242 (96.8)	0.001
Dry	250	59 (23.6)	191 (76.4)	

Similarly, no statistical relationship between the presence of *S. aureus* in the bovine udder skin and the development of subclinical mastitis was found; however, virulence factors that are key in both the development of infections and food poisoning were determined [37,38]. The most frequent enterotoxin gene in *S. aureus* strains was enterotoxin A (10.44 %), highlighting that no strains with the genes for enterotoxins B and C were found. As for toxins, 23 strains with the β hemolysin gene (34.32 %) were found; it is important to mention that this data is adjusted with the phenotypic test of hemolysis in ram's blood, which was considered because oligonucleotides designed for this gene do not amplify it completely and this gene is susceptible to the insertion of phages and the inclusion of genes such as enterotoxin A and staphylokinase (*saK*)[39,40]. In this study, no strains with one of the genes associated with Panton Valentine toxin were found.

Finally, only two strains with the *saK* gene (2.98 %) were found and three strains were defined as MRSA from the amplification of the *mecA* gene (4.47 %) (Table 4). In this sense, several studies have sought to establish a profile of virulence characteristic of the strains of *S. aureus* capable of causing subclinical mastitis, assuming that the microorganism must adapt to a certain environment when invading the bovine udder, the results have been diverse, however, as points in common, they converge in that hemolysin b and Panton Valentine toxin are virulence factors that could participate in the development of subclinical mastitis, having as their main function, the formation of pores or lysis of leukocytes[2,41,42]. Which could be confirmed with the results in this study, the strains having in low or no frequency, the genes of these toxins (both that of hemolysin and Panton Valentine toxin) were not associated with cases of subclinical mastitis. A relationship of these strains with particular clinical pictures in the bovine udder was not established; however, the strains have epidemiological importance due to the presence of enterotoxin genes, these could contaminate by dragging during milking and ultimately be found in the final dairy product and cause a problem of food poisoning[43].

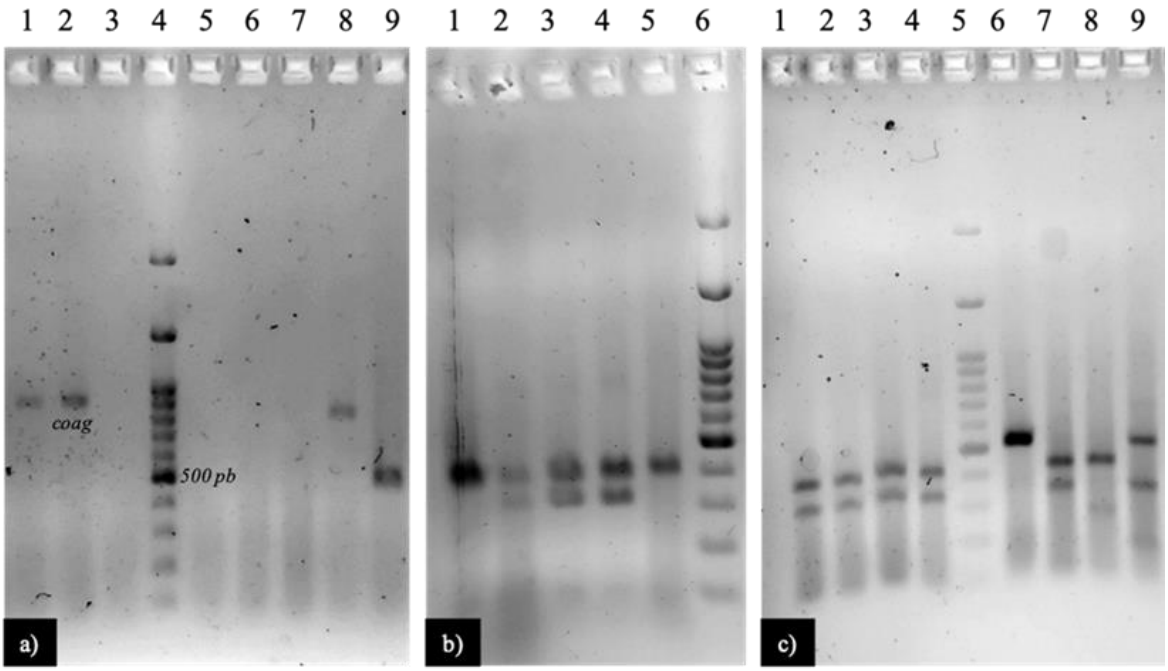
Table 4: Virulence factors of *S. aureus* strains isolated from cow udders

Virulence factor	n(%), N= 67
Enterotoxins	
seA	7 (10.44)
seB	-
seC	-
seD	2 (2.98)
seE	1 (1.49)
tsst-1	4 (5.97)
Toxins	
hlB	23 (34.32)
pvL	-
Enzymes	
saK	2 (2.98)
mecA	3 (4.47)

As for the molecular typing of the strains of *S. aureus*, the percentage of typing has shown important variations in relation to time; in the years close to the beginning of the use of the PCR-RFLP technique of the *coag* gene, the typing of 100 % of the strains was achieved[44-48]; however, in recent years this percentage decreases to 40 %[29,30,49], which is still higher than the percentage determined in this study (19.47 %) (Figure 1a). In this sense, two events must be considered, in the first instance it has been proposed that the decrease in the percentage of typing could be related to mutations in the coagulase gene that impact on the specificity of the proposed oligonucleotides, but do not affect the activity of the enzyme,

noting that oligonucleotides are the same as those used in the original technique and were established since 1992[22]; on the other hand, it is important to compare the results in relation to the type of host from which *S. aureus* was isolated, because the change of host could involve important changes in the strain including the variability of the *coag* gene; however, if the percentage of typing in studies of *S. aureus* in cows is compared, results vary considerably between typing percentages[29,30,44,49]. Although the percentage of typing is low, it was possible to identify a restrictotype (400 bp, 350 bp) that groups strains isolated from different cows from both barn A (Figure 2c, lane 4) and barn B (Figure 2b, lane 2, 3 and 4; Figure 2c, lane 1, 2 and 3), as well as the rainy season (Figure 2b, lane 2; Figure 2c, lane 1, 2 and 3) and dry season (Figure 2b, lane 3 and 4), which shows the infectious and contagious capacity of *S. aureus*, and this reaffirms the role of this microorganism in cases of mastitis of infectious type, which also emphasizes the importance of adopting measures that prevent the transmission of the microorganism in the dairy environment, being able to infect cattle, altering the quality of milk and cattle should be considered as a major source of contamination in the production of dairy products.

Figure 2: Electrophoresis of *coag* gene restriction of strains isolated from bovine udder skin



(A) 1, S528; 2, positive control (*S. aureus* ATCC25923); 3, negative control; 4, MPM; 5, S519 (PL); 6, S520 (PL); 7, S530 (PL); 8, S618; 9, S673 (PS).
(B) 1, S673 (PS); 2, S665 (PL); 3, 668 (PS); 4, 669 (PS); 671 (PS); 6, MPM
(c) 1, S522 (PL); 2, S528 (PL); 3, S529 (PL); 4, S521 (CL); 5, MPM (100 PB); 6, S517 (PL); 7, S643 (CS); 8, S650 (CS); 9, S618.
A= barn A, **B**= barn B; L= rainy; S= dry.

Conclusions and implications

In this study, no relationship was found between the presence of *S. aureus* in the teat skin of cattle udder and the development of mastitis; however, strains with genes for enterotoxins, which are a public health problem, were determined. In addition, the transmission of strains in dairy farms was evidenced, which highlights the importance of good milking practices.

Acknowledgments and conflicts of interest

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