

Abanico Veterinario. January-December 2020; 10:1-15 <http://dx.doi.org/10.21929/abavet2020.21>
Original Article. Received: 13/02/2020. Accepted: 18/08/2020. Published: 26/10/2020. Code:2020-12.

Genetic variation of *Staphylococcus aureus* causing mastitis in dairy cows in Jalisco

Variación genética de *Staphylococcus aureus* causante de mastitis en vacas lecheras en Jalisco

Castañeda-Vázquez Hugo*¹ , Padilla-Ramírez Francisco**² , Castañeda-Vázquez Martha¹ , Camacho-Palafox José¹ , Salas-Castañeda Erika¹ 

¹Universidad de Guadalajara, Laboratorio de Mastitis y diagnóstico Molecular. División de Ciencias Veterinarias, Guadalajara, México. ²Universidad de Guadalajara, Departamento de Producción Animal. División de Ciencias Veterinarias, Guadalajara, México. *Responsible author: Castañeda-Vázquez Hugo. **Author for correspondence: Padilla-Ramírez Francisco². Centro Universitario de Ciencias Biológicas y Agropecuarias, Camino Ramón Padilla Sánchez #2100 Nextipac, 45200, Zapopan, Jalisco, 37771150 Ext. 33177. hcastane59@yahoo.com.mx, fjavier.padilla@academicos.udg.mx, arcoiris2565@hotmail.com, ertytres333@hotmail.com, erika.salas@cucba.udg.mx.

Abstract

The genetic variability of *S. aureus* strains isolated from some cases of bovine mastitis was determined. 335 cows from 27 stables were sampled in 10 municipalities in Jalisco state. *S. aureus* strains were identified from milk samples of each mammary gland of each cow, which were grown in blood agar and based on culture characteristics, biochemical tests, and finally their molecular confirmation by PCR. The genetic variation in the strains was identified by pulsed-field electrophoresis technique. The images of the gels were analyzed using the Bionumerics® software. 2.26% of clinical mastitis and 40.45% of subclinical mastitis were diagnosed with the California test. A frequency of appearance of *S. aureus* of 9.8% of the total sampled glands was recorded. A genetic variation of 14.9% was observed. The 32 strains analyzed were grouped into pulsotypes with 95% or more of genetic similarity, resulting in 12 pulsotypes. It is concluded that there is great diversity in the genetic variability of *S. aureus* strains from different stables in the state of Jalisco and a great genetic similarity of strains within each stable.

Keywords: PFGE, *S. aureus*, typification, mastitis` frequency, genetic variation, pulsed field electrophoresis.

Resumen

Se determinó la variabilidad genética de las cepas de *S. aureus* aisladas de algunos casos de mastitis bovina. Se muestrearon 335 vacas de 27 establos en 10 municipios del estado de Jalisco. Las cepas de *S. aureus* se identificaron a partir de muestras de leche de cada glándula mamaria de cada vaca, las cuales se cultivaron en agar sangre y con base a las características del cultivo, las pruebas bioquímicas, y finalmente su confirmación molecular mediante PCR. La variación genética en las cepas identificadas se obtuvo con la técnica de electroforesis de campos pulsados. Las imágenes de los geles se analizaron mediante el software Bionumerics®. Se diagnosticó con la prueba de California un 2.26% de mastitis clínica y un 40.45% de mastitis subclínica. Se registró una frecuencia de aparición de *S. aureus* del 9.8% del total de las glándulas muestreadas. Se observó una variación genética de 14.9%. Las 32 cepas analizadas se agruparon en pulsotipos con 95% o más de similitud genética, resultando 12 pulsotipos. Se concluye que existe gran diversidad en la variabilidad genética de cepas de *S. aureus* de diferentes establos del estado de Jalisco y una gran similitud genética de cepas dentro de cada establo.

Palabras clave: *Staphylococcus aureus*, tipificación, frecuencia de mastitis, variación genética, electroforesis de campos pulsados.

INTRODUCTION

Mastitis is defined as mammary gland inflammation, caused mainly by pathogens ([Bedolla et al., 2008](#)), which causes a decrease in the production and milk quality in its subclinical form; however, it can turn into clinical mastitis, where it can reach the animal's waste due to loss of the mammary gland functionality ([Echeverri et al., 2010](#)). Likewise, subclinical mastitis tends to be present for a longer time and is more frequent than clinical mastitis ([Bedolla et al., 2007](#)). Economic losses can reach up to 5000 mexican pesos for each lactation of a cow per year ([Van et al., 2016](#)). Although around 137 microorganisms have been found in dairy cattle with mastitis, only a small number of pathogens are responsible for most of the cases (Wolter et al., 2004); for example, such is the case of the genus *Staphylococcus* spp, which express virulence factors that allow their persistence and distribution in the host ([Marqués et al., 2013](#)).

On the other hand, *Staphylococcus aureus*, described by the physician Alexander Ogston in 1880 ([Cervantes-García et al., 2014](#)), which is currently considered one of the main agents causing mastitis worldwide ([El-Sayed, 2006b](#)), with a frequency of almost 30% of samples from cows with mastitis ([Calderón and Rodríguez, 2008](#)). Invasive properties and extracellular factors are attributed to this pathogen, such as the production of toxins that lead to toxic shock syndrome, exfoliative toxins, and staphylococcal enterotoxins ([Vasconcelos and Ribeiro de Souza, 2010](#)). Additionally, it has a great capacity to acquire exogenous elements by horizontal transfer, which allows it to easily adapt to the environment and antimicrobial agents, through the acquisition of antibiotic resistance factors encoded by plasmids and transposons ([Baba et al., 2002](#)).

The molecular typing of microorganisms (genotyping) is very important to understand the evolution of pathogens and study their genetic relationship. In this way, a greater understanding is achieved during epidemiological investigations ([Amr El-Sayed, et al., 2017](#); [Castañeda et al., 2018](#), [García et al., 2019](#)). There are a variety of genotyping methods, each technique presenting strengths and weaknesses. Among the most prominent are pulsed field electrophoresis (PFGE), spa gene typing, multiple locus sequence typing (MLST), plasmid profile analysis, restriction fragment length polymorphism (RFLP), analysis (MLVA), and the analysis of the entire DNA genome sequence ([Struelens et al, 2009](#); [Kuroda et al., 2001](#)).

Pulsed Field Electrophoresis (PFGE) is a powerful genotyping technique used to separate large DNA molecules (complete genomic DNA), after macrorestriction (cutting with a restriction enzyme at various points in the genome) and fragment separation by applying an electric field, being this technique a good representation of the entire bacterial chromosome, because it provides clearly differentiated DNA fragments ([Sharma-Kuinkel et al., 2016](#)). One of the strengths of the technique is that it has a high discriminatory power ([Struelens et al, 2009](#)). [Castañeda et al., \(2011\)](#) showed a close relationship of field

strains of *S. aureus*, responsible for mastitis in western Mexico; suggesting that closely related clones of *S. aureus* are responsible for the majority of intramammary infections, caused Jalisco state ([Castañeda et al., 2018](#)). [Marqués et al., \(2013\)](#) observed in Brazil a marked diversity of *S. aureus* clones, detecting six different genetic profiles through pulsed field electrophoresis (PFGE). ([Kuroda et al., 2001](#); [Sato et al., 2017](#)) found genetic variations of up to 30% of 41 isolated *S. aureus* strains, in Japan from cows with mastitis, humans and in meat destined for sale. ([Can et al., 2017](#)) obtained a variation of around 25% of the totality of samples obtained from samples of meat, raw milk, cheese, meat and chicken in Turkey. The strains obtained from raw cow's milk showed a similarity of 90%.

Only a thorough investigation will help us understand the epidemiology of the pathogen *S. aureus*, and can help control the disease and/or produce a vaccine, minimizing its threat. For this purpose, several molecular biology tools have been developed for use in advanced molecular epidemiological studies, such as those used in the present *S. aureus* investigation.

MATERIAL AND METHODS

Sampling. A cross-sectional study was carried out with a random sampling in 27 stables belonging to the 10 municipalities: Acatic, Arandas, Atotonilco el Alto, Encarnación de Díaz, La Barca, Lagos de Moreno, Tepatitlán de Morelos, San Juan de los Lagos, San Miguel el Alto and Tototlán, considered the largest milk producers in Jalisco state, Mexico.

The number of animals sampled was proportional to the population of cows in each municipality, in a ratio of approximately 1 to 900. The size of the stables was classified by the number of cows in production, which at the time of sampling each stall had, and based on this the number of cows to be sampled in each one was determined (table 1). The total number of cows to be sampled was determined with the formula used for infinite or very large populations ([Gamboa, 2017](#)), resulting in 335 cows.

Mastitis detection. The California test was used as a field test to determine the frequency of subclinical mastitis; It was carried out before milking the animal, after eliminating the first jets of milk; taking an approximate 2 mL sample and applying a similar amount of the California Mastitis Test (CMT) reagent; Following the procedure proposed by ([Barnum and Newbould, 1961](#)), after some circular movements of the mixture, it was immediately interpreted.

Table 1. Classification of stables by size (number of cows in production)

Stables size	NU. of cows in production	Nu. of cows to sample
Small	≤ 25	≤ 11
Medium	26 a 99	12 a 15
Large	≥ 100	16 a 20

Bacteriological culture, biochemical tests, PCR technique; as well as pulsed field electrophoresis were carried out in the mastitis and molecular biology laboratory of the University Center for Biological and Agricultural Sciences (CUCBA) of the University of Guadalajara, which is located in Zapopan municipality in Jalisco state. The 1,325 milk samples were cultured in Petri dishes, with blood agar content added with 5% sheep blood, inoculating around 0.01 mL of milk for each sample.

Presumptive identification of *S. aureus*. Identification began by observing colony characteristics (presence and type of hemolysis, color and texture). The following 3 biochemical tests were then carried out: tube coagulase test ([Sperber and Tatini., 1975](#)), mannitol salt test ([Cervantes-García et al., 2014](#)) and DNase test ([Menzies, 1977](#)).

Molecular identification of *S. aureus*. The polymerase chain reaction (PCR) technique was used, since it allows the identification of pathogens providing high sensitivity and specificity, being the gold standard test for the identification of species ([El-Sayed et al., 2017](#)).

DNA extraction. The lysostafin extraction method proposed by [Chapaval et al., \(2008\)](#) was carried out, with some modifications. From the *S. aureus* cultures seeded on blood agar for 18-24 hours in incubation, 2 roasts were taken and placed in 200 μ L of a TE solution (10 mM Tris-1 mM EDTA), pH 8. Subsequently, it was added 2 μ L of lysostafin (1 mg/mL) and incubated for 2 hours at 37 °C, 200 μ L of the phenol-chloroform-isoamyl alcohol mixture (25-24-1) were added, it was stirred at high speed (3,000 rpm) in vortex for 3 seconds and centrifuged at 12,000 rpm for 10 minutes; 150 μ L of the supernatant was transferred to another tube and 60 μ L of 5 M NaCl and 300 μ L of 96% ethanol were added and the mixture was placed at -20 °C for 2 h to precipitate the DNA. Subsequently, it was centrifuged at 12,000 rpm for 15 min and the supernatant was discarded, taking care not to discard the pellet formed. Then 200 μ L of 70% ethanol was added and centrifuged at 12,000 rpm for 10 minutes, discarding the ethanol. The tubes were kept open in front of the burner for 30 min so that the traces of ethanol evaporated. Finally, 50 μ L of TE, pH 8, were placed.

Primers and PCR Program.

The following primers were used for the amplification of the 23 rDNA gene

Sequence 5 '(ACG GAG TTA CAA AGG ACG AC)

Sequence 3 '(AGC TCA GCC TTA ACG AGT AC)

And for the amplification of the gene the following program was used:

Stage 1 (1 cycle): step 1. Denaturation (94 °C for 5 min)

Stage 2 (37 cycles): step 1. Denaturation (94 °C for 40 s)

Step 2. Alignment (58 ° C for 60 s)

Step 3. Extension (72 ° C for 75 s)

Stage 3 (1 cycle): step 1. Extension (72 ° C for 5 min) ([El-Sayed et al., 2006b](#)).

Electrophoresis. For electrophoresis, a 1% agarose gel was used, which was run at 70 v for 40 min, using 1x TBE buffer (89 mM Tris-89 mM Borate-2 mM E.D.T.A), pH 8.

Determination of genetic variation. The pulsed field electrophoresis (PFGE) technique was used, the same technique used by [Sato et al., \(2017\)](#), to determine the genetic variability of *S. aureus*. Only the strains diagnosed positive for *S. aureus* by PCR were considered for the determination of genetic variation. In total 32 *S. aureus* strains were selected.

The technique used by [Castañeda et al., \(2011\)](#) was developed, with some modifications. The strains were inoculated on blood agar plates and incubated at 37 °C for 18 hours. From each bacterial strain a hoe was taken from the culture and suspended in 200 µL of the PIV solution (0.01 mM Tris, 1M NaCl) pH 8, and adjusted to an optical density of 1.5 with an absorbance level of 610 nm. From this solution, 60 µL of each sample were taken and mixed with 60 µL of 1% agarose (dissolved in TE), kept at a temperature of 45 °C. Subsequently, they were transferred to a compartment of the block mold and waited about 15 minutes for them to solidify. The blocks were transferred to a 1.5 mL tube containing: 0.5 mL of the lysis solution (6 mM Tris-1 M NaCl-100 mM EDTA-0.2% sodium deoxycholate - 0.5% sarcosyl), 25 µL of brij 58 (at 10%), 5 µL of lysozyme (50 mg/mL), and 5 µL of lysostafin (1 mg/mL); it was mixed and incubated at 37 °C for 21 hours. The solution was decanted and replaced by 0.5 mL of the ES solution (500 mM E.D.T.A-1% sarcosyl) and 25 µL of proteinase K (20 mg/mL) were added. They were incubated at 56 °C for 21 h. The ES solution was decanted and the blocks were washed 5 times, shaking gently for 20 min with 10 mL of TE (10 mM Tris-1 mM EDTA) for each wash. A 3 mm slice was cut from each block.

For digestion, 100 µL of the restriction solution were added: 87.75 µL water, 10 µL buffer (10x), 1.25 µL *Sma*I enzyme (12.5 U per sample) and 1 µL bovine serum albumin (BSA). It was left to incubate for 3 hours at 25 °C. Subsequently the slices were placed inside a

1% agarose gel in TBE 0.5x pH 8. Electrophoresis was carried out in 7 L 0.5x TBE pH 8 in the Chef-DR II apparatus (Bio-Rad).

The program used was: Voltage 6 V/cm, temperature 14 °C; initial time 5 s; final time 40 s; and duration 21 h. Subsequently, the gel was stained and the image was captured on a photodocumenter.

The determination of the genetic variation was carried out by analyzing the images of the gels, obtained using the PFGE technique, from dendrograms for the determination of kinship, with the help of the Bionumerics® software (Applied Maths, Kortrijk, Belgium). The Dice correlation coefficient and the unweighted pair group mathematical average grouping algorithm (UPGMA) were used. A dendrogram of similarity of the total of the samples and 3 other dendrograms (one for each sampled region) was elaborated. Genetic similarity percentages could be obtained with the elaboration of the dendrograms.

RESULTS

Mastitis frequency. Of the 1,340 mammary glands corresponding to the 335 cows sampled, the following results were obtained. Analyzing 1,325 functional mammary glands and 15 blind glands (non-functional), a frequency of 42.71% of mastitis was observed, of which clinical mastitis represented 2.26% and subclinical mastitis 40.45%.

Frequency of etiological agents. Of 1,325 samples collected from milk, a positive growth of *S. aureus* was obtained in 130 samples (corresponding to 9% of the total) and a growth of 261 of coagulase negative staphylococci (18% of the total). Therefore, it reflects the genus *Staphylococcus* spp importance, since it represented a growth of 27% of total samples

Genetic variation of *S. aureus* using the pulsed field electrophoresis (PFGE) technique. The following genetic profiles (pulsotypes) of 32 representative *S. aureus* isolated strains from stables were obtained using the PFGE technique (Figure 1).

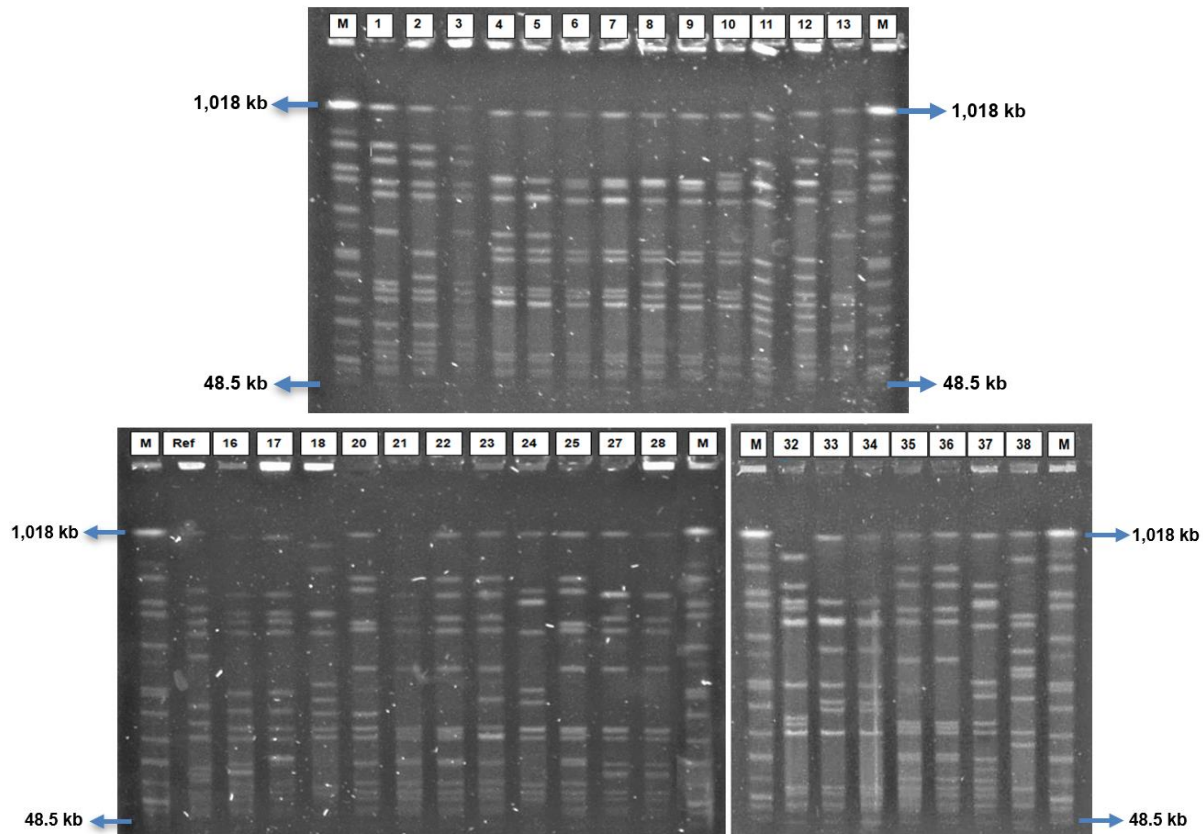


Figure 1. Pulsed field electrophoresis of DNA (after digestion with the restriction enzyme *smaI*) of the 32 isolated *S. aureus* strains from dairy cows in Jalisco state. Where M = Molecular weight marker from 48.5kb to 1,018kb (New England Biolabs), Ref = *S. aureus* reference strain (ATCC 25923) and number from 1 to 38 = the total number of isolates (32).

Dendrograms were prepared to determine *S. aureus* genetic variation. The 32 pulsotypes of the strains studied were grouped into 12 different pulsotype groupings, with 95% or more genetic similarity within each pulsotype group.

A percentage of genetic similarity of 85.1% was obtained, which corresponds to 14.9% of genetic variation of the 32 pulsotypes of isolated strains, of 10 municipalities sampled in Jalisco state. The grouping of pulsotypes (p3), was the most found grouping 25% (8/32) of total pulsotypes of analyzed strains (Figure 2).

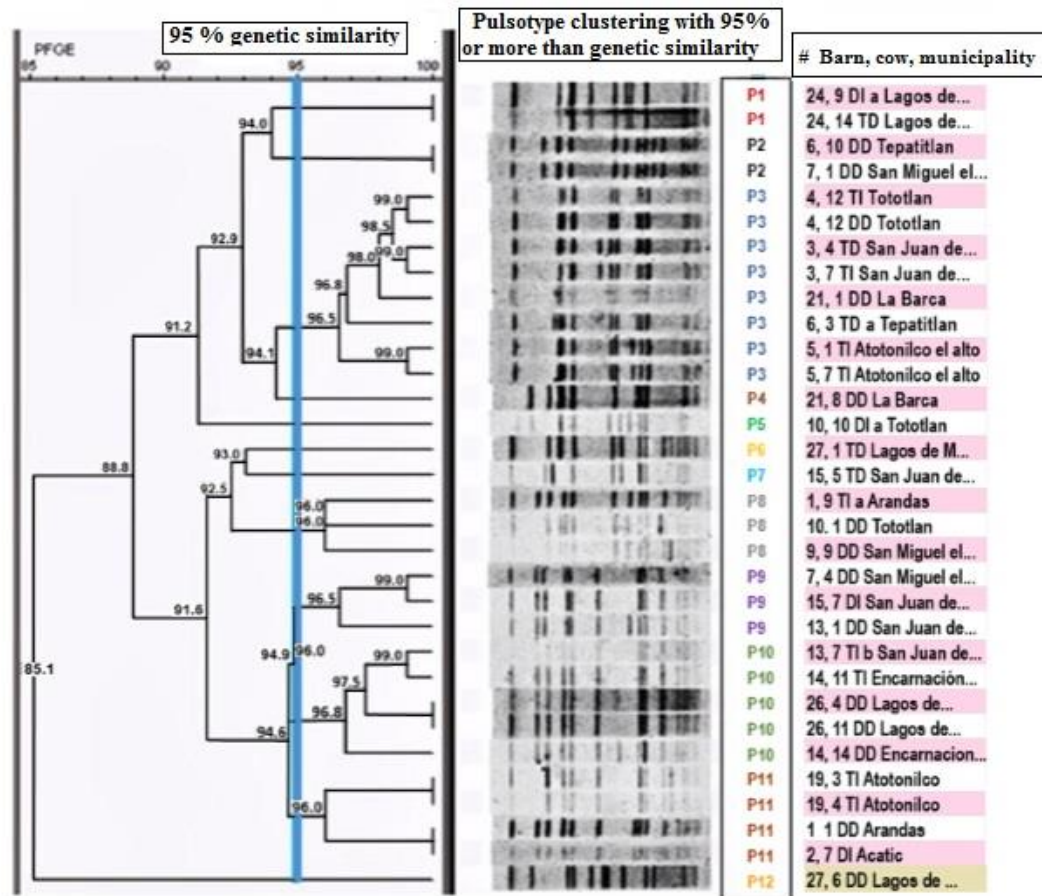


Figure 2. Pulsotype dendrogram of 32 *S. aureus* isolated strains in different regions from Jalisco state

In pulsotype dendrogram of isolated strains of Altos Norte region, 84.9% of genetic similarity was observed, which corresponds to 15.1% of genetic variation of 14 isolates from Encarnación de Díaz, Lagos de Moreno and San Juan de los Lagos municipalities (Figure 3).

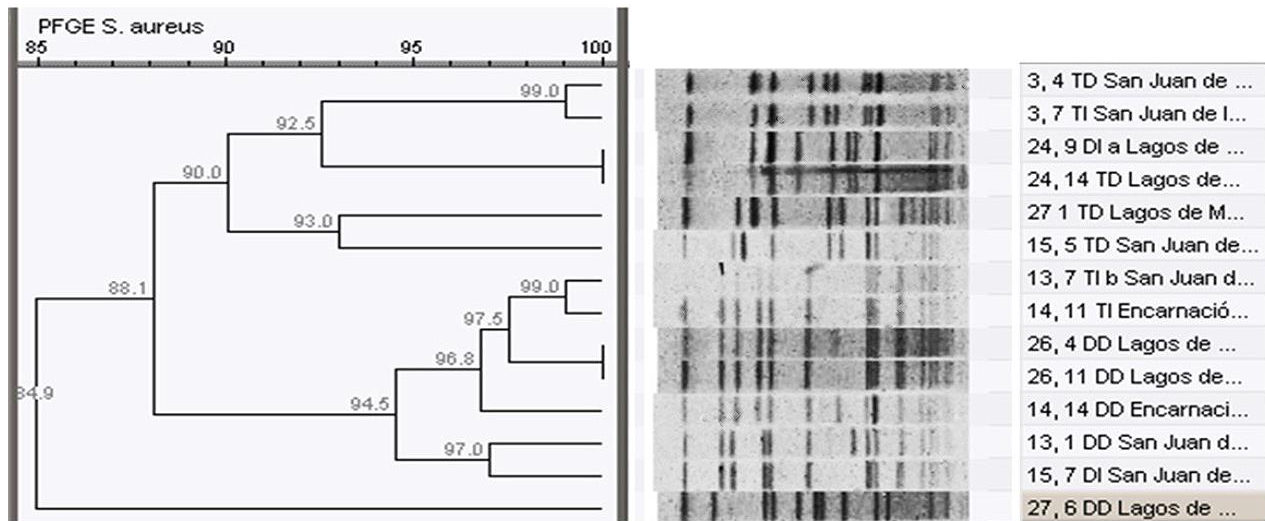


Figure 3. Pulsotype dendrogram of 14 isolated *S. aureus* strains in the Altos Norte region from Jalisco state

In pulsotype dendrogram of isolated strains from Altos Sur region, an 89.8% genetic similarity was observed, which corresponds to 10.2% of genetic variation of 8 isolated strains from of Acatic, Arandas, San Miguel el alto and Tepatitlán de Morelos municipalities (Figure 4).

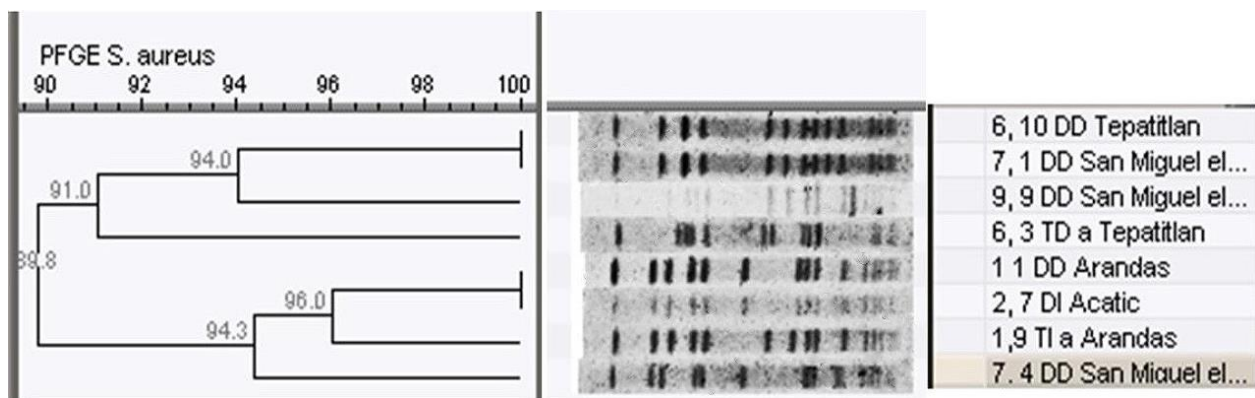


Figure 4. Pulsotypes dendrogram of 8 isolated *S. aureus* strains in Altos Sur region from Jalisco state

In pulsotype dendrogram of isolated strains from Ciénega region, a percentage of 90.2% of genetic similarity was observed, which corresponds to 9.8% of genetic variation of 10 isolates from Atotonilco el Alto, the Barca and Tototlán municipalities (Figure 5).

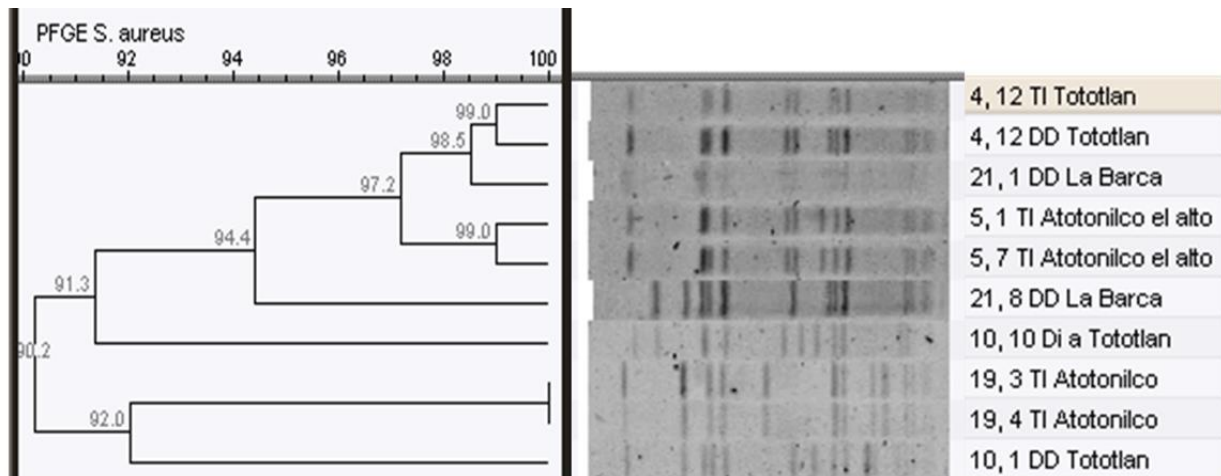


Figure 5. Dendrogram of the pulsotypes of 10 strains of *S. aureus* isolated in the Ciénega region of Jalisco state

Within the relationship of the morphological characteristics of the strains studied with their genetic profile (pulsotype) in the genetic variation technique, it was observed that the *S. aureus* strains that were identical in the PFGE technique (100% similarity) presented identical morphological characteristics. While the strain (38. 27, 6 DD) stood out from all the others in its culture, as it showed a pronounced beta hemolysis of 15 mm; in the same way, it was the strain that presented the pulsotype with the greatest genetic variation in the PFGE technique.

DISCUSSION

From the findings found, it is established that there are genetic variations greater than 10% of *Staphylococcus aureus* isolated from dairy cows in Jalisco state, finding 14.9% genetic variation among 32 strains analyzed in this study. The results related to the genetic variation observed in the present work are similar to those cited by [Can et al., \(2017\)](#), who found a genetic relationship of 90% of strains isolated from raw cow's milk in Turkey, which corresponds to the 10% genetic variation. In the same study, *S. aureus* strains obtained from raw cow's milk samples, meat, cheese and chicken samples in Turkey were analyzed together; where the genetic variation increased up to 25%, which means that there are types of genetically similar genetic profiles in the milk samples; perhaps because the *S. aureus* isolated from milk present a relatively similar genotype, since they require the same characteristics that allow them to be adapted, both inside and outside the mammary gland.

The results shown in this study are similar to the results made by [Castañeda et al., \(2011\)](#), who concluded that there is a close genetic relationship of *S. aureus*, responsible for mastitis; suggesting that closely related clones of *S. aureus* are responsible for most of the intramammary infections caused in Jalisco state. However, they differ from the results generated with those obtained by [Sato et al., \(2017\)](#), who found genetic variations of up

to 30% of 41 *S. aureus* strains isolated in Japan; perhaps because isolated strains from humans and meat destined for sale will also be taken. Therefore, the high variation percentage shown seems understandable, since the strains usually develop elemental characteristics that allow them to adapt to site conditions from which they are isolated.

Although a high percentage (42.71%) was obtained in the mastitis frequency in the sampled cows, only around 20% of the sampled mammary glands represented clinical mastitis and grades 2 and 3 of subclinical mastitis. Therefore, around 80% of glands were identified as negative for mastitis and grade 1 subclinical mastitis. On the other hand, in a study carried out by [Manjarrez et al., 2012](#), a higher frequency of mastitis was found in Mexico state; who cite 6.1% of clinical mastitis and 48.3% of subclinical mastitis, this being understandable since only small family-type stables were sampled, which presented little technification level. This represents deficiencies in the milking technique and little control of mastitis, differing from the sampling of our study in which, in addition to family stables, semi-technical and technical stables were chosen; therefore, the frequency of mastitis obtained in our study was lower.

On the other hand, [Yera y Ramírez, 2016](#) recorded a *S. aureus* appearance frequency of 8.75% in crossbred Holstein x Cebu cows, a frequency lower than that obtained which was 9.8%; perhaps due to a better resistance to infection in the animals sampled as they are hybrid animals. [Boscán et al., \(2009\)](#), observed a *S. aureus* appearance frequency of 12.15%, higher than that presented in this study, also differing from the sampling methodology, since milk samples were taken only in cows diagnosed with subclinical mastitis and that were at the beginning of the dry period. [Castañeda et al., \(2013\)](#) in Jalisco state, showed a *S. aureus* frequency at the barn level of 100%, the frequency being higher than that shown, it was 66.7%; probably due to the fact that in this study only milk belonging to glands diagnosed with mastitis was sown. This was the opposite of our study, in which all milk samples were seeded regardless of their result in the diagnosis of mastitis.

CONCLUSIONS

The grouping of *S. aureus* P3 pulsotypes represented 25% of the total genetic profiles (pulsotypes) of isolated strains (8/32), being present in all the regions that were sampled from Jalisco. The genetic variation compared to the morphological variation was identical. Likewise, the isolated strains within the same stable showed a remarkable genetic similarity, reaching 100% similarity in several stables. The results of the present study provide valuable information that may be complementary in the area of molecular epidemiology necessary for the control, treatment and production of the necessary vaccines for mastitis caused by *S. aureus*.

CITED LITERATURE

- BABA T, Takeuchi F, Kuroda M, Yuzawa H, Aoki KC, Oguchi A, Nagai Y, Iwama N, Asano K, Naimi T, Kuroda H, Cui L, Yamamoto K, Hiramatsu K. 2002. Genome and virulence determinants of high virulence community acquired MRSA. *Lancet*. 359: 1819-1827. [https://doi.org/10.1016/S0140-6736\(02\)08713-5](https://doi.org/10.1016/S0140-6736(02)08713-5)
- BARNUM DA, Newbould FHS. 1961. The use of the California mastitis test for the detection of bovine mastitis. *The Canadian Veterinary Journal*, 2(3), 83. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1585631/>
- BEDOLLA CC, Ponce de León MER. 2008. Pérdidas económicas ocasionadas por la mastitis bovina en la industria lechera. *Rev Electrón Vet*. 9: 1-26. <http://www.redalyc.org/articulo.oa?id=63611952010>
- BEDOLLA CC, Castañeda VH, Wolter W. 2007. Métodos de detección de mastitis bovina. *Rev Electrón Vet*. 8: 1-17. <http://agris.fao.org/agris-search/search.do?recordID=DJ2012036579>
- BOSCÁN J, Villaroel R, Oviedo A, Sánchez A, Pino D, García D, Hernández L, Pérez M. 2009. Bacterias patógenas potenciales al inicio del período seco de vacas doble propósito con mastitis subclínicas. *Revista Científica de Maracaibo*. 19: 277- 283. <http://www.redalyc.org/articulo.oa?id=95911669010>
- CALDERÓN A, Rodríguez V. 2008. Prevalencia de mastitis bovina y su etiología infecciosa en sistemas especializados en producción de leche en el altiplano cundiboyacense (Colombia). *Revista colombiana de ciencias pecuarias*. 21: 582-589. <https://dialnet.unirioja.es/servlet/articulo?codigo=2897973>
- CAN HY, Elmalı M, Karagöz A. 2017. Molecular typing and antimicrobial susceptibility of *Staphylococcus aureus* strains isolated from raw milk, cheese, minced meat, and chicken meat samples. *Korean J Food Sci An*. 37: 175. <https://doi.org/10.5851/kosfa.2017.37.2.175>
- CASTAÑEDA VH, Jäger S, Wolter W, Zschöck M, Castañeda VMA, El-Sayed A. 2011. Genotyping of *Staphylococcus aureus* isolated from dairy herds in México. *Rev Científic*. 21: 308-316. <http://www.redalyc.org/articulo.oa?id=95918727005>
- CASTAÑEDA VH, Jäger S, Wolter W, Zschöck M, Castañeda VMA, El-Sayed A. 2013. Isolation and identification of main mastitis pathogens in Mexico. *Arq Bras Med Vet Zoo*. 65:377-382. <https://doi.org/10.1590/S0102-09352013000200012>

CASTAÑEDA VH, Wolter W, Serratos JM, Castañeda VM, Salas CEP, Alvarez-Moya C. 2018. Avances en las investigaciones de *Staphylococcus aureus* como agente patógeno causante de mastitis bovina, mediante biología molecular. En: Bienestar animal en la práctica, en producciones lecheras, desde la perspectiva europea. Edited and published by Frank JCM, Van Eerdenburg. Pp. 108-121.

<https://www.dropbox.com/s/q9jdztauo43io26/Bienestar%20animal%20en%20la%20practica.pdf?dl=0>

CERVANTES-GARCÍA E, García-González R, Salazar-Schettino PM. 2014. Características generales del *Staphylococcus aureus*. *Rev Latinoam Patol Clin Med Lab*. 61: 28-40. <https://www.medigraphic.com/cgi-bin/new/resumen.cgi?IDARTICULO=48300>

CHAPAVAL L, Moon DH, Gomes JE, Duarte FR, Tsai SM. 2008. An alternative method for *Staphylococcus aureus* DNA isolation. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*: 60(2):299-306. https://www.scielo.br/scielo.php?pid=S0102-09352008000200004&script=sci_arttext

ECHEVERRI JJ, Jeramillo MG, Restrepo LF. 2010. Evaluación comparativa de dos metodologías de diagnóstico de mastitis en un hato lechero del departamento de antioquia. *Rev. Lasallista investig.* 7: 49-57. <http://www.redalyc.org/articulo.oa?id=69514965007>

EL-SAYED A, Alber J, Lämmle C, Abdulmawjood A, Zschöck M, Castaneda VH. 2006a. Comparative sequence analysis of spa gene of *Staphylococcus aureus* isolated from bovine mastitis: characterization of an unusual spa gene variant. *J dairy Res*. 73: 322-327. <https://doi.org/10.1017/S002202990600183X>

EL-SAYED A, Alber J, Lämmle C, Jäger S, Wolter W, Castañeda-Vázquez H. 2006b. Comparative study on genotypic properties of *Staphylococcus aureus* isolated from clinical and subclinical mastitis in Mexico. *Vet Mex*. 37: 165-179. <https://www.medigraphic.com/cgi-bin/new/resumenI.cgi?IDREVISTA=39&IDARTICULO=8854&IDPUBLICACION=976>

EL-SAYED A, Walid Awad, Nadra-Elwgoud Abdou, Hugo Castañeda Vázquez. 2017. Molecular biological tools applied for identification of mastitis causing pathogens, *International Journal of Veterinary Science and Medicine*. 5(2):89-97. <https://www.tandfonline.com/doi/full/10.1016/j.ijvsm.2017.08.002>

GAMBOA ME. 2017. Estadística aplicada a la investigación científica. En J.C. Arboleda (Ed.). Apropiación, generación y uso solidario del conocimiento (pp. 59-76). Las Tunas, Cuba: Editorial Redipe-Edacun. ISBN 9781945570148 <http://roa.ult.edu.cu/handle/123456789/3667>

GARCÍA GM, Montoya GN, López VM, Aguilar MSW, Salvador Lagunas BS, Valladares CB, Vázquez ChJC, Castañeda H, Velázquez OV. 2019. Caracterización de los ecotipos de *Staphylococcus aureus* en hatos lecheros de producción familiar en el Valle de Toluca, México. Pp. 455-492. En: Temas selectos en la Innovación de las Ciencias Agropecuarias, Ciudad de México: Alfaomega Grupo Editor: Ciudad de México: Universidad del Estado de México. 2019. ISBN 9786075384115

KURODA Makoto Toshiko Ohta, Ikuo Uchiyama, Tadashi Baba, Harumi Yuzawa, Ichizo Kobayashi, Longzhu Cui, Akio Oguchi, Ken-ichi Aoki, Yoshimi Nagai, JianQi Lian, Teruyo Ito, Mutsumi Kanamori, Hiroyuki Matsumaru, Atsushi Maruyama, Hiroyuki Murakami, Akira Hosoyama, Yoko Mizutani-Ui, Noriko K Takahashi, Toshihiko Sawano, Ryu-ichi Inoue, Chikara Kaito, Kazuhisa Sekimizu, Hideki Hirakawa, Satoru Kuhara, Susumu Goto, Junko Yabuzaki, Minoru Kanehisa, Atsushi Yamashita, Kenshiro Oshima, Keiko Furuya, Chie Yoshino, Tadayoshi Shiba, Masahira Hattori, Naotake Ogasawara, Hideo Hayashi, Keiichi Hiramatsu. 2001. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet*. 357:1225-1240. [https://doi.org/10.1016/S0140-6736\(00\)04403-2](https://doi.org/10.1016/S0140-6736(00)04403-2)

MANJARREZ LAM, Díaz ZS, Salazar GF, Valladares CB, Gutiérrez CADC, Barbabosa PA, Talavera RM, Alonso FMU, Velázquez OV. 2012. Identificación de biotipos de *Staphylococcus aureus* en vacas lecheras de producción familiar con mastitis subclínica en la región centro-este del Estado de México. *Rev Mex Cienc Pecu*. 3:265-274. http://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S2007-11242012000200008

MARQUÉS VF, De Souza MMS, Mendonça ECL, Alencar TA, Pribul BR, Coelho SMO, Lasagno M, Reinoso EB. 2013. Análise fenotípica e genotípica da virulência de *Staphylococcus* spp. e de sua dispersão clonal como contribuição ao estudo da mastite bovina. *Pesq. Vet. Bras*. 33:161-170. http://www.scielo.br/scielo.php?pid=S0100-736X2013000200005&script=sci_arttext

MENZIES, R. E. 1977. Comparison of coagulase, deoxyribonuclease (DNase), and heat-stable nuclease tests for identification of *Staphylococcus aureus*. *Journal of clinical pathology*, 30(7), 606-608. <https://jcp.bmj.com/content/30/7/606.abstract>

SATO T, Usui M, Konishi N, Kai A, Matsui H, Hanaki H, Tamura Y. 2017. Closely related methicillin-resistant *Staphylococcus aureus* isolates from retail meat, cows with mastitis, and humans in Japan. *PloS one*. 12: e0187319. <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0187319>

SCHUKKEN YH, Günther J, Fitzpatrick J, Fontaine CM, Goetze L, Holst O, Leigh J, Petzl W, Schuberth JH, Sipka A, Smith EDG, Quesnell R, Watts J, Yancey R, Zerbe H, Gurjar A, Zadoks RN, Seyfert HM. 2011. Host-response patterns of intramammary infections in dairy cows. *Vet Immunol Immunop.* 144: 270-289. <https://doi.org/10.1016/j.vetimm.2011.08.022>

SCHALM OW, Noorlander DO. 1957. Experiments and observations leading to development of the California Mastitis Test. *JAVMA.* 130:199-204.

SHARMA-KUINKEL BK, Rude TH, Fowler VG. 2016. Pulse-field gel electrophoresis. En Bose JL. *The Genetic Manipulation of Staphylococci: Methods and Protocols.* Pp. 117-130. <https://link.springer.com/book/10.1007/978-1-4939-3158-3>

SPERBER, W. Z., y Tatini, S. R. 1975. Interpretation of the tube coagulase test for identification of *Staphylococcus aureus*. *Appl Microbiol.* 29: 502-505. <https://aem.asm.org/content/29/4/502.short>

STRUELENS MJ, Hawkey PM, French GL, Witte W, Tacconelli E. 2009. Laboratory tools and strategies for methicillin-resistant *Staphylococcus aureus* screening, surveillance and typing: state of the art and unmet needs. *Clin Microbiol infect.* 15: 112-119. <https://doi.org/10.1111/j.1469-0691.2009.02698.x>

VAN SFJ, Santman-Berends IM, Lam TJ, Hogeveen H. 2016. Failure and preventive costs of mastitis on Dutch dairy farms. *J Dairy Sci.* 99:8365-8374. <https://doi.org/10.3168/jds.2015-10561>

VASCONCELOS NG, Ribeiro de Souza da Cunha MI. 2010. Staphylococcal enterotoxins: Molecular aspects and detection methods. *Journal of Public Health and Epidemiology.* 2(3):29-42. ISSN 2141-2316. <http://www.academicjournals.org/jphe>

WOLTER W, Castañeda H, Kloppert B, Zschock M. 2004. Mastitis Bovina. Ed., Editorial Universitaria. Pp. 18-48. ISBN. 3-7862-0099-8.

YERA PG, Ramírez W. 2016. La Prevalencia de mastitis clínica en vacas mestizas Holstein x Cebú. *REDVET.* 17:1-7. <http://www.redalyc.org/articulo.oa?id=63646040004> - <http://www.veterinaria.org/revistas/redvet/n030316.html>

ZSCHÖCK M, Sommerhaeuser J, Castaneda VH. 2000. Relatedness of *Staphylococcus aureus* isolates from bovine mammary gland suffering from mastitis in a single herd. *J Dairy Res.* 67: 429-435. <https://doi.org/10.1017/S0022029900004325>