

Análisis de los factores de virulencia de *Staphylococcus aureus* aislados de mastitis bovina en México

Virulence factors analysis of *Staphylococcus aureus* isolated from bovine mastitis in México

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RESUMEN. Un total de 117 aislados bacterianos obtenidos de casos de mastitis bovina positivos a las pruebas de catalasa, coagulasa, termonucleasa, manitol y PCR fueron analizados para determinar la producción de los siguientes factores de virulencia: hemolisinas (alfa y beta), factor de agregación y cápsula. Adicionalmente fue también evaluada la sensibilidad a oxitetraciclina, penicilina, oxacilina y estreptomycin. La producción de hemolisinas y el factor de agregación fueron los 2 factores de virulencia expresados en la mayoría de los aislados (78%) y (83%) respectivamente, mientras que la formación de cápsula se detectó en 31% de los aislados bovinos. Una fuerte correlación entre ausencia de cápsula y producción de hemolisinas fue observado, ya que 72 de un total de 92 cepas hemolíticas no mostraron cápsula. La ausencia de cápsula fue asociada a la producción del factor de agregación en 89% de los aislados bacterianos positivos a este factor. El 68% y 69% de los aislados bacterianos resultaron positivos a penicilina y oxacilina respectivamente. La mayoría de los aislados (116) expresaron al menos 1 factor de virulencia (hemolisinas, cápsula o factor de agregación). Finalmente, el papel de los factores de virulencia y su relación en la patogenicidad de *S. aureus* en la mastitis bovina en México es discutido.

Palabras clave: *Staphylococcus aureus*, mastitis, hemolisinas, cápsula, factor de agregación.

Abstract. A total of 117 bacterial isolates obtained from bovine mastitis and positives to catalase, coagulase thermonuclease, mannitol and PCR tests were analyzed for virulence factor production: hemolysins (alpha and beta), clumping factor, and capsule. Additionally penicillin, oxacillin, streptomycin and oxytetracycline sensitivity were determined. Hemolysins and clumping factor production were two virulence factors expressed in the majority of the isolates (78 %) and (73 %) respectively, whereas that capsule formation was detected in 31 % of the bovine isolates. A strong relationship between capsule absence and hemolysins production was observed since 72 of the 92 hemolytic strains were non capsulated. The absence of capsule was associated to Clfa production in 89 % of the isolates positive to this factor. Sixty eight and sixty nine percent of the bacterial isolates were resistant to penicillin and oxacillin, respectively. Most of the isolates (116) expressed at least one virulence factor (hemolysins, capsule, clumping factor). The role of virulence factors and their relationship in the pathogenicity of *S. aureus* in bovine mastitis in México is under discussion.

Keywords: *Staphylococcus aureus*, mastitis, hemolysins, capsule, clumping factor.



Introduction

Bovine mastitis an inflammation of mammary glands usually due to a microbial infection [1], is the most important cause of losses in the dairy industry. These losses are mainly due to low milk yield, low milk quality, and high production costs. Although several bacterial pathogens can cause the disease, *Staphylococcus aureus* is the main agent responsible for contagious bovine mastitis, and is very difficult to eradicate given the wide range of virulence factors that contribute to the ability of the bacteria to survive in the host [2, 3]. Virulence factors may be divided in three functional categories: Factors that mediate adhesion of bacteria to host cells; those that produce tissue damage; and those that protect the bacteria against the host's immune system and antibiotics. Adhesion to epithelial cells is the initial step in the process of infection by *S. aureus* [4]; binding to various components of the extracellular matrix, such as fibrinogen, fibronectin and collagen allow the bacteria to colonize damaged tissues [5, 6, 7]. Clumping factor A (Clfa) is considered one of most important adhesion factors and has been identified as a virulence factor in an endocarditis model [8]. Additionally, *S. aureus* produce a wide variety of exoproteins, including hemolysins that contribute to their ability to colonize and cause disease in mammalian hosts. Alpha hemolysin is the most studied of the *S. aureus* cytotoxins [9]. Beta toxin is a sphingomyelinase expressed by most strains isolated from bovine intra-mammary infections, but rarely by human isolates [10]. It has been reported that both toxins increase *S. aureus* adherence to mammary epithelial cells [10, 11]. The capsule has been shown to promote *S. aureus* virulence in several animal infection models [12]. Capsulated *S. aureus* strains are more resistant to phagocytosis than non-capsulated strains, and allow the bacteria to remain in the infected hosts [13] Even though virulence factor production by *S. aureus* has been widely described [3, 14], very few studies have been done that describe the main virulence factors phenotypes in *S. aureus* strains isolated from bovine mastitis [15]. The identification of virulence factors produced by bovine *S. aureus* increases our knowledge about target antigens for vaccination purposes. The objectives of this study were to isolate strains of *S. aureus* in bovine mastitic milk samples from Mexico, and to characterize virulence factors as a preliminary step for the development of efficient vaccines.

Materials and Methods

1. Cultures.

Milk samples were tested for the presence of *S. aureus*. The samples were collected from farms at seven different locations in Mexico (Aguascalientes, Chihuahua, Durango, Hidalgo, Jalisco, Querétaro, and Veracruz.) For the isolation of strains, 0.1 ml of milk was streaked on Baird-Parker medium (Sigma) with acriflavin 50 µg/ml. After incubation for 24 to 48 h, all the colonies formed on the plates were examined applying the following tests.

2. Biochemical and molecular tests.

A total of 117 bacterial isolates obtained from clinical and sub-clinical bovine mastitis positives in the catalase, coagulase thermonuclease, and mannitol tests (National Mastitis Council, 1990) were selected to perform confirmatory tests for species determination *Staphylococcus aureus* by PCR amplification of DNA coding for 23S rRNA using the primers: forward (cgattcccttagtagcgcg) and reverse (ccaatcgcacgcttcgcta) [16]. Loopfuls of 117 *S. aureus* strains were suspended in 50 µl of distilled water into eppendorf tubes. The cellular suspension was brought to a boil during 10 min, and immediately was centrifuged at 14,000 RPM for 5 min. The supernatant was directly used for the PCR assay.

The PCR conditions were: 94°C for 45 seg; followed by 25 cycles of 1 min at 94°C, 1 min at 70°C, and 1 min at 72°C; finally 10 min at 72°C.



3. Virulence factors determination

Hemolysin production: The isolates were assayed for hemolysis after overnight incubation at 37°C onto sheep agar blood (Becton Dickinson). A sample was taken from each pure culture of *S. aureus* in tripticase soy agar, and was inoculated in a flask containing 20 ml of tripticase soy broth which was incubated at 37°C at 250 rpm for a period of 18 h. 1 ml of the culture was centrifuged at 14,000 RPM, for 5 min, to obtain the supernatant. This supernatant was filtered through 0.45µm millipore membranes and 10 µl of supernatant was placed onto 4mm diameter filter paper disc previously placed on blood agar. These plates were incubated for 24 h at 37°C. Hemolytic activity was recorded as alpha hemolysis, beta hemolysis, double hemolysis (alpha + beta), and no hemolysis.

Serum Soft-Agar (SSA) technique: Appropriately diluted suspensions of each strain were added to medium BHI with normal rabbit serum (1% v/v), and incubated at 37°C up to 18 h, after which colonial morphology was evaluated. Diffuse colony morphology were considered to be capsulated, and compact growth represented non-capsulated organisms [17].

Antibiotic susceptibility testing was performed using the disk diffusion method on Muller-Hinton agar plates [18]. The antibiotics utilized were oxytetracycline, oxacillin, streptomycin and penicillin.

Clumping Factor Determination: The clumping factor was determined by using the technique described by Kosaku [19]. Loopfuls of 117 *S. aureus* strains were grown in a tripticase soy broth for 18 to 24 h from a sample of a pure colony previously isolated in a solid medium. A positive result usually occurs in 5-20 seconds, and a negative result is given if the coagulation does not occur in 3-4 minutes.

Results

A total of 117 isolates positive to biochemical tests were selected to perform confirmatory tests for *S. aureus* by PCR amplification of DNA coding for 23S rRNA. The presence of a band of 1267 bp on agarose gel was an indicative of *S. aureus*. DNA of the *S. aureus* strain CECT 5192 was used as positive control (Fig. 1). In order to characterize *S. aureus* isolates, three of the main virulence factors were analyzed: hemolysin production, clumping factor, and capsule formation; additionally, antimicrobial resistance was determined.

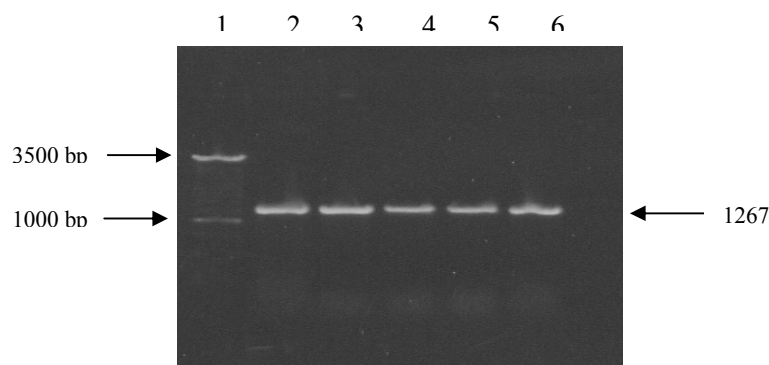


Figure 1. Specificity of the molecular probe amplification by PCR using DNA of *S. aureus* isolates from bovine mastitis. Lane: 1) 250-bp DNA ladder; 2) *S. aureus* CECT 5192; 3) *S. aureus* HT-7; 4) *S. aureus* HT-29; 5) *S. aureus* HT-45; 6) *S. aureus* HT-48; 7) Negative control

Hemolysin production was detected in 78 % of the *S. aureus* isolates. Fourteen isolates (12 %) were alpha hemolysin, thirty-four (29 %) beta hemolysin, and forty-four (37 %) showed both hemolysins; whereas 21 %, was non-hemolytic. Beta hemolysin was most frequently synthesized by *S. aureus* strains, 78 of the isolates produced the toxin, either alone or combined with alpha hemolysin (Table 1). Capsule formation was evaluated using the (SSA) technique; according to the results, a high percentage of the isolates (69 %) were not capsulated, whereas in 31 % this structure was detected. Of the 36 capsulated isolates, we detected a subset of 12 bovine *S. aureus* isolates that expressed a weak diffuse morphology (data not shown). Clumping factor was detected in 85 (73 %) of the *S. aureus* isolates, whereas in 32 (27 %) this protein was not observed (Table 1). Four compounds which are frequently used in mastitis therapy were tested for their antimicrobial activity against bovine strains of *S. aureus*. Eighty strains of *S. aureus* were resistant to penicillin. Eighty-one strains showed resistance to oxacillin, while only 27 and 24 strains were resistant to streptomycin and oxytetracyclin, in that order (Table 1).

In order to assess the potential role of the three virulence factors in the pathogenesis of *S. aureus* in bovine mastitis, an analysis of the factor combinations detected in the isolates was performed. Hemolysins-Clfa production was the combination most frequently found in the isolates (55%), while that hemolysins-capsule production was detected in only 8.5% of the isolates (Table 2). Additionally, a strong relationship between the absence of a capsule and hemolysins production was observed, as 71 of a total of 92 hemolytic isolates did not show a capsule (Table 2).

A significant relationship between the absence of a capsule and Clfa production was observed, 89% of the strains positive to Clfa did not show capsule formation (Table 2). The virulence factors analysis revealed that most of the isolates (116) expressed at least one virulence factor (hemolysins, capsule, or clumping factor). Virulence factors expression was observed in 66% of the isolates associated with the synthesis of two factors. Only 8.5% of the isolates showed the presence of the 3 virulence factors simultaneously (Table 2).

Discussion

An efficient vaccine against bovine mastitis is not yet available, and prevention and control of mastitis requires identification of the main antigenic determinants for the design and development of more efficient vaccines. In this work, the frequency of *S. aureus* alpha and beta hemolysins was evaluated. Beta hemolysin was the main toxin detected in the bovine mastitis isolates. This result is consistent with other studies [15]. *S. aureus* isolates from peracute and acute mastitis have been reported to produce larger amounts of Beta toxin than *S. aureus* isolated from chronic infections [20]. Alpha hemolysin is considered a main pathogenic factor because its hemolytic, dermonecrotic, and neurotoxic activity [9]. In this study we observed a low frequency of alpha hemolysin producing isolates. In the majority of the strains the synthesis of alpha hemolysin was associated with beta hemolysin (Table 1). These results agree with Da Silva et al [21], but contrast with the high proportion of alpha hemolytic strains described by Kenny et al [22].

Table 1. Virulence factors determination of *S. aureus* isolated from bovine mastitis in México.

	Hemolysins				Capsule		Clumping Factor		Antibiotic Resistance			
	α	β	$\alpha\beta$	None	Positive	Negative	Positive	Negative	Penicillin	Oxacillin	Streptomycin	Oxitetracycline
Strains number	14	34	44	25	36	81	85	32	80	81	27	24
Frequency (%)	12	29	37	22	31	69	73	27	68	69	23	20

Serologic evaluation has been performed on identified capsular polysaccharide in human and bovine isolates [23, 24] Nevertheless, considerable variability has been detected in the prevalence of serotype 5 and 8 capsules among bovine isolates of *S. aureus* from different countries [12, 25]. Diffuse colony morphology in SSA, considered a criterion of capsulation, seems to be a characteristic of most *S. aureus* strains from domestic animal milk [26]. However, a significant number of *S. aureus* isolates from mastitis do not produce capsule [25]. In the current study, only 36 isolates (31%) showed diffuse morphology in the SSA. Nevertheless, compact morphology does not exclude capsulation, since some capsulated strains can not be identified with this technique [26]. Expression of the capsule is greatly influenced by various environmental signals *in vitro* and *in vivo* [27, 28], and transcription of the *cap* operon has been shown to be affected by regulatory elements such as *agr*, *sarA*, *mgr*, σ^B [29-31] and *ccpA* [32].

In this study, the absence of capsule was associated to Clfa production in 89 % of the isolates positive a this factor (Table 2); Clumping factor reaction has been used for the identification of encapsulated strains of *S. aureus* from human sources [33], however in isolates from bovine mastitis this is unusable. The absence of reactivity in the Clfa detection test in some isolates may be due to a masking of Clfa by the capsule [34]. Risley et al (2007) found, that one acapsular *S. aureus* mutant strain showed a two-fold increase in platelet binding, compared to the isogenic encapsulated strains. Acapsular *S. aureus* cells may have an advantage over encapsulated strains in subclinical disease because they can persist in the mammary gland [35]. Since fibronectin-binding protein plays a crucial role in *S. aureus* adherence and internalization in bovine epithelial cells [36], masking of staphylococcal surface adhesins by the staphylococcal capsule may influence the pathogenesis in bovine mastitis. Blockage of the primary stages of *S. aureus* infection, specifically, bacterial adhesion to the cells may be the most effective strategy for preventing infections [37]. One important observation in our study was the close relationship between the absence of a capsule and hemolysin production. Seventy-two of a total of 92 hemolytic strains did not show a capsule. However, this relationship could be consequence of a normal process of genetic variability and adaptive evolution of the isolates to very special habitats that allowed that certain combinations of virulence factors were conserved.

Table 2. Combinations of detected virulence factors of *S. aureus* isolated from bovine mastitis in México

Combinations of detected factors	Isolates (n)	Isolates (%)
Hemolysins, Clumping factor, Capsule	10	8.5
Hemolysins, Clumping factor	64	55
Hemolysins, Capsule	10	8.5
Clumping factor, Capsule	3	2.5
Hemolysins	8	7
Clumping factor	8	7
Capsule	13	11
Negative	1	0.8
Total number	117	100

Among the four antibiotics tested, penicillin and oxacillin were the less effective. The high percentage observed of resistance strains to these antibiotics differ from results obtained by Pengov and Ceru [38]. The discovery of a high percentage of resistance strains to penicillin and oxacillin has important implications for

bovine mastitis control since these drugs represent the main antibiotics group recommended for staphylococcal mastitis treatment, and the emergence of resistant strains in the dairy herds could be related to the emergence of virulence strains [39]. However, in this study, no relationship between the prevalence of virulence factors and resistance to antibiotics was detected. The prevalence of antibiotic resistant strains was rather related to the region from which the isolates were obtained.

In conclusion, the high percentage of hemolysin and Clfa producing strains obtained in this work; suggest an important role of both virulence factors in the pathogenesis of bovine mastitis in Mexico. The low percentage of capsulated isolates observed in this study does not dismiss the important role played by the capsule in bovine mastitis. The presence of two or more virulence factors could increase the pathogenic ability of isolates in relation to those that express only one virulence factor, however, further research must be performed.

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