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Research Article

Staphylococcus agnetis: An Emergent Pathogen Isolated from Subclinical Mastitis with Capacity to Internalize into Bovine Mammary Epithelial Cells

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ABSTRACT

Bovine mastitis is a multifactorial disease that produces important losses in livestock and dairy industry. One of the main factors that causes this disease are microorganisms such as *Staphylococcus aureus*, which has multi-resistance to antibiotics and virulence factors such as its ability to internalize into bovine Mammary Epithelial Cells (bMECs), which allows bacteria to evade the host's immune system and persist inside the cell. However, Coagulase-Negative *Staphylococci* (CNS) have a significant role as bovine mastitis pathogens. *S. agnetis* is an emergent CNS pathogen isolated from bovine mastitis, whose capacity to internalize into bMECs is unknown. In the present work we characterized an isolate of *S. agnetis* from bovine mastitis, which was resistant to penicillin, dicloxacillin and ampicillin; also, the isolate showed resistance to methicillin through a mechanism that may be related to the presence of the *mecA* gene. In addition, *S. agnetis* internalized into bMECs (10% relative to *S. aureus* (ATCC 27543), which could be related to the presence of the *fnbpA*, *clfB* and *spa* genes. This is the first study that demonstrates the ability of internalization of *S. agnetis* into bMECs, which is a virulence factor that may represent a major problem in the management of bovine mastitis.

Keywords: Mastitis bovine; *Staphylococcus agnetis*; Internalization

INTRODUCTION

Mastitis is defined as the inflammatory response resulting of the infection and/or damage of the udder tissue, which is reported in numerous species, mainly in domestic dairy animals [1]. This pathology is the most frequent disease of dairy cattle and can be potentially fatal [2]. Mastitis is one of the diseases costliest to dairy industry associated with a reduced milk production, changes in milk composition and quality [3]. *Staphylococcus aureus* is a coagulase-positive bacterium, which is the main etiological pathogen of contagious bovine mastitis [4]. This microorganism is well known for its tolerance to wide range of antimicrobial agents and its ability to internalize and persist into bovine mammary epithelial cells, which allow it to evade the host immune system and survive inside a wide variety of mammalian cells that difficult the antimicrobial therapy [5]. Recently, coagulase-negative *Staphylococci* (CNS) have become the most common bovine mastitis isolates and are now predominant over *S. aureus* [6] and have been considered as emerging mastitis pathogens. In CNS group different microorganisms as *S. sciuri*, *S.*

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haemolyticus, *S. chromogenes*, *S. epidermidis*, *S. saprophyticus*, *S. simulans and S. agnetis*, among others, are included [7]. However, the information about the capacity of internalization of microorganisms of this group into bovine mammary epithelial cells is still limited. In particular, *S. agnetis* is a CNS bacterium isolated for the first time from bovine mastitis in 2012 [8] and later in 2015 in México [9]. Currently, the presence of *S. agnetis* in mastitis cases has been reported in various countries around the world including Brazil, USA, Canada and India [10-13]. However, to date there is no reported information on its ability to internalize into bovine mammary epithelium cells. The internalization of the bacteria in the host cell, is a virulence hallmark that makes difficult to treat the infection with conventional antibiotics, it also allows the bacteria to evade the host's immune system, as well as to persist inside the cell, which leads to the chronicity of infection and promotes its recurrence [14]. In order to increase the knowledge on this bacterium, in this work we characterized an isolate of *S. agnetis* obtained from subclinical bovine mastitis. Also, the internalization ability of this isolate into bovine mammary epithelium cells was evaluated.

MATERIALS AND METHODS

Strain and microbiological culture

Staphylococcus aureus (ATCC 27543) previously isolated from a case of bovine clinical mastitis was used in this study as strain control. This strain has recognized capacity to internalize into bovine mammary epithelial cells [15]. *S. agnetis* 394696 isolate used in this work (NCBI/EMBL accession JQ 394696) was reported by León-Galván et al. [9]. Bacteria were grown at 37°C overnight in Luria-Bertani broth (DIBICO) for conducting experiments. The Colony Forming Units (CFU) were adjusted by optical density determination (O.D. 600 nm).

Primary culture of bovine mammary epithelial cells (bMECs)

The isolation of bMECs was performed from alveolar tissue of lactating cow udders as described by Anaya-López et al. [16]. Cells from passages 4^{th} to 7^{th} were cultured in growth medium composed by DMEM medium/nutrient mixture F-12 Ham (DMEM/F-12K, Sigma) supplemented with 10% fetal calf serum (Gibco), 10 µg/mL insulin (Sigma), 5 µg/mL hydrocortisone (Sigma), 100 U/mL penicillin, streptomycin (100 µg/mL) and 1 µg/mL amphotericin B (Invitrogen). Subsequently, cells were grown in 5% CO_2 atmosphere at 37°C.

Morphological and biochemical characterization of *Staphylococcus agnetis* 394696 isolate

In a previous work, *S. agnetis* 394696 was identified by colonial morphology, Gram staining and amplification of the 16S rDNA [9]. Additionally, in this work we carried out a biochemical characterization that included the coagulase test, mannitol fermentation and hemolysis production [17-19].

Antimicrobial susceptibility testing

The antimicrobial susceptibility test of *S. agnetis* 394696 was done using the Kirby-Bauer disk diffusion method in Muller-Hinton agar. The antimicrobial used were: Penicillin (PE) 6 µg, Dicloxacillin (DC) 30 µg, Pefloxacin (PEF) 5 µg, Cefuroxime (CXM) 30 µg, Gentamicin (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 Cephalothin (CF) 30 µg (BIORAD). Oxacillin resistance was determinate by agar dilution method in Muller-Hinton agar, supplemented with 4% NaCl and 6 µg/mL of Oxacillin (Sigma) after 48 h incubation a 37°C using ~ 5.4×10^6 CFU [20].

Internalization assays of *Staphylococcus agnetis* 394696 into bMECs

Internalization assays (gentamicin protection assay) were carried out as described by Ochoa-Zarzosa et al. [5]. bMEC monolayers (≈ 50,000 cells/well) were challenged with *S. aureus* or *S. agnetis* (MOI 30:1 bacterium per cell, to be able to compare with a bacterium with recognized internalization capacity). Briefly, bMECs were inoculated with bacterial suspensions and incubated for 2 h in 5% CO_2 at 37°C. After, bMEC monolayers were washed three times with PBS (pH 7.4) and incubated in grow media without serum, supplemented with 40 µg/mL gentamicin for 30 min at 37°C to eliminate extracellular bacteria. *bMEC* monolayers were detached with trypsin-EDTA (Sigma) and lysed with 200 µL of sterile distilled water. Then, bMEC lysates were diluted 100-fold, plated on LB agar for triplicate and incubated overnight at 37°C. The number of total CFU was determined by the standard colony counting technique. Data are presented as the percentage of internalization in relation to *S. aureus* strain.

Genomic DNA isolation

Bacteria were cultured in LB media for 16-18 h at 37°C; then, 1 mL of culture was mixed with lysis solution (SDS 10%, 0.6% Proteinase K) by vortexing and was incubated 1 h at room temperature. Genomic DNA was extracted by addition of 600 µL of phenol:chloroform: isoamyl alcohol (25:24:1); gently mixed for 2 min and centrifuged for 10 min at 16,000 g at 4°C. The aqueous phase was recovered, and the nucleic acids were precipitated with 600 µL of isopropyl alcohol. Finally, genomic DNA was diluted in 100 µL of sterile distilled water and stored at -20°C until use.

Amplification of *fnbpA*, *clfB* and *spa* genes of *Staphylococcus agnetis* 394696

Amplification of *fnbpA*, *clfB* and *spa* genes was carried out with a proof fidelity enzyme (Invitrogen). Oligonucleotides used and alignment temperatures are shown in Table 1. Amplification conditions were as follow: 3 min at 94°C; 30 cycles of 1 min at 95°C, 30 s at 59°C -62°C, 30 s at 72°C; and finally, 7 min at 72°C. An aliquot of 10 L of the PCR products was subjected to

electrophoresis in 1.5% (w/v) agarose gels and stained with SYBR Green (Invitrogen) to amplicon visualization.

Table 1: Primers used for the amplification of genes involved in methicillin resistance and internalization.

Primer	Sequence	Alignment temperature (°C)
mecA	5´GTAGAAATGACTGAACGTCCGATGA 3´ 5´CCAATTCCACATTGTTTCGGTCTAA 3´	62
fnbpA	5´CGACACACCTCAAGACAATAGCGG 3´ 5´TGTGGCTTACTTTCTGCTGCCGTTC 3´	59
clfB	5´TGAAAGTGCAGATTCCGAAAAAAAC 3´ 5´CCGTCGGTTGAGGTGTTTCATTTG	60
spa	5´ATATCTGGTGGCGTAACACCTGCTG 3´ 5´CGCATCAGCTTTTGGAGCTTGAGAG 5´	60

RESULTS AND DISCUSSION

Biochemical and morphological characteristics of *S. agnetis* 394696 isolated from subclinical bovine mastitis

S. aureus is one of the most common etiological pathogens of contagious bovine mastitis, but in the last years, different bacteria have also become emerging pathogenic microorganisms of subclinical bovine mastitis including *S. agnetis*. In this sense, it was observed that *S. agnetis* 394696 isolate growth as circular colonies, convex, smooth, shiny and white that reach a diameter of 1-3 mm. Also, the *S. agnetis* 394696 isolate was negative for mannitol fermentation, non-hemolytic on bovine blood agar and coagulase-negative after 24 h (Figure 1). The colony morphology of *S. agnetis* 394696 showed differences respect to the strain previously reported by Taponen [8], who reported that bacterium formed opaque and light gray colonies. This colony differences suggest that *S. agnetis* 394696 should has different characteristics and genetic variability that the strain reported previously [8]. This is not a strange phenomenon as several works have reported genetic variability and different properties between bacterium of the same genus and different species or same genus and species. For example, has been reported that different isolates of *S. aureus* show variability in growth rate, colony size, and colony pigment; these differences have been associated with its virulence and antibiotic sensitivity [21]. On the other hand, the results of mannitol fermentation, production of hemolysis and coagulase test agree with a previous report [8].

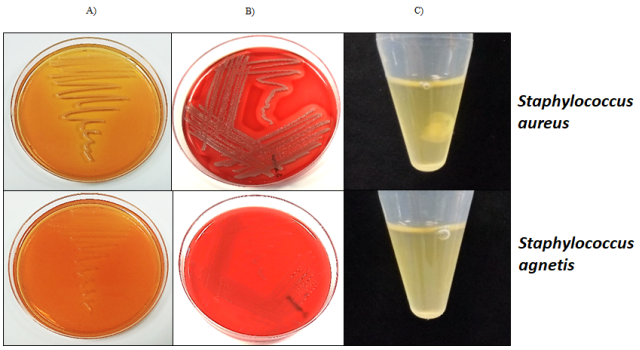

Figure 1: Biochemical testing of *Staphylococcus agnetis*; A) Mannitol fermentation test; B) Hemolysis test; C) Coagulase test. *Staphylococcus aureus* ATCC 27543 was used as reference strain.

Antimicrobial susceptibility patterns of *Staphylococcus agnetis* 394696

Due to the increase in the number of isolates of *S. agnetis* in subclinical bovine mastitis [8-13] the need to analyze its characteristics, virulence and pathogenicity is highlighted. To provide information about antibiotic susceptibility of *S. agnetis* 394696, an antibiogram was performed. *S. agnetis* 394696 was resistant to penicillin, dicloxacillin and ampicillin. This result differs from data reported in India, where two collected isolates were resistant to only one antibiotic [13]. These profiles of resistance can be associated with the management practices and production systems present in the region. This multi-resistance is an important factor that hinders the resolution of infection with conventional antibiotics. In this sense, infections caused by methicillin resistant *Staphylococci* (MRS) are more harmful due to prolonged treatments and limited drug options because is intrinsically cross-resistant to virtually all of the β-lactam antibiotics [22]. In this sense, *S. agnetis* 394696 showed resistance to methicillin (oxacillin, 6 μg/mL) and harbor the *mecA* gene. Several studies suggested that *mecA* gene is present in all of the MRS [23]. However, some clinical isolates of *S. agnetis* [13] and other *staphylococci* like-*S. aureus* are mecA-positive but oxacillin-susceptible [24]. It has already been described that methicillin resistance is related to the expression of the protein penicillin-binding protein (PBP2), which is regulated by the mecA, mecR1-MecI system; however, if there is any failure in system activation, expression of the PBP2 protein decreases, thus, some isolates are methicillin-sensitive despite carrying *mecA* gene [23]. In *S. agnetis* 394696, the methicillin resistance can be attributed to classical mechanism of resistance related to this gene. Nevertheless, further studies are required to confirm the expression of PBP2 protein in *S. agnetis* 394696 (Figure 2).

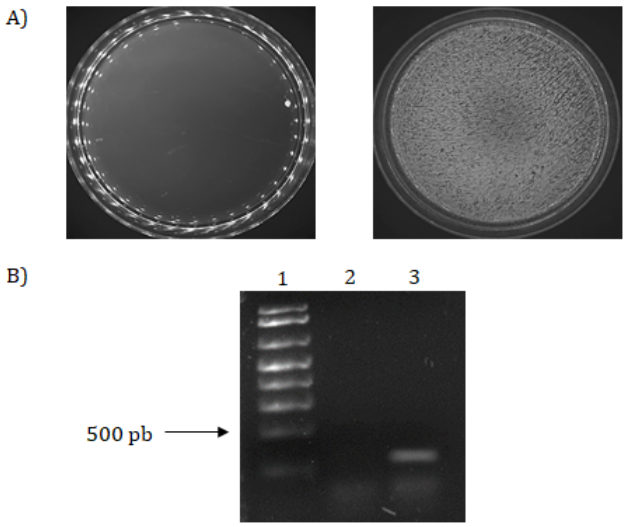

Figure 2: Methicillin resistance of *Staphylococcus agnetis* A) Image representative of a *S. aureus* plate (left) and *S. agnetis* plate (right). Bacteria were grown on LB agar plates with oxacillin (6 µg/ml) and 4% NaCl. B) Amplification of mecA gene using genomic DNA as template. Lane 1, molecular size marker (Invitrogen 100 pb). Lane 2, *S. aureus* and lane 3, *S. agnetis*.

Internalization of *Staphylococcus agnetis* 394696 into bovine mammary epithelial cells (bMECs)

S. aureus is one of the main causative agents of subclinical bovine mastitis due, among other factors, to the fact that some strains have the capacity to internalize into bovine mammary epithelium cells [15]. This phenomenon has already been described in *Staphylococci* coagulase-negative as *S. epidermidis*, which is associated with bovine intramammary infections [25]. However, previous to this work it was unknown whether *S. agnetis* has this characteristic. In the present work, internalization assays were carried out in a primary culture of bMECs and we used *S. aureus* ATCC 27543 as a control strain. The results showed that *S. agnetis* 394696 internalized into bMEC 10% relative to control (Figure 3).

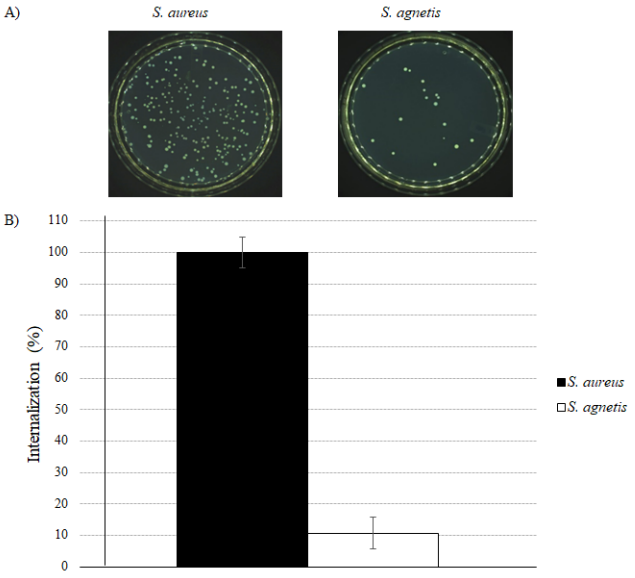

Figure 3: Internalization of *Staphylococcus agnetis* into bovine mammary epithelial cells (bMEC). A) Representative 1:100 dilution plate of internalization assays. The CFU recovered in the test are shown for *S. aureus* (left) *S. agnetis* (rigth). B) *S. agnetis* internalization is represented by the percentage of CFU recovered after bMEC lysis. Values were determined considering the control (*S. aureus* ATCC 27543) as 100% internalization. Each bar shows the mean of triplicates ± SE of three independent experiments.

With the above results, it is confirmed that, although with a lower percentage, *S. agnetis* has the ability to internalize into *bMECs*. The most studied mechanism of internalization of *S. aureus* into nonprofessional phagocytic cells (c.a. *bMECs*) is through a process of receptor-mediated endocytosis (zipper mechanism) [14]. In this mechanism, the fibronectin binding protein (*FnbpA*) present on the bacterial surface binds to the fibronectin of the extracellular matrix, and this complex is recognized by the integrins of the host cell; on the other hand, clumping factor B (*ClfB*) is a bifunctional protein that binds to cytokeratin and fibrinogen, and protein A (*SpA*), is an abundant surface protein of *S. aureus*, which activates TNF receptors and EGF receptor (*EGFR*) signaling cascades that can perturb the cytoskeleton [26-29]. However, it is unknown if this mechanism is utilized by S. agnetis to internalize into *bMECs*. Using in silico analysis, it has been showed the presence of these genes in the genome of S. agnetis [30-32]. For this reason, we searched if *S. agnetis* 394696 harbor the *fnbpA*, *clfB* and *spa* genes. Interestingly, the expression of these genes was detected in *S. agnetis* isolate (Figure 4). According to these results, *S. agnetis* has the ability to internalize into bovine mammary epithelium cells, which could be mediated by *fnbpA*, *clf*B and *spa* genes; however, further studies are necessary to demonstrate the role of these genes in this process.

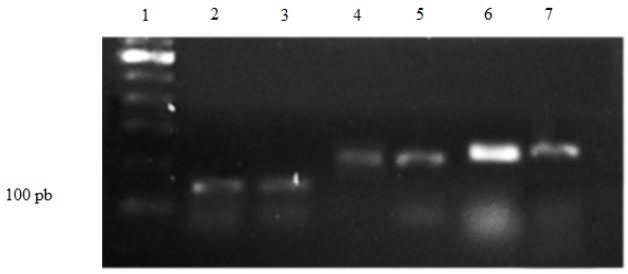

Figure 4: Expression of *fnbA*, *clfB* and *spa* genes in *Staphylococcus agnetis*. Analysis was performed using genomic DNA and PCR endpoint. fnbA gene (lane 2, *S. aureus* and lane 3 *S. agnetis*); clfB gene (lane 4, *S. aureus* and lane 5 *S. agnetis*); spa gene (lane 6, *S. aureus* and lane 7 *S. agnetis*). Lane 1, molecular size marker (Invitrogen 100 bp).

CONCLUSION

Bovine mastitis remains as a problem of difficult treatment and resolution, mainly due to the variety of factors involved in the disease development (multidrug resistance and diversity of virulence factors of microorganisms). Here we showed that *S. agnetis* 394696 isolate is resistant to different antibiotics such as penicillin, dicloxacillin, ampicillin and methicillin. Also, to our knowledge this is the first report showing that this bacterium has the ability to internalize into bovine mammary epithelial cells. The resistant to different antibiotics and the capacity of *S. agnetis* to invade host cells are two crucial factors to consider in the management of bovine mastitis generated by this microorganism.

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COMPLIANCE WITH ETHICAL STANDARDS

Animal studies were carried out humanely and according with national and international Animal Care and Use Committee protocols.

CONFLICT OF INTERESTS

All the authors declare no conflict of interest regarding this manuscript.

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