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Detection of *Staphylococcus aureus* adhesion and biofilm-producing genes and their expression during internalization in bovine mammary epithelial cells

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ABSTRACT

Staphylococcus aureus is one of the most prevalent pathogens isolated from bovine mastitis, causing chronic intramammary infections (IMI) that limit profitable dairying. The course of infection is often associated with factors both related to the host and the bacterium. Aims of this study were to select *S. aureus* isolates from bovine IMI with different genotypic profiles harboring genes involved in adherence and biofilm production, to determine the behavior of these strains in contact with bovine mammary epithelial cells (MAC-T) and the expression of those genes during bacterial-cell early interactions. The genetic diversity of 20 *S. aureus* strains that were isolated from milk samples taken from cows with persistent-P and non-persistent-NP IMI was high, discriminated into 13 fingerprint groups. The occurrence of genes coding for *S. aureus* surface proteins (*clfA*,*clfB*, *fnbA*, *fnbB*, *fib*, *cna*) and biofilm formation (*icaA*, *icaD*, *icaC*, *bap*) and *in vitro* biofilm-forming ability was not related to strain clinical origin (NP or P). Internalization of *S. aureus* into MAC-T cells was strain-dependent and internalized bacteria overexpressed adherence and biofilm-forming genes compared with those that remained in the supernatant of co-cultures; particularly those genes encoding FnBPs and IcaD. Strains yielding highest invasion percentages were those able to overexpress *fnBP*, irrespectively of the presence of other evaluated genes. Strains from NP IMI showed a greater multiplication capacity *in vitro* compared with strains from P IMI. These results provide new insights about *S. aureus* differential gene expression of adhesion-internalization factors during early interaction with mammary epithelial cells.
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1. Introduction

Staphylococcus aureus is one of the most prevalent major pathogens causing intramammary infections (IMI) in cattle (Zecconi et al., 2006). Commonly, an acute episode of mild to moderate clinical mastitis occurs, but in many cases the infection is not successfully eliminated, resulting in development of chronic subclinical mastitis. Persistence of *S. aureus* in the mammary gland and poor response of the pathogen to antibiotic therapy makes *S. aureus* IMI a common cause of culling (Hebert et al., 2000; Zecconi et al., 2006). Both early interactions between *S. aureus* and host cells, as well as the events that lead to establishment of chronic mastitis are not fully understood, but persistent infections are often associated with an impairment of the immune response due to factors related both to the bacterium and the host. *S. aureus* has a variety of virulence factors, which favor bacterial survival and multiplication in the mammary gland (Zecconi and Scali, 2013).

Previous studies found associations between *S. aureus* genotypes and severity of mastitis clinical signs (Haveri et al., 2007). More recent studies showed that genotypes of *S. aureus* isolated

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from bovine IMI were highly related to their virulence gene pattern (Graber et al., 2009; Piccinini et al., 2012). Furthermore, some subtypes were highly associated with selected epidemiological features like within-herd prevalence and persistence; however, the relationship to pathogenic properties was less evident (Graber et al., 2009). In this regard, in a study comparing subclinical and clinical isolates from *S. aureus* ovine mastitis, Le Maréchal et al. (2011) found no genotypic, but proteomic and transcriptomic differences. While the strain from ewe subclinical mastitis showed overexpression of genes encoding surface molecules, the strain causing lethal gangrenous mastitis mainly overexpressed genes encoding exoproteins (Le Maréchal et al., 2011). Studies characterizing gene expression of *S. aureus* from bovine IMI in the presence of host cells have not yet been carried out. Information about potential differential gene expression during bacterial-cell interactions can contribute to increase the knowledge of both bacteria and cell mechanisms that lead to establishment of infection.

In vitro and *in vivo* studies have demonstrated that *S. aureus* binds to cells and extracellular matrix components and invades bovine mammary epithelial cells, as well as other cells from mammary tissue (Almeida et al., 1996; Hebert et al., 2000) displaying several virulence factors that mediate adhesion to the host cells. The main mechanism for host cell adhesion is mediated by fibronectin binding proteins (FnBP) A and B, that allow bacterial-cell interaction *via* a fibronectin bridge with fibronectin receptors of mammal cells ($\alpha 5\beta 1$ integrins) (Sinha et al., 1999). Another pathogen adhesion mechanism is through clumping factors (Clf) A and B; these are fibrinogen binding proteins that contribute to initiate infection (Zecconi and Scali, 2013). This attachment also prevents pathogen destruction via opsonophagocytosis, promoting cleavage and inactivation of complement components that mediate opsonization (Hair et al., 2010). Furthermore, biofilm formation, a highly organized multicellular complex, is associated not only with epithelial adhesion but also with evasion of host immune defense (Melchior et al., 2009). Biofilm production requires the presence of the gene cluster *icaADBC* (intracellular adhesion locus). Both the high prevalence of *S. aureus* isolated from bovine IMI harboring the *ica* locus (Vasudevan et al., 2003) and the proportion of biofilm producers among isolates belonging to pulsotypes associated with milk rather than to bovine extramammary sites (Fox et al., 2005), suggest its potential role as a virulence factor in the pathogenesis of mastitis.

The multiplicity of virulence factors and the evidence that potential key elements are not equally expressed *in vivo* by different isolates (Klein et al., 2012) has to be taken into account for the development of effective vaccines against *S. aureus*. An ideal immunotherapeutic agent should prevent bacterial adhesion, promote phagocytosis-induced bacterial elimination and neutralize toxic exoproteins. Therefore, among strategies for vaccine development, antigen selection to generate a protective immune response is probably the most critical and challenging step. Antigen candidates must be conserved and expressed by most strains and, theoretically, adhesion factors should be prime targets for vaccine development (Zecconi and Scali, 2013). Nevertheless, immunization attempts using individual antigens have shown only partial protection against intramammary staphylococcal infections in murine models (Tuchscherr et al., 2008). This suggests that formulations comprising more than one target antigen should increase defensive functions against IMI. Therefore, achievement of more effective anti-staphylococcal vaccines requires a better understanding of the mechanisms by which *S. aureus* can colonize and persist inside its host. Aims of this study were to select *S. aureus* isolates from bovine IMI with different genotypic profiles harboring genes involved in adherence and biofilm production, to determine the behavior of these strains in contact with mammary epithelial cells and the expression of those genes during bacterial-cell early interactions.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Staphylococcus aureus ($n = 20$) were isolated from milk samples taken from Holstein cows with clinical and subclinical mastitis belonging to different dairy farms located in Santa Fe, Córdoba and Buenos Aires provinces (Argentina) collected between 2000 and 2014. Bacteria were identified as *S. aureus* according to standard phenotypic methodology and stored at −80 °C in trypticase soy broth (TSB) (Britania, Buenos Aires, Argentina) with 15% glycerol until use. Fifteen *S. aureus* isolates were from clinical mastitis and 5 from subclinical mastitis. Mastitis were characterized as subclinical (milk somatic cell count of $>250 \times 10^3$/mL, no macroscopic changes) or clinical (visual abnormalities in milk and/or swelling or tenderness in the udder). Isolates from clinical mastitis belonged to 15 different unrelated dairy farms, 9 from Santa Fe province, 2 from Buenos Aires province and 4 from Córdoba province. Samples from clinical cases were obtained by the veterinary practitioners that acted as advisor of the dairy farm ($n = 11$) or by trained personnel during a visit that included sampling of every lactating cow at the farm ($n = 4$). Cows with clinical IMI were treated with standard treatments based of beta lactam or macrolide antibiotics for 3 days. Strains from clinical IMI isolated only once from a mammary quarter and not re-isolated in two consecutive milk samplings after antibiotic therapy, were considered with low adaptation to bovine mammary gland and designated as nonpersistent-NP. Strains from subclinical IMI belonged to two different dairy farms located in Santa Fe province. Two isolates were obtained each from the same mammary quarter before and 15 and 21 days following a standard treatment with beta lactam antibiotics for 3 days (Farm A), while the remainder three isolates were obtained from the same mammary quarter from three different cows in three or more consecutive monthly milk samplings over a period of six months (Farm B). These isolates were tested by pulsed field gel electrophoresis (PFGE) showing the same pattern and were considered highly adapted to bovine mammary gland and designated as persistent-P.

2.2. DNA isolation and PCR

Bacteria were activated from frozen stocks by overnight culture at 37 °C on trypticase soy agar (TSA) (Britania) under aerobic conditions. Bacterial suspensions were grown overnight in TSB at 37 °C, harvested by centrifugation, and incubated for 2 h at 37 °C in 10 mM Tris–HCl pH 8 and 2.5 mg/mL lysozyme (Genbiotech SRL, BA, Argentina). Lysis was achieved by incubating with lysis buffer (50 mM Tris, 100 mM EDTA, 1% SDS, pH 8) and 1 mg/mL of proteinase-K (Genbiotech SRL) at 50 °C for 1 h. After phenol: chloroform extraction and ethanol precipitation, DNA was resuspended in Milli-Q sterile water and quantified spectrophotometrically using an UV/vis Lambda 20 (PerkinElmer) spectrophotometer.

For *S. aureus* characterization, species-specific oligonucleotide primers were used to amplify a 108 bp-segment of a glutamate synthetase family protein gene previously described by Martineau et al. (1998) (Table 1). PCR amplification was performed using 20 μL of reaction mixture containing 1X *Taq* buffer, 250 μM of each deoxynucleotide, 1 μM of each primer (Invitrogen, Life technology, CA, USA), 2.4 mM $MgCl_2$, 1.5 U of *Taq* DNA polymerase (Promega, WI, USA), and 100 ng of template DNA. The PCR reaction was carried out in a thermocycler (Ivema T-18, Ivema Desarrollos SRL) using the following program: an initial step at 96 °C for 3 min and

Table 1
PCR primers and conditions for identification of *Staphylococcus aureus* genes.

Primer	Sequence (5′–3′)	aT (°C)	Amplified fragment	Amplicon size (bp)	Reference
clfA[a,b]	F-TTACGAATCAGTTGACGAATGTG R-AGGCACTGAAAAACCATAATTCA	55	*Clumping factor A*	104	Le Maréchal et al. (2011)
clfB[a,b]	F-TGCAAGTGCAGATTCCGAAAAAAAC R-CCGTCGGTTGAGGTGTTTCATTTG	62	*Clumping factor B*	194	Klein et al. (2012)
fnbA[a,b]	F-CGACACAACCTCAAGACAATAGCGG R-CGTGGCTTACTTTCTGATGCCGTTC	62	*Fibronectin binding protein A*	133	Klein et al. (2012)
fnbB[a,b]	F-ACGCTCAAGGCGACGGCAAAG R-ACCTTCTGCATGACCTTCTGCACCT	62	*Fibronectin binding protein B*	197	Designed[c]
icaA[a,b]	F-CTTGCTGGCGCAGTCAATAC R-CCAACATCCAACACATGGCA	55	*Intercellular adhesion A*	178	Designed[c]
icaD[a,b]	F-CGCTATATCGTGTGTCTTTTGGA R-TCGCGAAAATGCCCATAGTT	55	*Intercellular adhesion D*	164	Designed[c]
icaC[a]	F-CTTGGGTATTTGCACGCATT R-GCAATATCATGCCGACACCT	55	*Intercellular adhesion C*	209	Designed[c]
fib[a]	F-CGTCAACAGCAGATGCGAGCG R-TGCATCAGTTTTCGCTGCTGGTTT	62	*Fibrinogen binding protein*	239	Atshan et al. (2013)
cna[a]	F-AATAGAGGCGCCACGACCGT R-GTGCCTTCCCAAACCTTTTGAGCA	62	*Collagen Adhesin*	156	Atshan et al. (2013)
bap[a]	F-CCCTATATCGAAGGTGTAGAATTGCAC R-GCTGTTGAAGTTAATACTGTACCTGC	60	*Biofilm-associated protein*	971	Cucarella et al. (2001)
gyrB[a,b]	F-CCAGGTAAATTAGCCGATTGC R-AAATCGCCTGCGTTCTAGAG	55	*DNA gyrase subunit B*	121	Rudkin et al. (2012)
sa442[a]	F-AATCTTTGTCGGTACACGATATTCTTCACG R-CGTAATGAGATTTCAGTAGATAATACAACA	55	*Glutamate synthetase family protein*	108	Martineau et al. (1998)

aT: annealing temperature.
[a] Standard PCR.
[b] Quantitative RT-PCR.
[c] Designed with Primer Blast software.

35 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. An extension step at 72 °C for 2 min was added. The amplified fragments were visualized by electrophoresis in agarose gel (3%) stained with 0.5 mg/L of ethidium bromide (Biodynamics, BA, Argentina).

For adhesion genes (*clfA*, *clfB*, *fnbA*, *fnbB*, *fib*, *cna*) and biofilm-producing genes (*icaA*, *icaD*, *icaC*, *bap*) PCR was performed according to above protocol, using 1.2 U *Taq* DNA polymerase, 2 mM $MgCl_2$ and 0.4 μM sense and anti-sense oligonucleotides (Invitrogen) (Table 1). Cycling conditions were the same used for molecular identification, using specific annealing temperature for each pair of primers. Amplified products were analyzed by electrophoresis in an agarose gel (concentration according to product length).

The accessory gene regulator (*agr*) groups were determined by a multiplex PCR described previously by Gilot et al. (2002). Briefly, purified nucleic acids were amplified in a 25 μL reaction mixture containing 1X *Taq* buffer, 0.4 U of *Taq* DNA polymerase (Invitrogen), 2.5 mM $MgCl_2$, 250 μM of each deoxynucleotide and the following primers (0.3 μM): Pan (5′-ATG CAC ATG GTG CAC ATG C-3′), agr1 (5′-GTC ACA AGT ACTATA AGC TGC GAT-3′), agr2 (5′-TAT TAC TAA TTG AAA AGT GGC CAT AGC-3′), agr3 (5′-GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G-3′), and agr4 (5′-CGA TAA TGC CGT AAT ACC CG-3′). Multiplex PCRs were performed in a thermocycler (Ivema T-18, Ivema Desarrollos SRL) for 1 cycle at 94 °C for 3 min; 26 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min; and finally 1 cycle at 72 °C for 10 min. Amplification products were subjected to electrophoresis in a 1.5% agarose gel containing ethidium bromide and visualized by transillumination under UV.

2.3. Pulsed field gel electrophoresis

The genetic relationships of 20 bovine *S. aureus* isolates were established by PFGE. In brief, *S. aureus* were cultured and plugs were prepared. Chromosomal DNA was digested with the endonuclease *Sma* I (Promega) at 25 °C for 17 h. Then, electrophoresis was carried out in a CHEF DR-III apparatus (Bio-Rad, Germany) in 1% agarose gels and was run for 23 h at 12 °C at 6 V/cm with pulses from 5 to 35 s. A standard pattern (Lambda Ladder PFGE Marker, New England Biolabs) was included in the gels to allow comparison of the digitally normalized PFGE profiles. Gels were stained with 0.5 mg/L ethidium bromide (Biodynamics) and scanned with Bio-Rad Gel Doc system by using the Molecular Analyst Software (Bio-Rad). For final band analysis, relative positions were established visually on thermal paper prints of the gels and were compared with those generated with bacteriophage lambda ladder DNA concatemers (New England Biolabs). Macrorestriction patterns were analyzed by Pyelph software (Pavel and Vasile, 2012) to calculate Dice coefficients of correlation and to generate a dendrogram by the unweighted pair group method using arithmetic averages (UPGMA) clustering.

2.4. Biofilm formation

Quantification of biofilm production was assessed by a microtiter plate assay (MPA) described by Stepanović et al. (2007) with modifications. Isolates were cultivated in Columbia agar at 37 °C for 24 h under aerobic conditions. After verifying purity of the strain, two colonies were inoculated into 5 mL of TSB (Britania) and incubated at 37 °C for 24 h. Stationary-phase culture was vortexed and diluted 1:100 in medium for biofilm production (TSB supplemented with 1% glucose-TSB_{glc}). The suspension was added into sterile flat-bottomed 96-well polystyrene microtiter plates (Gbo, BA, Argentina), 200 μL per well, and incubated at 37 °C for 24 h under aerobic conditions. After discarding the content of the wells, they were washed three times with 300 μL of sterile phosphate-buffered saline (PBS; pH 7.2) and drained by inversion. The adherent bacterial cells were fixed with 150 μL of methanol for 20 min, and then plates were emptied and left to dry overnight. The adherent cells were stained with 150 μL of 2% Hucker crystal violet solution for 15 min and were washed twice with water. Dye bound to the adherent cells was dissolved with 150 μL of 95% ethanol and optical density (OD) was measured at 570 nm using a spectrophotometer (SpectroStar Nano BMG Labtech). The experiment was performed in triplicate and sterile TSB_{glc} was used as negative control. An OD_{570} value of 0.3 was taken as the cutoff point to

differentiate between biofilm producer from non-biofilm-producer strains [cut-off (ODc) = mean OD plus 3 standard deviation (SD) of negative control]. Strains were considered as strong biofilm producers +++ ($OD_{570} > 1.2$), moderate biofilm producers ++ ($1.2 > OD_{570} > 0.6$), weak biofilm producers + ($0.6 > OD_{570} > 0.3$), and no biofilm producers – ($OD_{570} < 0.3$). As positive control, bovine *S. aureus* strain V329 (Genbank accession AY220730, kindly provided by Dr J.R. Penadés, Spain) was used.

2.5. Cell invasion assay

Invasion assays were based on a protocol previously described by Almeida et al. (1996) with modifications. An established bovine mammary epithelial cell line (MAC-T) was used for all the experiments. Bacteria were activated from frozen stocks (−80 °C) by culture on Columbia agar, incubated overnight at 37 °C and co-cultured with confluent monolayer of MAC-T cells in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) at a multiplicity of infection (MOI, ratio of *S. aureus* organisms to cells) of 100:1 for 2 h at 37 °C in 5% CO_2. In parallel, bacterial suspensions were incubated for 2 h at 37 °C in 5% CO_2 in DMEM, to use as control groups. After incubation, supernatants of the co-culture were recovered as non-internalized bacteria; MAC-T monolayers were washed three times with PBS 1X (pH 7.4) and treated with gentamicin (100 μg/mL, Sigma Chemical Co., St. Louis, MO) in DMEM at 37 °C in 5% CO_2 for 2 h to kill extracellular bacteria. Supernatants were then collected and plated on TSA added with 5% calf blood to verify killing by gentamicin. Finally, MAC-T monolayers were washed three times with PBS 1X, treated with 0.25% trypsin 0.1% EDTA (Gibco, BRL) and further lysed with Triton X-100 (Amersham, Arlington Heights, IL, USA) at a final concentration of 0.025% (v/v) in sterile distilled water to release intracellular staphylococci. MAC-T lysates were 10-fold serially diluted, plated on TSA added with 5% calf blood and incubated overnight at 37 °C. Colony forming units per mL (CFU/mL) of *S. aureus* internalized in MAC-T cells and *S. aureus* suspensions alone (control group) were determined by standard colony counting techniques. A non-invasive *Escherichia coli* strain (DH5α) was used as negative control (0% invasion). The invasion assay was performed in triplicate and experiments were repeated five times. Data were expressed as percentage of internalization relative to control group (CFU/mL of parallel cultured *S. aureus* after 2 h of incubation). Additionally, bacterial growth of control group was calculated by difference between CFU/mL after 2 h of incubation in DMEM and CFU/mL that were initially in contact with MAC-T cells.

2.6. RNA extraction and relative quantitative real-time PCR (qPCR)

For expression studies total RNA extraction was performed from 3 experimental groups: *S. aureus* present in the supernatant of co-cultures with MAC-T (group 1, not internalized), internalized bacteria (group 2), and *S. aureus* cultured alone (group 3) used as control. The RNA was extracted from a sample pool of five independent experiments per group. Total RNA was isolated using RiboPure™-Bacterial RNA purification kit (Ambion Inc., Austin, TX, USA) following manufacturer's instructions. Then DNA was removed using turbo DNAse (Ambion). RNA from group 2 was further purified using MICROBEnrich™ kit (Ambion). Quantity and quality of purified RNA was determined using a fluoroscopic method (Qubit, Invitrogen, CA, USA) and by visualization on a denaturating agarose gel. Purified RNA was immediately retro transcribed to avoid RNA degradation using SuperScript II Reverse Transcriptase (Invitrogen) according to manufacturer's instructions.

Quantitative real time PCR was carried out using 5X HOT FIREpol EvaGreen qPCR kit (Solis BioDyne) on a StepOne v2.1 thermocycler (Applied Biosystem). The reaction components were 1X DNA Master Mix, and 0.4 μM of specific primers (Invitrogen) (Table 1). The amplification protocol consisted in a holding stage at 95 °C for 15 min, 40 cycles of 15 s at 95 °C, 15 s at the corresponding annealing temperature (T_a) and 10 s at 72 °C, with another holding stage for 10 min at 72 °C. The stability of the housekeeping gene expression was assessed by using BestKeeper© Software as described by Pfaffl et al. (2004), taking into account coefficient of variation (CV) and standard deviation (SD) parameters. The lowest CV and SD value for *DNA gyrase subunit B* (*gyrB*), demonstrates that the expression is stable under the conditions used for *in vitro* cell infection. Thus *gyrB* was used as endogenous control for normalization of the relative mRNA levels. Relative gene expression was calculated by the $\Delta\Delta^{Ct}$ method including the efficiency of amplification (Pfaffl, 2001), using the REST-2009 software (Relative Expression Software Tool, Qiagen, Hilden, Germany, http://www.REST.de.com). The transcript quantities were expressed as changes (*n*-fold) relative to the values of the control.

2.7. Growth curves analysis

Isolates kept at −80 °C were reactivated in TSA (Britania) and incubated at 37 °C for 24 h under aerobic conditions. After verifying purity, two colonies were inoculated into 5 mL of TSB (Britania) and incubated overnight at 37 °C. Starting from the overnight culture, 50 mL of fresh medium were inoculated with 0.5 mL of each bacterial suspension, fractioned in 9 sterile tubes and cultivated on rotary shaker (150 rpm) at 37 °C. Every hour, 100 μL aliquots of cell cultures were removed to make 1:100 fold serial dilutions in sterile PBS, and plate onto TSA for CFU counts. Each assay was repeated three times. After obtaining the growth curves, values of time and Log_{10} CFU/mL corresponding to the exponential growth phase were taken. Bacterial generation time (GT) for each strain was calculated as the inverse of the lineal regression slope of the graphic Log_{10} CFU/mL *versus* time (min); taking into account that one logarithmic unit corresponded to 3.3 generations.

2.8. Statistical analysis

A statistical software package (SPSS 11.0 for Windows; SPSS, Inc., Chicago, IL) was used to perform statistical analysis. Percentages of internalization among of *S. aureus* strains were compared by analysis of variance (ANOVA) with subsequent Duncan *post-hoc* test. All data obtained were expressed as mean ± standard error of the mean (SEM). Nonparametrical data were analyzed with the Mann–Whitney test. Values of $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Pulsed field gel electrophoresis typing

All *S. aureus* isolates ($n = 20$) were discriminated into 13 fingerprint groups by PFGE typing (Fig. 1) which were arbitrarily designated with letters (A–M). A dendrogram that included all patterns was constructed on the basis of the levels of similarity, and a cut-off point of 80% was considered to define the groups. The pulsotype (PT) identified with the letter F was the most common accounting for 35% of the strains tested and consisted exclusively of strains isolated from clinical NP IMI from dairy herds belonging to Santa Fe and Córdoba provinces. The PT identified with the letter C included two *S. aureus* strains (isolated from clinical NP IMI), and remaining PT included one strain.

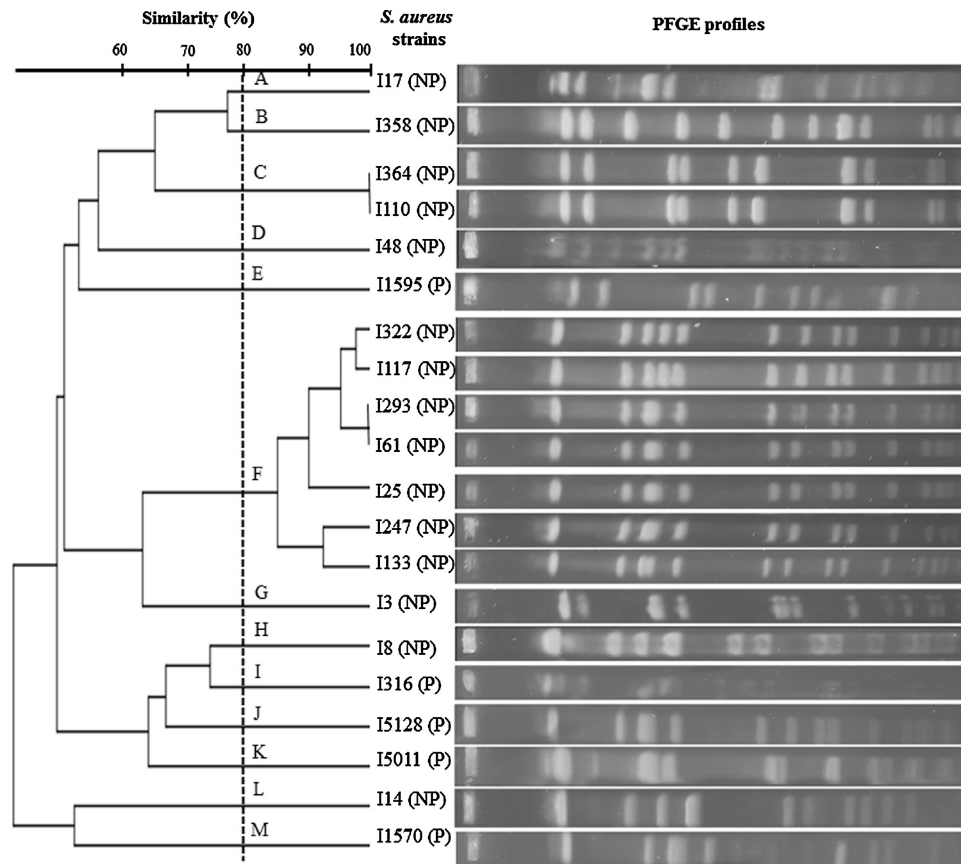

Fig. 1. PFGE genotyping of *S. aureus* strains isolated from clinical nonpersistent (NP) and subclinical persistent (P) bovine IMI. In the left panel, dendrogram obtained from cluster analysis of amplified band profiles (right panel) of *S. aureus* strains. The unweighted pair group method using average linkages and a Dice coefficient (with a tolerance limit of 1%) were used to build the dendrogram. A dashed line indicates the cut-off value chosen to determine the 13 clusters indicated A–M.

3.2. Identification of S. aureus adhesion and biofilm-associated genes

Prior to invasion assays, the presence of *S. aureus* adhesion and biofilm-associated genes was evaluated by PCR. Genes *clfA*, *clfB*, *fnbA* and *icaD* were found in 100% of the 20 strains isolated from clinical NP and subclinical P bovine IMI (Table 2). Genes *icaA* and *icaC* were found in 95% of strains ($n = 19$). Genes *fnbB* and *fib* were found in 90% of strains ($n = 18$). The gene *cna* was detected in 20% of strains ($n = 4$), while *bap* gene was not present in any strain (Table 2).

Due to their high prevalence, *clfA*, *clfB*, *fnbA*, *fnbB*, *icaA* and *icaD* were chosen to compare gene expression of 9 isolates during invasion assays. Isolates I17 (PT-A), I358 (PT-B), I247 (PT-F), I48 (PT-D), I61 (PT-F) from clinical NP IMI and isolates I316 (PT-I), I5128 (PT-J), I1595 (PT-E), I5011 (PT-K) from subclinical P IMI were selected based on the dendrogram groups in such a way that the entire genetic diversity of the studied isolates was represented (Fig. 1). Two strains from PT-F were selected since this PT included the largest number of strains.

3.3. Determination of agr group

All *S. aureus* strains ($n = 20$) isolated from P and NP bovine IMI belonged to *agr* group I (100%).

3.4. Biofilm production

In MPA, 100% of *S. aureus* strains from clinical NP and subclinical P bovine IMI showed ability to produce biofilm, although at different intensities (Table 3). Two strains obtained from clinical NP IMI (10% of total strains), were able to produce biofilm weakly (Table 3). 8/20 strains (40%) showed moderate biofilm formation; 6 of these strains (75%) were obtained from clinical NP IMI, while 2 (25%) from subclinical P IMI (Table 3). 10/20 strains (50%) showed strong biofilm formation; 7 of these strains (70%) were obtained from clinical NP IMI, while 3 (30%) from subclinical P IMI (Table 3).

Table 2
Occurrence in percentages (%) of adhesion and biofilm-associated genes in *S. aureus* strain from clinical nonpersistent (NP) and subclinical persistent (P) bovine IMI.

S. aureus strains	*clfA*	*clfB*	*fnbA*	*fnbB*	*fib*	*cna*	*icaA*	*icaC*	*icaD*	*bap*
I25 (NP)	+	+	+	+	+	–	+	+	+	–
I14 (NP)	+	+	+	–	+	–	+	+	+	–
I117 (NP)	+	+	+	+	+	+	+	+	+	–
I364 (NP)	+	+	+	+	–	–	+	+	+	–
I61 (NP)	+	+	+	+	+	–	+	+	+	–
I110 (NP)	+	+	+	+	+	–	+	+	+	–
I17 (NP)	+	+	+	+	+	–	+	+	+	–
I293 (NP)	+	+	+	+	+	–	+	+	+	–
I247 (NP)	+	+	+	+	+	–	+	+	+	–
I322 (NP)	+	+	+	+	+	–	–	–	+	–
I8 (NP)	+	+	+	+	+	–	+	+	+	–
I3 (NP)	+	+	+	+	+	+	+	+	+	–
I133 (NP)	+	+	+	+	+	–	+	+	+	–
I358 (NP)	+	+	+	+	–	–	+	+	+	–
I48 (NP)	+	+	+	+	+	+	+	+	+	–
I316 (P)	+	+	+	+	+	–	+	+	+	–
I1595 (P)	+	+	+	+	+	–	+	+	+	–
I5128 (P)	+	+	+	+	+	–	+	+	+	–
I5011 (P)	+	+	+	+	+	+	+	+	+	–
I1570 (P)	+	+	+	–	+	–	+	+	+	–
Occurrence (%)	100	100	100	90	90	20	95	95	100	0

Table 3
Biofilm formation in *S. aureus* strains isolates from clinical nonpersistent (NP) and subclinical persistent (P) bovine IMI tested by microtiter plate method.

S. aureus isolates	Biofilm production assay (SD[a, c])	Biofilm production ability
I25 (NP)	0.599 (0.110)	+
I14 (NP)	0.609 (0.174)	+
I117 (NP)	0.916 (0.144)	++
I364 (NP)	0.972 (0.257)	++
I61 (NP)	0.943 (0.282)	++
I110 (NP)	1.023 (0.128)	++
I17 (NP)	1.108 (0.105)	++
I293 (NP)	1.093 (0.260)	++
I247 (NP)	1.326 (0.364)	+++
I322 (NP)	1.246 (0.304)	+++
I8 (NP)	1.317 (0.100)	+++
I3 (NP)	1.349 (0.391)	+++
I133 (NP)	1.234 (0.443)	+++
I358 (NP)	2.448 (0.731)	+++
I48 (NP)	3.326 (0.157)	+++
I316 (P)	0.879 (0.142)	++
I1595 (P)	1.061 (0.092)	++
I5128 (P)	1.340 (0.062)	+++
I5011 (P)	1.333 (0.166)	+++
I1570 (P)	1.393 (0.222)	+++
V329[b]	3.488 (0.029)	+++

[a] Standard deviations.
[b] Positive control.
[c] Quantification of biofilm formation by optical density (OD) determination: (+++): strong biofilm producers ($OD_{570} > 1.2$), (++): moderate biofilm producers ($1.2 > OD_{570} > 0.6$), (+): weak biofilm producers ($0.6 > OD_{570} > 0.3$), and (−): no biofilm producers ($OD_{570} < 0.3$).

3.5. Cell invasion assay

The ability to internalize in host cells of *S. aureus* belonging to different clonal types causing clinical NP and subclinical P bovine IMI was evaluated by the epithelial MAC-T cell invasion assay. A high variability in percentages of internalization between *S. aureus* strains from clinical NP and subclinical P IMI was observed (Fig. 2). Two strains showed the highest internalization percentages ($P < 0.05$); one of these (I17) was obtained from clinical NP IMI, while the other one (I5011) from subclinical P IMI (Fig. 2). Six strains showed the lowest internalization percentages ($P < 0.05$; Fig. 2), with similar number of isolates from clinical NP and subclinical P IMI. One strain obtained from clinical NP IMI (I61) showed percentages of internalization in-between the other evaluated strains ($P < 0.05$, Fig. 2).

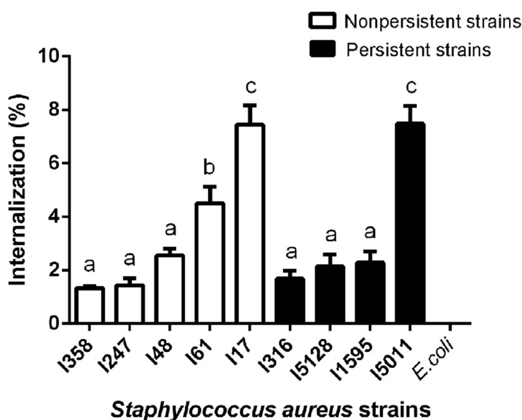

Fig. 2. Percentages of invasion to MAC-T cells of *S. aureus* strains isolated from clinical nonperistent (NP) and subclinical persistent (P) bovine IMI. Each bar represents the arithmetic mean ± standard error of the mean (SEM) for percentages of invasion. Average of triplicates of five independent experiments is presented. *Escherichia coli* DH5-α strain was used like noninvasive control. Different letters correspond to statistically significant differences ($P < 0.05$).

Fig. 3A shows the means of Log_{10} CFU/mL obtained after 2 h of incubation of 9 *S. aureus* strains from clinical NP and subclinical P bovine IMI in the culture medium of MAC-T cells. Strains from clinical NP IMI showed a higher growth rate than strains from P IMI ($P = 0.015$).

3.6. Expression levels of S. aureus *adhesion and biofilm-producing genes by qPCR*

In general, relative expression of adherence and biofilm-producing genes in *S. aureus* strains that failed to internalize in MAC-T cells did not change or decrease significantly compared with controls (Table 4). However, the relative expression of *clfB* for I5011 strain, of *fnbB* for I48 and I17 strains and of *icaD* for I358 and I5011 strains showed significant increments compared with controls (Table 4). In the group of bacteria that failed to internalize in MAC-T cells, no significant differences were observed in the expression of *clfA* ($P = 0.402$), *clfB* ($P = 0.742$), *fnbA* ($P = 0.200$), *fnbB* ($P = 0.057$), *icaA* ($P = 0.571$) and *icaD* ($P = 0.857$) between strains from clinical NP and subclinical P IMI. Relative expression of adherence and biofilm-producing genes for not internalized I5128 and I61 strains could not be calculated due to variations in housekeeping expression in these particular cases, which did not allow a correct normalization. It should be mentioned that similar results were obtained with other reference genes tested (data not shown). In the group of *S. aureus* strains that internalized in MAC-T, relative expression of adherence and biofilm-producing genes was higher than in the group of strains that did not

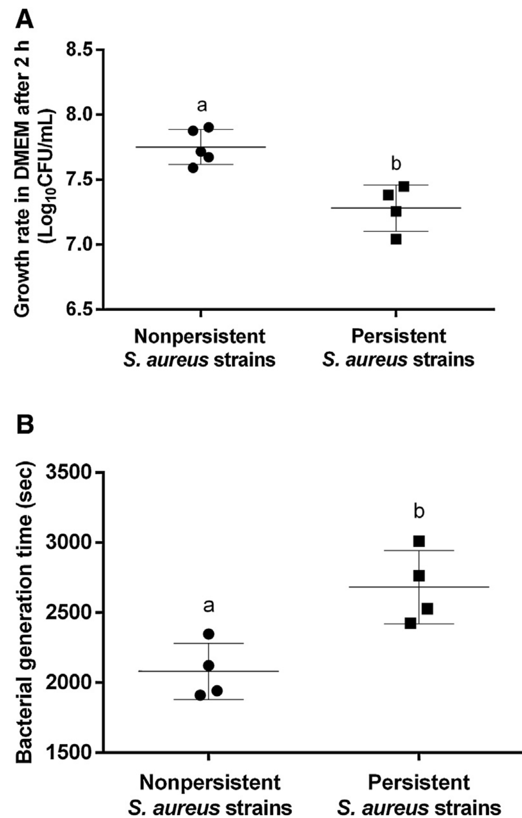

Fig. 3. (A) Growth rate of *S. aureus* strains from nonpersistent and persistent bovine IMI in MAC-T culture medium after 2 h of incubation. The horizontal lines represent the median values. Levels of significance for comparisons of a *versus* b were $P = 0.015$ (Mann–Whitney test). (B) Bacterial generation time (GT) of *S. aureus* strains isolated from nonpersistent and persistent bovine IMI. The horizontal lines represent the median values. Levels of significance for comparisons of a *versus* b were $P = 0.0286$ (Mann–Whitney test).

Table 4
Fold change in mRNA levels of adhesion and biofilm forming genes of *S. aureus* strains isolated from clinical nonpersistent (NP) and subclinical persistent (P) bovine mastitis after 2 h of co-incubation with MAC-T cells. Not internalized: *S. aureus* supernatant of co-culture with MAC-T. Internalized: *S. aureus* internalized to MAC-T.

	S. aureus isolated	*clfA*	*clfB*	*fnbA*	*fnbB*	*icaA*	*icaD*	*gyrB#*
Not internalized	I247 (NP)	0.583	0.717	0.444	1.016	0.236	0.403	1.0
	I358 (NP)	0.997	0.930	0.963	0.618	1.206	1.523↑	1.0
	I48 (NP)	0.627↓	1.105	0.462↓	4.815↑	0.976	0.661↓	1.0
	I17 (NP)	0.081↓	0.086↓	0.753↓	5.746↑	0.350↓	0.235	1.0
	I1595 (P)	0.327	0.646	0.708	0.342↓	0.343↓	0.138↓	1.0
	I316 (P)	0.105↓	0.073↓	0.072↓	0.026↓	1.167	1.302	1.0
	I5011 (P)	4.976	3.934↑	0.072↓	0.021↓	0.191↓	5.293↑	1.0
Internalized	I247 (NP)	1.075	0.818	53.446↑	29.243↑	2.136	6.678↑	1.0
	I61 (NP)	2.021	0.945	97.006↑	23.425↑	0.829	3.296↑	1.0
	I358 (NP)	57.88↑	4.918↑	97.006↑	23.507↑	11.080↑	88.982↑	1.0
	I48 (NP)	2.662↑	3.730↑	42.921↑	37.987↑	5.374↑	18.059↑	1.0
	I17 (NP)	3.537↑	1.828	2.848↑	406.61↑	5.903↑	17.352↑	1.0
	I1595 (P)	21.706↑	1.313	56.298↑	17.753↑	1.814	4.236↑	1.0
	I5128 (P)	1.094	1.166	5.426↑	1.064	1.205	2.821↑	1.0
	I316 (P)	2.454↑	16.718↑	18.700↑	11.043↑	13.215↑	28.457↑	1.0
	I5011 (P)	81.572↑	30.890↑	71.755↑	157.586↑	5.774↑	192.661↑	1.0

clfA and *B:* clumping factors A and B; *fnbA* and *B:* fibronectin binding proteins A and B; *icaA* and *D:* intercellular adhesion A and D; *gyrB:* DNA gyrase subunit B, # reference gene. Downward arrows indicate significantly decreased and upward arrows indicate significantly increased (target sample is different to control; $P < 0.05$).

internalize in the MAC-T cells (*clfA*, $P = 0.005$; *clfB*, $P = 0.016$; *fnbA*, $P < 0.001$; *fnbB*, $P < 0.001$; *icaA*, $P = 0.002$ and *icaD*, $P = 0.001$). However, among internalized bacteria, only four isolates overexpressed all the genes evaluated, namely I48 and I358 (from clinical NP IMI) and I316 and I5011 (from subclinical P IMI). Genes with higher relative expression level were the encoding Fn-binding proteins and icaD. Specifically, the highest level of relative expression was for *fnbB* on isolate I17, reaching an expression 406.61 higher than the control (Table 4). In the group of internalized bacteria, no significant differences were observed in the expression of *clfA* ($P = 0.714$), *clfB* ($P = 0.412$), *fnbA* ($P = 0.666$), *fnbB* ($P = 0.190$), *icaA* ($P = 0.893$) and *icaD* ($P = 0.513$) between strains from clinical NP and subclinical P IMI.

3.7. Bacterial generation time (GT)

Bacterial generation time (GT) was calculated for 4 strains from clinical NP IMI and 4 strains from subclinical P IMI. Since isolate I61 had very slow growth in subsequent cultures, it was not possible to build an exponential growth curve to calculate GT. All strains reached a maximum cell density after 5 h of culture with an average cell count of 1×109 CFU/mL (data not shown). However, the GT for clinical NP strains were significantly lower than those observed for subclinical P strains ($P = 0.028$; Fig. 3B).

4. Discussion

The genetic diversity found in *S. aureus* isolated from clinical NP and subclinical P IMI was consistent with other reports (Lundberg et al., 2014); however, in Argentina this variability is scarcely documented. Buzzola et al. (2001) performed a molecular epidemiological analysis of 112 *S. aureus* isolated from 21 districts and observed four different clusters by PFGE. Isolates belonging to the most prevalent cluster were widely distributed in the country. In the present work, the majority of the isolates represented a unique PT and the most common PT (F) gathered 35% of the strains tested including strains from Santa Fe and Córdoba provinces, indicating a wide distribution of this PT. For co-culture assays, we selected 9 isolates with different PT, but all carrying adhesion and biofilm-encoding genes and with moderate or strong biofilm production capability.

Regarding the occurrence of genes that encode for *S. aureus* surface proteins, results obtained in this study have shown similarities and few differences with previous research. The *clfA* gene was detected in 100% of the isolates, which is similar to Ote et al. (2011) findings (96.6%). The *clfB* gene was identified in all isolates tested (100%), which is consistent with previous studies (Ote et al., 2011; Klein et al., 2012). The *fnbA* gene was detected in 100% of isolates, which is in accord with Ikawaty et al. (2010) findings (96%). The presence of *clfA*, *clfB* and *fnbA* genes in all isolates evaluated is a strong indicator of the importance of these genes in the pathogenesis of *S. aureus* IMI. The *fnbB* gene was identified in higher percentage (90%) than observed by Ikawaty et al. (2010) (43%). The occurrence of *cna* gene was low compared with the other factors studied (20%). A similar prevalence (22.4%) was observed by Klein et al. (2012), while Ote et al. (2011) and Ikawaty et al. (2010) observed a higher prevalence (31.9% and 49%, respectively). The *fib* gene encodes the extracellular fibrinogen-binding protein (Efb), known to bind C3 protein (Tang et al., 2013). In the present study *fib* was identified in 90% of isolates tested, similar to results obtained from human origin (Tang et al., 2013), but lower than those reported for other bovine mastitis isolates (Zecconi et al., 2006; Ikawaty et al., 2010).

Synthesis of polysaccharide intercellular adhesin (PIA), necessary for biofilm development, requires enzymes encoded in the *icaADBC* operon (Vasudevan et al., 2003). In the present study, *icaD* was identified in all strains tested, while *icaA* and *icaC* genes in 95% of them. These finding were associated with biofilm formation, assessed by MPA. These results agree with previous studies that detected *icaA* and *icaD* genes in all bovine mastitis isolates (Vasudevan et al., 2003; Szweda et al., 2012). However, the *bap* gene could not be amplified in any sample, which agrees with previous reports on *S. aureus* isolates from bovine origin (Melchior et al., 2009; Szweda et al., 2012).

In vitro and *in vivo* studies have shown that *S. aureus* is able to invade and survive within different cell types in the mammary gland (Almeida et al., 1996; Hebert et al., 2000). In the present study, all *S. aureus* isolates were internalized in MAC-T cells and no differences in the invasion ability between strains from clinical NP and subclinical P IMI were observed. Moreover, invasiveness of the selected strains was not associated with the ability to form biofilm, as reported by Oliveira et al. (2011) on a similar number of strains tested. In contrast, Bardiau et al. (2014) did observe correlation, although using a larger number of *S. aureus* strains and different test conditions. In addition, they found an association between isolates belonging to *agr* group I and high invasion ability in MAC-T

cells (>2%) and biofilm production compared with isolates from *agr* group II. This association was also previously documented by Buzzola et al. (2007). In the present study, all strains evaluated belonged to *agr* group I and showed different invasion ability (<2% and >2%). Discrepancies between studies may rely in different MOI used, which was lower in the preceding study.

Although previous studies have investigated the presence of several genes involved in adhesion and biofilm production in *S. aureus* strains from NP and P IMI (Haveri et al., 2007; Veh et al., 2015); there is no information about quantitative expression of relevant genes involved in adhesion-internalization following pathogen-host interaction *in vitro* . In this study, all strains carrying *clfA*, *clfB*, *fnbA*, *fnbB*, *icaA* and *icaD* genes were able to express these genes *in vitro*. In general, the relative expression of adherence and biofilm-producing genes in bacteria that failed to invade MAC-T cells did not change or decreased showing no difference between strains from clinical NP and subclinical P IMI. In contrast, in those that achieved cellular internalization, the expression was significantly higher, although only two isolates from clinical NP IMI and two from subclinical P IMI overexpressed all genes evaluated. Furthermore, no significant differences were observed between strains from NP and P IMI. Lack of association between strains with different clinical origin (subclinical P or clinical NP) in the expression of adhesion and biofilm-producing genes suggests that early interactions explored by this *in vitro* experimental system are not involved in bacterial characteristics that determine further persistence within the mammary gland.

Regarding bacteria that achieved MAC-T internalization, we observed that most isolates tended to overexpress *fnbA* and *fnbB* to a greater extent than *clfA* and *clfB*, regardless of the origin (clinical NP or subclinical P IMI). Previous research demonstrated that the majority of 9 genetically different *S. aureus* strains from subclinical IMI exhibited lower expression of *clfB* compared with *fnBP* genes under *in vitro* culture conditions during the growth phase (Klein et al., 2012). In addition, Atshan et al. (2013) during *in vitro* biofilm development in methicillin resistant *S. aureus* observed greater relative expression levels of *fnbA* and *fnbB* compared with *clfA* and *clfB* genes at 12, 24 and 48 h of biofilm formation.

Previous works have demonstrated that FnBPs promote adhesion to the surface of bovine mammary epithelial cells and subsequent internalization (Brouillette et al., 2003). In the present study, genes overexpressed in all strains were those encoding FnBPs and icaD, although with variations among strains. The highest *fnbB* overexpression was observed in isolates I17 from clinical NP IMI and I5011 from subclinical P IMI; being 407 and 158 times higher than control samples, respectively. Interestingly, both isolates reached the highest internalization percentages in MAC-T cells. Brouillette et al. (2003) demonstrated that the invasive abilities of *S. aureus* *fnb*-mutants *in vitro* were severely compromised when FnBPs were not present in the surface protein complex. In the present study, strain I5128 from subclinical P IMI internalized in MAC-T cells at low percentage overexpressing only *fnbA* and *icaD* genes. Our findings are in agreement with those of Brouillette et al. (2003), who showed that absence of one type of adhesion protein severely reduces, but does not eliminate binding and internalization of *S. aureus* into mammary epithelial cells *in vitro.*

All evaluated isolates were able to overexpress *icaA* and *icaD* or both genes at different levels after internalization in MAC-T cells, which indicates a potential role of biofilm-production during early bacteria-cells interactions. Particularly, strains I5128 and I5011 (from subclinical P IMI) showed the greatest ability to form biofilm in MPA; however, following invasion to MAC-T cells, I5011 overexpressed both genes but I5128 only overexpressed *icaD.* This could be related to time-dependent expression, according with Atshan et al. (2013).

In order to determine whether the replication capacity was associated with strains origin (clinical NP and subclinical P), we evaluated their multiplication rate *in vitro*. All strains reached a maximal density after 5 h of culture; however, strains from clinical NP IMI showed significantly lower GT than strains from subclinical P IMI. This could indicate an increased rate of bacterial replication in the early infection process for strains from clinical NP IMI, which was also corroborated during invasion assay after 2 h of incubation. However, these differences in growth rates were not related to the invasive capacity to MAC-T cells. Since there is no information about growth rate of *S. aureus* strains from clinical NP and subclinical P IMI, further research will be needed to clarify this association.

In conclusion, neither presence of genes associated with adherence and biofilm formation and *in vitro* biofilm-forming ability nor internalization capacity of *S. aureus* were related to strain clinical origin (clinical NP or subclinical P IMI). Bacteria internalized in MAC-T cells overexpressed adherence and biofilm-forming genes, particularly those encoding FnBPs and IcaD. Highest invasion percentages were achieved by those isolates able to overexpress *fnBP*, irrespectively of the presence of other evaluated genes. Overall, results of this study provide new insights about *S. aureus* gene expression of adhesion-internalization factors during early interaction with the host cells.

Conflict of interest

None of the authors have any conflict of interest.

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