Interaction of primary mammary bovine epithelial cells with biofilm-forming staphylococci associated with subclinical bovine mastitis

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Summary

Background: Staphylococci are recognized worldwide as one of the most important etiological agents of bovine mastitis due to their virulence factors such as their ability to penetrate inside mammary epithelial cells and their ability to form biofilm. **Aims:** The objectives of this study were to establish a model of primary mammary epithelial cells originating from the secretory tissue of the bovine udder in order to evaluate the invasion ability of 42 staphylococci isolated from subclinical bovine mastitis cases. **Methods:** Two techniques were used to establish a model of primary mammary epithelial cells, the explant technique and the enzymatic method. Biofilm formation was detected using a quantitative spectrophotometric assay. When compared with the enzymatic digestion method, the epithelial cells obtained by the explant technique grew faster and reached quickly to confluence. **Results:** The results showed that 60% of *Staphylococcus aureus* isolates (n=12) were able to invade the epithelial cells and 72.7% of coagulase negative staphylococci (CNS) isolates were invasive (n=16). *Staphylococcus xylosus* isolates showed higher invasion values compared to *S. aureus* isolates and non-biofilm forming staphylococci were able to invade primary epithelial cells, but no significant difference was found between the internalization capabilities of biofilm positive and negative isolates. **Conclusion:** The results show that the explant technique is a valuable method for developing primary epithelial cells without damaging the cells, and provides new insights regarding the ability of staphylococci to penetrate inside primary mammary epithelial cells.

Key words: Biofilm, Bovine mastitis, Epithelial cells, Invasiveness, Staphylococci

Introduction

Bovine mastitis is a major disease affecting dairy cattle worldwide; it took attention due to its complexity, and important economic losses (Oliveira *et al*., 2001). Staphylococci are considered one of the most important pathogens in bovine mastitis (Barkema *et al*., 2006; Haran *et al*., 2012). Internalization into bovine mammary immortalized epithelial cell lines by staphylococci isolates has been reported for *Staphylococcus aureus*, *S. xylosus*, *S. epidermidis*, *S. fleurettii* and *S. chromogenes* (Almeida *et al*., 2001; Pereyra *et al*., 2016; Souza *et al*., 2016). This could explain the frequent inability of antibiotic treatments to overcome these infections and the chronic character of intramammary *Staphylococcus* infections. However, little is known about the capacity of staphylococci to penetrate inside primary mammary epithelial cells. Moreover, very few studies are interested in the role of biofilm as a potential virulence factor facilitating staphylococci colonization of the mammary gland epithelium. In other bacterial genera, such as *Pseudomonas* spp. and *Salmonella* spp. the ability to form biofilm appears to be associated with invasiveness (Berlutti *et al*., 2005; Latasa *et al*., 2005).

The invasive ability of bacteria can be evaluated *in vitro* by measuring their capacity to invade isolated bovine mammary epithelial cells (BMEC). The appropriate choice of research model is decisive. One of the most popular approaches is the selection of established cell lines, such as the mammary epithelial cell line T (MAC-T) (Huynh *et al*., 1991), and Penn State Bovine Mammary Epithelial cell line (PS-BME) (Gibson *et al*., 1991). However, serial passages of cell lines can further cause genotypic and phenotypic variation over an extended period of time. Therefore, they may not adequately represent primary cells and yield different results (Kaur *et al*., 2012; Buehring *et al*., 2014). These factors renewed interest in primary cells that can maintain many of the important markers and functions seen *in vivo* (Alge *et al*., 2006; Pan *et al*., 2009). There are generally two techniques that have been used to cultivate primary epithelial cells:

(i) The explant technique

(ii) The enzymatic method

To the authors' knowledge, no studies have compared the explant technique and the enzymatic method based on their capacity to isolate and cultivate bovine primary mammary epithelial cells. The objectives of this investigation were, therefore

(i) to isolate and cultivate bovine primary mammary

epithelial cells (PMECs) *in vitro* from a healthy lactating cow
(ii) to compare the two different isolation techniques
(iii) to evaluate the intracellular invasion ability of staphylococci from subclinical bovine mastitis
(iv) to determine the *in vitro* correlation between biofilm formation and invasiveness

Materials and Methods

Bacterial strains and growth conditions

Staphylococcus spp. were isolated from milk samples taken from Holstein cows with subclinical mastitis belonging to different dairy farms in the Batna province, Algeria. One isolate was taken from one infected quarter of each cow. Three parameters were used to identify an infected quarter; high somatic cell count (SSC) (>200,000 cells/ml), the absence of clinical signs of diseased cows, and positive California mastitis test (CMT) (Owens *et al*., 1997). Preliminary strain identification was performed based on conventional methods using the tube coagulase test according to Quinn *et al*. (2002). Specific identification was made using the ApiStaph® system (BioMérieux, France). Forty-two staphylococci belonging to 7 species were isolated and used for the internalization assay as follows:
Staphylococcus aureus (n=20)
Staphylococcus xylosus (n=12)
Staphylococcus epidermidis (n=4)
Staphylococcus scuiri (n=2)
Staphylococcus lugdunensis (n=2)
Staphylococcus simulans (n=1)
Staphylococcus capitis (n=1)

Reference strain *S. aureus* ATCC 27543 was included as a positive control.

For the invasion assay, isolates were grown overnight on Trypticase soy agar (TSA, Difco, France) at 37°C. A single colony was inoculated in 5 ml Trypticase soy broth (TSB, Difco, France), and grown at 37°C without shaking for 24 h. The overnight culture was centrifuged (2500 × g for 15 min at room temperature), the supernatant was discarded and the pellet was washed once with sterile phosphate buffered saline (PBS, pH = 7.2) and re-suspended in Dulbecco modified eagle medium (DMEM, Sigma, UK). Bacterial concentrations in subcultures were estimated by spectrophotometric measurements at 600 nm to give a cell density of 2×10^6 CFU/ml. All the strains were sensitive to gentamicin.

Biofilm assay

The biofilm assay was performed as previously described by Stepanovic (2007). *Staphylococcus* isolates were grown overnight at 37°C on blood agar. A single colony was inoculated in 5 ml TSB and incubated for 18 h at 37°C. After that, the turbidity of the bacterial suspension was adjusted to obtain turbidity comparable to that of the 0.5 McFarland standards. This suspension was then diluted in 1:100 in TSB supplemented with 0.25% glucose (TSBg). This dilution was used as the inoculum in the microtiter plate test. For each staphylococci isolated, 200 µL aliquots of prepared suspension were inoculated into three wells of the 96-well tissue culture plates. Each culture plate included a negative control composed of TSBg. The plates were incubated at 37°C for 24 h. Afterwards, the content of each well was removed by aspiration and the wells were rinsed 3 times with 300 µL PBS. The plates were then dried in an inverted position. The attached bacteria were fixed for 20 min at room temperature by adding 150 µL methanol in each well. The plates were then stained with 150 µL aqueous solution of crystal violet 2% (Sigma) for 15 min at room temperature. After staining, the plates were rinsed with tap water and later, the stain bound to the bacteria was dissolved by adding 150 µL of 95% ethanol (Sigma). The plates were then left at room temperature for at least 30 min, and the optical density (OD) of each well was measured using a microplate ELISA reader at 570 nm (Metertech Σ 960). The experiment was performed in triplicate and sterile TSBg was used as a negative control. An OD_{570} value of 0.2 was taken as the cut off point according to Stepanovic's protocol (2007) to differentiate between biofilm and non-biofilm-producers.

Isolation of primary mammary cells

Primary cell culture was carried out as described by Huynh (1991), with some modifications. Mammary parenchyma tissues were aseptically derived from a healthy slaughtered Holstein lactating cow with an infection-free udder, according to the principles described by Cifrian (1994). To minimize contamination with myoepithelial cells, the parenchyma tissue was then transported with 1 × Dulbecco's phosphate-buffered saline (DPBS), and preserved at 4°C until cell isolation. The mammary tissue pieces were then washed three times with DPBS, and minced using scalpels and surgical scissors. Minced samples were finally incubated in aseptic Hanks balanced salt solution (HBSS) for 1.5 h at 37°C.

Preparation of bovine mammary cell culture

For the disaggregation of bovine mammary tissues, two different methods (explant and enzymatic digestion) were used and the results were compared in terms of yield and confluency (Harrison *et al*., 1907; Carrel *et al*., 1912). In the primary explant technique, unwanted tissue such as necrotic material was dissected and transferred to a second dish. Then chopped very finely with crossed scalpels, fragments of secretory tissue were washed with DPBS and transferred to polystyrene Petri dishes (100 mm), with about 20 pieces spread evenly over the growth surface. Once an outgrowth had formed, the remaining explants were collected with a scalpel.

In the enzymatic digestion technique, the prepared tissue was digested with Trypsin-EDTA (0.25%, Sigma) solution for 45 min at 37°C. The digest was then filtered through a nylon mesh (100 µm) and the filtrate was centrifuged at 2000 × g for 10 min, the supernatant was discarded and the pellet was diluted with the growth medium and plated in 25 cm^2 flasks.

Media and cell culture conditions

Primary cell inoculums were plated in DMEM with 4.5 g/L glucose (Sigma), supplemented with 20% fetal bovine serum (FBS), insulin (1 µg/ml, Sigma), hydrocortisone (5 µg/ml, Sigma), amphotericin B (2.5 µg/ml, Sigma), and penicillin-streptomycin (50 IU/ml, Sigma), they were then incubated at 37°C, 5% CO_2 and 95% humidity. The medium was changed every 2 days, and the primary cell cultures were passaged at ~80% confluency (Trypsin/EDTA 0.25%).

Fibroblast and debris were eliminated as described by Pal (1983). In all invasion assays, epithelial cells were used after the second passage, their viability was determined by Trypan Blue exclusion (0.4%, Sigma) using a Haemocytometer and the nuclei were visualized by Giemsa coloration (Freshney, 2010).

The morphology of the cells was observed using a light microscope and a phase-contrast microscope.

The internalization assay

The internalization assay was performed as described by Almeida (1996), with some modifications. The cells were grown to confluence after the second passage in flat bottom microplates (96-wells), washed with PBS and then incubated for 24 h with the invasion medium (growth medium without FBS and antibiotics), at 37°C and 5% CO_2.

The cell monolayers (approximately 2×10^5 cells/well) were washed with PBS and inoculated with approximately 2×10^6 CFU/well of staphylococci at a multiplicity of infection (MOI) of 10:1. Plates were incubated for 2 h at 37°C. In parallel, bacterial suspensions were incubated to be used as control groups for 2 h at 37°C in 5% CO_2 in DMEM. The cells were washed with PBS to remove unattached bacteria and extracellular bacteria were treated with gentamicin (100 µg/ml in DMEM) for 2 h. Cells were washed again with PBS, incubated with EDTA/Trypsin (0.25%) for 5 min at 37°C, followed by incubation in Triton X-100 solution (5 min) to release intracellular staphylococci. The cell lysates, and the control group were carefully suspended and serially diluted. The invasion assay was performed in triplicate and internalized bacteria were quantified on TSA plates.

Statistical analysis

Statistical significance was determined by One-way ANOVA, using SPSS software version 20, and graphs were made using Excel (2007). Each experiment was carried out in triplicate (biological repeats) and all data obtained were expressed as mean±SD. Values of $P<0.05$ were considered as statistically significant.

Results

Biofilm formation

Results from the microtiter plate test show that 83.3% of staphylococci isolates were able to produce biofilm (n=35), while 16.7% of the staphylococci were non-biofilm producers (n=7) (OD_{570} <0.2). 17.6% of *S. aureus* isolates were found to be strong biofilm producers (n=3) (OD_{570} >0.8), 58.9% were moderate biofilm producers (n=10) (0.8> OD_{570} >0.4), and 23.52% were weak biofilm producers (n=4) (0.4> OD_{570} >0.2), while 50% of the CNS isolates were found to be strong biofilm producers (n=9), 38.9% were moderate biofilm producers (n=7) and 11.1% were weak biofilm producers (n=2) (Table 1).

Isolation of bovine primary mammary epithelial cells

One day after culturing, a small number of adherent cells were observable in the Petri dishes used for the explant technique and in the 25 cm^2 flasks used for the enzymatic technique (Fig. 1a). After 3 days, small islands started to form (Fig. 1b), and it took 12 days for cells digested enzymatically to reach 60% confluency (Fig. 1c) and 12 days to arrive at 80% confluency, and only 7 days for the primary cells in the Petri dishes to reach 80% confluency (Fig. 1d). Domes were observed in the confluent monolayers, with cobblestone shaped cells in the flasks and the Petri dishes. No bacteria or fungi contamination was observed, and the purification procedure was effective in eliminating elongated cells from the PMEC cultures.

Table 1: Quantification of biofilm formation of staphylococci by optical density (OD)

S. aureus isolates (n=17)	Biofilm production assay (SD [a])	Biofilm production ability*	CNS isolates (n=18)	Biofilm production assay (SD [a])	Biofilm production ability*
SA1	0.55 (0.43)	++	S1: *S. xylosus*	0.93 (0.12)	+++
SA2	0.52 (0.23)	++	S2: *S. xylosus*	1.05 (0.34)	+++
SA3	0.61 (0.01)	++	S3: *S. xylosus*	0.82 (0.02)	+++
SA4	0.70 (0.11)	++	S4: *S. xylosus*	0.60 (0.05)	++
SA5	0.91 (0.08)	+++	S5: *S. xylosus*	0.67 (0.32)	++
SA8	1.17 (0.34)	+++	S6: *S. xylosus*	1.09 (0.09)	+++
SA9	1.12 (0.91)	+++	S7: *S. xylosus*	0.24 (0.08)	+
SA11	0.46 (0.08)	++	S8: *S. xylosus*	0.42 (0.23)	++
SA12	0.61 (0.07)	++	S9: *S. xylosus*	0.23 (0.01)	+
SA13	0.23 (0.34)	+	S12: *S. xylosus*	1.07 (0.12)	+++
SA14	0.22 (0.23)	+	S14: *S. epidermidis*	0.92 (0.34)	+++
SA15	0.20 (0.56)	+	S15: *S. lugdunensis*	1.04 (0.18)	+++
SA16	0.70 (0.53)	++	S16, S17: *S. epidermidis*	0.77 (0.23)	++
SA17	0.38 (0.21)	+	S18: *S. sciuri*	0.70 (0.04)	++
SA18	0.44 (0.02)	++	19: *S. sciuri*	0.56 (0.07)	++
SA19	0.72 (0.45)	++	S20: *S. lugdunensis*	1.12 (0.21)	+++
SA20	0.72 (0.12)	++	S21: *S. simulans*	0.95 (0.12)	+++

* (+++): Strong biofilm producers, (++): Moderate biofilm producers, and (+): Weak biofilm, [a] Standard deviations

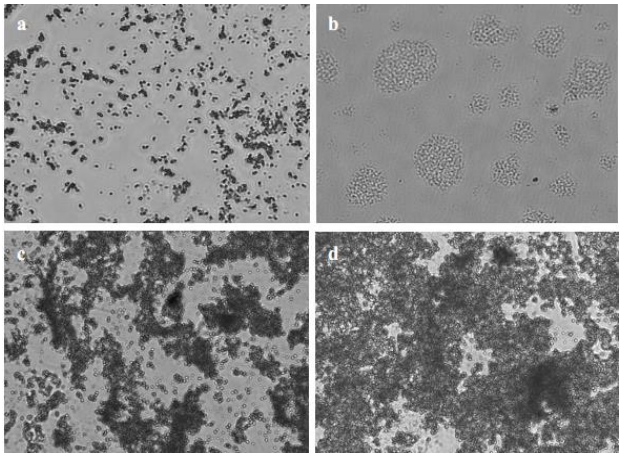

Fig. 1: Morphology and confluence of PMECs. (**a**) Contrast observation of PMECs cultured *in vitro* (×40), (**b**) PMECs formed islands (×40), (**c**) PMECs at 60% confluency after 7 days in 25 cm^2 flasks (×40), and (**d**) PMECs at 80% confluency after 7 days in the Petri dishes (×40)

The internalization assay

Our isolates showed different levels of invasion. 60% of *S. aureus* isolates (n=12) were able to invade the PMECs, while 40% were non-invasive (n=8). Two biofilm forming isolates showed the highest internalization numbers (SA2: 4.22 ± 0.40, SA11: 4.04 ± 0.08). Among the biofilm-negative isolates, three were able to invade the epithelial cells, one of which showed the highest invasive ability into PMECs among *S. aureus* isolates (SA10: 4.72 ± 0.05), and two had a low-level of invasiveness (Fig. 2).

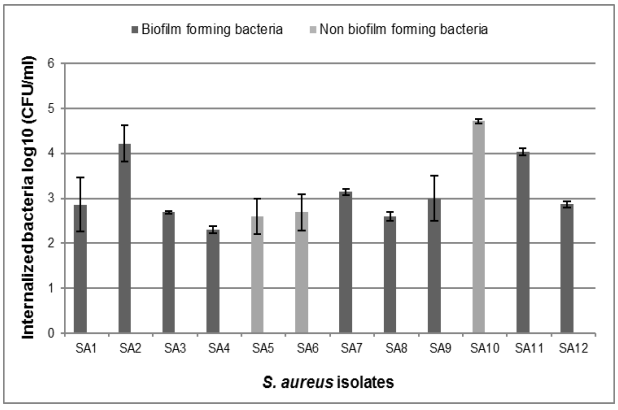

Fig. 2: Survival of biofilm-positive *Staphylococcus aureus* isolates compared with biofilm negative isolates after 2 h. These data represent the means and standard deviations of three independent experiments performed in triplicates

Based on CFU results, 72.7% of the CNS isolates were able to internalize into PMECs (n=16), while 27.3% were non-invasive (n=6). Two biofilm forming *S. xylosus* isolates showed the highest internalization numbers (S1: 5.33 ± 0.20; S5: 5.27 ± 0.03) (Fig. 3), while *S. lugdunensis* isolates were not able to invade the PMECs. Additionally, CNS biofilm-negative isolates were all able to invade primary cells except for one of the *S. epidermidis* isolates (S22), which was not able to produce a biofilm and penetrate inside the epithelial cells. Internalization ability between biofilm-forming and non biofilm-forming isolates was compared and no significant difference was observed (P=0.419). Moreover, we did not observe a significant difference between the internalization ability of *S. aureus* isolates and CNS isolates (P=0.415).

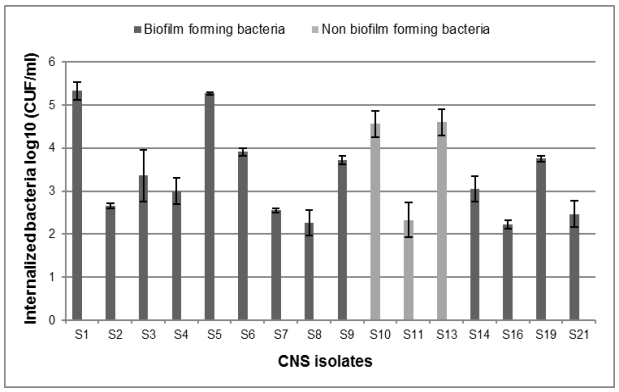

Fig. 3: Survival of biofilm-positive CNS compared with biofilm negative isolates. These data represent the means and standard deviations of three independent experiments performed in triplicates. S1-S11: *S. xylosus*, S13: *S. capitis*, S14, S16: *S. epidermidis*, S19: *S. sciuri*, and S21: *S. Simulans*

Discussion

The main aims of this study were to isolate and cultivate primary bovine mammary epithelial cells using two different techniques to determine the intracellular invasion ability of staphylococci isolated from bovine mastitis cases.

The isolation of mammary cells by mechanical digestion and selective digestion with Trypsin/EDTA resulted in two distinct populations with different viability and growth capacity. Cells obtained by the explant technique were more morphologically homogenous and arrived quickly to confluency (7 days) compared to cells obtained after the enzymatic digestion that were more mixed with elongated cells and debris, and took 12 days to form a confluent monolayer. However, cobblestone shaped cells were observed in both cases. Experiments on explant are the closest model resembling mammary tissue because the cellular composition of the mammary tissue is similar to the *in vivo* conditions (Rose *et al*., 2006). The Explant method has also been successfully used to isolate human gingival epithelial cells (Kedjarune *et al*., 2001), mesenchymal stem/stromal cells from different tissues (Yang *et al*., 2007; Ishige *et al*., 2009; Spath *et al*., 2010), and to isolate rabbit limbal epithelial cells (Zhang *et al*., 2005). Using this method, mammary epithelial cells were isolated with simplicity and relative ease. However, more time was required before subculture in the enzymatic method. The use of another enzyme such as collagenase may be more effective in the digestion of mammary epithelial cells (Huynh *et al*., 1991).

The intracellular invasion ability of the isolates varied for the 42 bovine mastitis isolates tested in our

model. *Staphylococcus aureus* isolates were able to invade the PMECs. Similar observations were also reported by Almeida *et al*. (1996), Hensen *et al*. (2000), and Brouillette *et al*. (2003). However, not all of the *S. aureus* isolates were able to invade PMECs. These findings may suggest that bacterial invasion is not a necessary mechanism for the establishment and persistence of mastitis (Anaya-López *et al*., 2006). Moreover, similar to the observations reported by Almeida (2001), CNS species were able to internalize into the PMECs; nonetheless, the *S. lugdunensis* isolates that we tested were not able to invade the PMECs.

When internalization of *S. aureus* and CNS isolates was compared, no significant differences were observed, although internalization values differed from strain to strain, which indicate that invasion capacity is strain-dependent. Moreover, *S. xylosus* isolates showed higher invasion values compared to *S. aureus* isolates. This may be due to the use of the "trigger" mechanism, which is an alternative signal transduction pathway by the CNS (Almeida *et al*., 2001). The internalization appears to occur through a bacterial induced endocytosis, which involves host cell cytoskeleton elements (Almeida *et al*., 1995), eukaryotic nucleic acid, and bacterial proteins synthesis (Almeida *et al*., 1997). However, Brouillette (2003) indicated that adherence to MAC-T cells was reduced for fibronectin-binding protein ($FnBPs^-$) deficient bacteria suggesting that the absence of one type of adhesion protein severely reduces, but does not eliminate internalization into mammary epithelial cells *in vitro*.

Similar to the results reported by Oliveira (2011) and Pereyra (2016), our data indicate that the invasiveness of the selected isolates was not associated with the ability to form biofilm. In contrast, Buzzola (2001) and Bardiau (2014) found that biofilm-forming ability influences the invasion capacity of the *S. aureus* mastitis isolates. They studied phylogenetic characteristics such as accessory gene regulator (*agr*) typing, and found that all the *S. aureus* isolates belonging to *agr* group 'I' had the ability to form biofilm and to invade the MACt cells, while strains belonging to group 'II' were non-invasive and did not have the ability to form biofilm.

The internalization of primary mammary epithelial cells by staphylococci was highly effective. Both the explant technique and the enzymatic method effectively isolated mammary epithelial cells, but the explant technique appeared to be more successful. The internalization ability of staphylococci varied among species and the invasiveness was not associated with the ability to form a biofilm. Overall, the results of this study show that the explant technique is a valuable method for developing bovine primary mammary epithelial cells without damaging the cells, and providing new insights about the ability of staphylococci to penetrate inside primary mammary epithelial cells.

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