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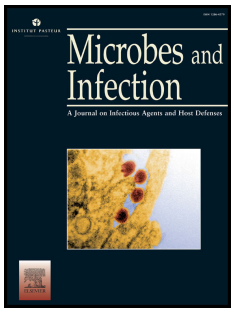

Evaluation of the relative expression of genes associated with adherence after different hours of co-culture between *Streptococcus uberis* and MAC-T cells

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TITLE: Evaluation of the relative expression of genes associated with adherence after different hours of co-culture between *Streptococcus uberis* and MAC-T cells.

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ABSTRACT

Streptococcus uberis is an environmental pathogen associated with subclinical and clinical IMI in both lactating and non-lactating cows. RC19 strain was isolated from a cow with subclinical mastitis, qualitatively classified as moderate biofilm producer in Todd Hewitt medium (THB), and it showed a high value of the adhered bacteria (CFU/ml). Hence, the aims of this study were (a) to determine ability to adhere to and internalize into epithelial cells MAC-T for 1, 2 and 3 h, (b) to evaluate the relative expression of adherence-associated genes from co-cultures of *S. uberis* with MAC-T cells at 1, 2 and 3 h. We hypothesized that upon contact with bovine mammary epithelial cells, *S. uberis* upregulates adherence-associated genes encoding adhesins, which enable it a higher adherence to and/or internalization into host cells. Four to six genes increased their R with regard to the control after initial contact with MAC-T cells (group 1) at 1, 2 and 3 h. The highest value of R was observed at 2 h after co-culture between RC19 and MAC-T cells.

1. INTRODUCTION

Bovine mastitis is an inflammation of the mammary gland and the most prevalent disease in dairy cattle since it affects dairy herds worldwide [1]. It is an expensive disease for the dairy industry, since it reduces milk yield and quality, and is responsible for significant losses in dairy farms [2,3]. *Streptococcus uberis* is an environmental pathogen associated with subclinical and clinical bovine intramammary infections (IMIs) in both lactating and non-lactating cows [4], which can persist in the udder and cause chronic infection in the mammary gland [5]. The capability to adhere to mammary epithelial tissue has been accounted an important strategy in many bovine pathogens, including *S. uberis*, which might afford an advantage to colonize the lactating mammary gland [6–10]. Several *S. uberis* adhesins involved in binding to host cells surface and extracellular matrix components have been described and the environmental and growth conditions would regulate their expression [7,9,11,12]. There were currently no studies characterizing gene expression in *S. uberis* from bovine mastitis in the presence of host cells, so we investigated the expression of adherence-associated genes in one strain at different hours of co-culture between *S. uberis* and MAC-T cells. In a previous study made in our laboratory, a total of 34 isolates collected from clinical and subclinical bovine mastitis from 17 herds located in the central dairy region of Argentina, were identified as *S. uberis* by biochemical and molecular tests, and confirmed by MALDI-TOF (MS system Bruker Daltonics, Bremen, Germany) [13]. Later, we observed a high prevalence and a high degree of similarity in the nucleotide and amino acid sequences of six adherence-associated genes (*acd*A SUB_RS03245, *lmb* SUB_RS04460, *scp*A SUB_RS05795, *sua* SUB_RS08150, *fbp* SUB_RS05580 and *lbp* SUB_RS00865) among field strains, despite the wide clonal heterogeneity detected [14]. Recently, we investigated the capability of adherence to and internalization into

MAC-T cells and the expression profile of adherence genes among nine *S. uberis* strains with different ability to form biofilm [15]. We detected that the strains were capable of adhering to and internalizing into MAC-T cells at different levels, and we concluded that did not find out a single profile of relative expression values (R) both in bacteria after the initial contact with MAC-T cells (G_1) and in adhered and internalized bacteria (G_2). However, one strain (RC19) showed higher R values in G_1 and lower values in G_2 with respect to control in all adherence genes, which agrees with our hypothesis. This strain isolated from subclinical mastitis was qualitatively classified as moderate biofilm producer in Todd Hewitt medium, and it showed a high value of adhered bacteria (CFU/ml) [15]. According to these results, we selected the RC19 strain to extend our knowledge about early bacterial pathogen-host interactions. Hence, the aims of this study were (a) to determine ability to adhere to and internalize into epithelial cells MAC-T for 1, 2 and 3 h, (b) to evaluate the relative expression of adherence-associated genes from co-cultures of *S. uberis* with MAC-T cells at 1, 2 and 3 h. We hypothesized that upon contact with bovine mammary epithelial cells, *S. uberis* upregulates adherence-associated genes encoding adhesins, which enable it a higher adherence to and/or internalization into host cells.

2. MATERIALS AND METHODS

2.1. Adherence assays

For adherence assays, the established bovine mammary epithelial cell line (MAC-T) [16] was used. Epithelial cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Natocor), 5 µg/ml bovine insulin (Sigma-Aldrich, MO, USA), 1 µg/ml hydrocortisone (Sigma-Aldrich, MO, USA), antibiotic-antimycotic (Gibco BRL, Grand Island, NY), 2 mM glutamine (Emeve, BA, Argentina), 40 mM

Hepes (Gibco BRL), and 1 mM sodium pyruvate (Sigma-Aldrich, USA). For each experiment, MAC-T cells were seeded at 1 x 10^5 cells/well in 24-well plates at 37°C in 5% CO_2:95% air (v/v) until 100% confluence. The bacterial adherence assays were performed in standardized conditions according to Almeida et al. (2006) and Fessia et al. (2020). The bacterial suspension was co-cultured with a confluent monolayer of MAC-T cells in DMEM at a multiplicity of infection (MOI) of 10, for 1, 2 and 3 h at 37°C in 5% CO_2:95% air (v/v). Then, MAC-T cell lysates were 10-fold serially diluted, plated in triplicate on trypticase soya agar and incubated overnight at 37°C. Colony-forming units *S. uberis* associated with MAC-T cells per ml (CFU/ml) were determined by standard colony counting techniques. Each assay was run in triplicate with four observations per assay, and means were compared by analysis of variance (ANOVA). Means showing statistically significant differences ($p < 0.05$) were consecutively evaluated by Tukey's post-hoc test.

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

To study relative expression, the total RNA extraction was realized from three experimental conditions: *S. uberis* in the supernatant of co-cultures for 1, 2 and 3 h with MAC-T cells (bacteria that were in contact with MAC-T cells, group 1), *S. uberis* in the lysate of MAC-T cells after 1, 2, and 3 h of co-culture (associated bacteria, i.e., adhered and internalized bacteria, group 2), and *S. uberis* without contact with MAC-T cells as a control group. The RNA isolation and cDNA synthesis were carried out as previously described [15]. Real-time qRT-PCR was performed to quantify the relative gene expression of adherence-associated genes, *acdA, lmb, scpA, sua, fbp* and *lbp* (See table Table 1 supplemented), and was normalized to the *ddlA* gene. Each cDNA was amplified under thermal cycling protocol according to Fessia *et al*. (2020). The reactions were performed in a MX3000 Multiplex Quantitative PCR system

(Stratagene-Agilent) by using iTaq Universal SRYB Green 2X SuperMix kit (Bio-Rad) in duplicate in two independent experiments. The quantification of mRNA was determined using the delta Ct method [17] and the transcript quantities were expressed as changes (n-fold) relative to the values of the control.

3. RESULTS AND DISCUSSION

Results showed that RC19 strain was able to adhere to and internalize into MAC-T bovine mammary epithelial cells after 1, 2 and 3 h. Fig. 1 shows the highest arithmetic means expressed as Log10 CFU/ml at 2 and 3 h ($2.2.10^5 \pm 6.9.10^4$ CFU/ml and $1.4.10^5 \pm 3.5.10^4$ CFU/ml, respectively), which are significantly higher than those at 1 h (p=0.0008). We observed that RC19 strain showed an average percentage of 0.40%, 11.70% and 5.79% of associated bacteria to MAC-T cells after 1, 2 and 3 h of co-culture, respectively, with respect to the number of bacteria detected in the initial inoculum. Adherence to and internalization into the epithelium of the mammary gland are two important events in early *S. uberis* pathogenesis and have been extensively investigated in *in vitro* studies by several authors [18–22]. However, these abilities have not been determined in the *in vivo* challenges carried out to date since that it has been difficult to study. In Argentina, there are not previous studies about the adherence ability of *S. uberis* to MAC-T cells at different hours of co-culture, but our observations are in concordance with other studies [9,22,23]. Almeida et al. (1996) reported that *S. uberis* UT101 and UT102 were able to adhere to MAC-T cells at 1 h of co-culture. In this sense, Almeida *et al*. (1999) showed that UT888 evidenced higher values of adhered bacteria than UT366 strain after 2 h of co-culture with MAC-T cells. In coincidence with Tassi et al. (2015), RC19 strain was able to adhere to MAC-T cells after 3 h of co-incubation. These authors demonstrated that FSL Z1-048, a clinically virulent strain, exhibit 1000-fold higher levels of adherence than FSL Z1-124, avirulent

strains, after 3 h of co-culture with BME-UV1 cells. Previously, Tamilselvam et al. (2006) indicated that *S. uberis* can survive within MAC-T cells for an extended time without causing apparent cell damage or death. As of yet, little is known about the expression relative of adherence-associated genes in *S. uberis* strains from IMIs. Recently, Kerro Dego *et al*. (2018) showed that 10 genes of *S. uberis* were upregulated during early stages of host-bacterial interactions, after 2 h or 4 h of co-culture with primary bovine mammary epithelial cells. These genes were associated with bacterial adhesion to and internalization into host cells, two-component regulatory systems, sugar transport, signal transduction, regulation of gene transcription, and pathogenicity to the host. In our previous study, we evaluated the expression relative of the *acdA, lmb, scpA, sua, fbp* and *lbp* genes involved in bacterial adherence events [15]. Four genes, *acdA, lmb, fbp* and *lbp* increased their R values with regard to the control after initial contact of RC19 strain with MAC-T cells (group 1) at 1, 2 and 3 h of co-culture. Genes *lmb, fbp* and *lbp* showed significantly higher values than the control group. The relative expression of *scpA* and *sua* showed increased values of R with regard to the control after initial contact only at 2 h after co-culture. In general, the relative expression of adherence-associated genes decreased after 1, 2 and 3 h of co-culture in associated bacteria (group 2). Four (*acdA, scpA, sua, fbp*) and all six genes exhibited significantly lower values than the control at 1 h and 2 h, respectively, after co-incubation between RC19 and MAC-T cells. We observed a increase in R values of *lmb, fbp* and *lbp* genes in group 2 bacteria after 3 h of co-culture between the RC19 strain and the MACT cells in comparison to 1 and 2 h, and with respect to the control group, could be attributed to a potential role of these genes in some event subsequent to adherence process. In conclusion, the results obtained in this study suggested that *acdA, lmb, fbp* and *lbp*

could have a role in early interaction between pathogen-host cells, and contribute to the adherence of *S. uberis* to MAC-T cells after 2 h of co-culture.

4. CONCLUSION

Until this moment, this is the first study to demonstrate the relative expression of adherence-associated genes from co-cultures between *S. uberis* and MAC-T cells at 1, 2 and 3 h. More extensive studies are needed to investigate the relative expression of potential genes involved in adhesion, internalization, and intracellular survival processes into host cells to advance our understanding of the pathogenicity of *S. uberis*.

Conflict of interest

None of the authors of this paper has any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Figure captions

253 **Figure 1.** Mean value of bacteria number belonging to *Streptococcus uberis* RC19
254 strain associated with MAC-T epithelial cells at different hours of co-culture. Each bar
255 represents the arithmetic mean ± standard error (SEM) of the mean of four independent
256 experiments performed in triplicate, expressed as Log10 CFU/mL.*The nominal *p*-
257 value for statistical significance was $p < 0.05$.

258

259 **Figure 2.** Fold change expressed as Log (2) R for adherenced-associated genes at
260 different experimental conditions by *Streptococcus uberis* RC19 strain associated to
261 MAC-T epithelial cells. **A.** Bacteria in contact with MAC-T cells (group 1) at 1, 2 and 3
262 h. **B.** Associated bacteria with MAC-T cells (group 2) at 1, 2 and 3 h .*The nominal *p*-
263 value for statistical significance was $p < 0.05$, ** $p<0.01$, *** $p<0.001$.

264