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Assessment of the potential utility of different regions of *Streptococcus uberis* adhesion molecule (SUAM) for mastitis subunit vaccine development

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ABSTRACT

Streptococcus uberis is one of the most prevalent pathogens causing clinical and subclinical mastitis worldwide. Among bacterial factors involved in intramammary infections caused by this organism, *S. uberis* adhesion molecule (SUAM) is one of the main virulence factors identified. This molecule is involved in *S. uberis* internalization to mammary epithelial cells through lactoferrin (Lf) binding. The objective of this study was to evaluate SUAM properties as a potential subunit vaccine component for prevention of *S. uberis* mastitis. B epitope prediction analysis of SUAM sequence was used to identify potentially immunogenic regions. Since these regions were detected all along the gene, this criterion did not allow selecting a specific region as a potential immunogen. Hence, four fractions of SUAM (-1fr, 2fr, 3fr and 4fr), comprising most of the protein, were cloned and expressed. Every fraction elicited a humoral immune response in mice as predicted by bioinformatics analysis. SUAM-1fr generated antibodies with the highest recognition ability towards SUAM native protein. Moreover, antibodies against SUAM-1fr produced the highest proportion of internalization inhibition of *S. uberis* to mammary epithelial cells. In conclusion, SUAM immunogenic and functionally relevant regions were identified and allowed to propose SUAM-1fr as a potential candidate for a subunit vaccine for *S. uberis* mastitis prevention.

1. Introduction

Bovine mastitis is the most significant limiting factor for profitable dairying worldwide. Total costs of mastitis in Argentina, including direct production losses and disease control expenses, have recently been estimated to be ≥ 1 US$/milking cow/day [1]. In addition to decreased milk production and quality, extensive antibiotic usage for treatment and prevention of intramammary infections (IMI) is a serious concern for dairy industry and public health [2].

Several streptococcal species, including *Streptococcus uberis*, are capable of causing IMI. This organism, ubiquitous in the cow's environment, causes IMI both during lactation and the nonlactating period and accounts for the majority of environmental streptococcal mastitis cases in heifers within the first week of lactation [3]. Continuous mammary gland exposure to *S. uberis* and poor control of IMI caused by this organism through recommended control procedures, has led to a search for enhancing cow's resistance to infection through vaccination [4].

To develop an effective vaccine for the control of *S. uberis* mastitis, knowledge of its virulence factors and their capability to generate a protective immune response is a critical step. Several virulence factors that contribute to *S. uberis* pathogenesis have been described. Among these, *S. uberis* adhesion molecule (SUAM) is involved in bacterial adherence, internalization and persistence in bovine mammary epithelial cells [5]. The extraordinary diversity of *S. uberis* strains [3] has also been a great difficulty in designing a

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vaccine that could confer protection against field strains [6]. Recent research conducted at our laboratory determined that 97.8% of *S. uberis* isolated from bovine IMI in Argentina harbored the *sua* gene [7]. In addition, we found that SUAM nucleotide and amino acid sequences showed an identity between 95% and 100% with respect to all reference sequences registered in GenBank [7]. The high prevalence of SUAM among *S. uberis* isolated from bovine IMI as well as its conservation among isolates makes it an attractive candidate for development of a subunit recombinant vaccine for mastitis prevention. However, production of recombinant proteins with coding sequences >1000 bp poses technical difficulties [8]. Considering that SUAM molecular weight is 112 KDa with a nucleotide sequence of approximately 2700 bp, the objectives of this study were to examine by in silico and experimental criteria SUAM regions rich in B epitopes in order to adjust the recombinant protein fractions size containing these regions, to produce selected molecule fractions and to characterize their immunogenicity.

2. Methods

2.1. Bacterial strains and growth conditions

Two strains of *S. uberis* designated SU05 and SU42 isolated from cows with mastitis were used. These strains were characterized previously in our laboratory [7]. These strains are not epidemiologically related and were isolated from different types of mastitis, SU42 from subclinical mastitis and SU05 clinical mastitis [7]. The isolates were initially identified using standard conventional biochemical tests [9] and further confirmed using restriction fragment length polymorphism analysis of 16S rDNA as previously described [10]. Streptococcal stocks were stored at −80 °C in Todd−Hewitt broth (THB) (Sigma-Aldrich Co., St. Louis, MO) with 20% glycerol (Promega, Madison, USA) until further use. *Escherichia coli* BL21 (DE3) strain (Novagen, Madison, WI, USA) was grown in Luria-Bertani (LB) medium (Britania) supplemented with ampicillin (Amp) (100 μg/mL) (Sigma Chemical Co., St.Louis, USA) as needed for plasmid maintenance.

2.2. Epitope prediction

The amino acid sequence of SUAM from *S. uberis* UT888 (GenBank accession number, ABB52003.1), a strain originally isolated from a cow with chronic mastitis, was used for all analyses [11,12]. The epitope prediction study was carried out using two online prediction methods, ABCpred and AAPPred. The first (http://www.imtech.res.in/raghava/abcpred/), allows to predict B epitopes, of defined length (10, 12, 14, 16° 20 aa) and the antigenicity analysis is based on an artificial neural network, using a database of known epitopes [13]. In this work a length defined between 10 and 20 aa was used. The second method, AAPPred (http://www.bioinf.ru/aappred/), determines the antigenicity of amino acid pairs (AAP), evaluating the frequency at which they appear next to a scale of hydrophobicity propensity, flexibility, accessibility within a protein and polarity [14].

2.3. Design, expression and purification of recombinant proteins

To obtain the recombinant SUAM fractions, five pairs of primers were designed (Table 1) using the Primer Select (DNAstart®) software. This software selects primers with the highest score, resulting in some cases the superposition of the template sequences. The oligonucleotide sequences included sites for restriction enzymes *Eco*RI and *Hind*III. PCRs were performed, from SU05 of *S. uberis* strain bacterial genome, by running 35 cycles with a temperature profile of 1 min at 94 °C, 1 min at 57 °C, and 1 min at 68 °C. The purified PCR products were digested with *Eco*RI and *Hind*III, and ligated into the pET-32a plasmid (Novagen, Madison, WI, USA) at the corresponding restriction sites. The resultant recombinant vectors were referred to as SUAM-1fr/pET-32a, SUAM-2fr/pET-32a, SUAM-3fr/pET-32a, SUAM-4fr/pET-32a and SUAM-5fr/pET-32a, respectively for each fraction. The identity of the cloned fragments was confirmed by sequencing, yielding a percentage higher than 98% homology to the sequence available in GenBank DQ232760.1. The pET-32a constructions were transformed into the *E. coli* strain BL21 (DE3) for protein expression. *E. coli* BL21 (DE3) cells were transformed with the different construction plasmids and grown in LB medium supplemented with Amp at 37 °C. When the culture optical density $(OD)_{600}$ reached 0.6, protein expression was initiated by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) (Promega). After additional 3 h cultivation, cells were harvested by centrifugation, resuspended in buffer (50 mM Na_2HPO4 pH 8, 300 mM NaCl, 10 mM Imidazol, 1 mM phenyl methyl sulfonyl fluoride) and sonicated with 50% pulses for 2 min at 600 W. The whole cell lysate was then centrifuged at 13,000 rpm for 10 min to separate soluble and insoluble portions and the supernatant was recovered. Proteins were purified from supernatant with a nickel pseudo-affinity IDA-Sepharose column (Novagen, Madison, WI, USA). Purity and concentration of the proteins were evaluated with 15% polyacrylamide gel electrophoresis under denaturing conditions (SDS−PAGE) [15] and subsequent Coomassie Brilliant Blue (Sigma) staining [16]. Quantification was performed by densitometry using Qubit (Invitrogen®) fluorometer.

2.4. Native SUAM extraction

Native SUAM (SUAM*wt*) was extracted as described previously [5]. Briefly, SU42 was grown in THB overnight at 37 °C, washed with sterile PBS (pH 7.4) and resuspended in 0.2% sodium dodecyl sulfate (Bio-Rad, Hercules, CA, USA) for 1 h at 37 °C with intermittent mixing. After incubation, bacterial suspensions were aliquoted, microfuged for 5 min and stored at −70 °C.

2.5. Production of SUAMwt, SUAM-1fr, SUAM-2fr, SUAM-3fr and SUAM-4fr antibodies

For production of antibodies against SUAM*wt* and recombinant fractions, 6 weeks-old CF-1 female mice were immunized by intraperitoneal route. Groups of 6 mice were inoculated with 0.2 mg of the corresponding purified recombinant SUAM fraction and SUAM*wt* using complete Freund's adjuvant for the initial injection and incomplete Freund's adjuvant for the following doses. After the first doses, mice were boosted two times every two weeks. Control group was inoculated with PBS. Serum samples were obtained before initial inoculation and one week after each additional injection. Serum was separated by centrifugation and stored at −20 °C. All procedures used in this study were approved by the Institutional Ethic Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral) being performed according to the recommendations of the Guide for the Care and Use of Laboratory Animals [17].

2.6. Measurement of antibodies

Antibody levels (total IgG) in all serum samples, were measured by indirect ELISA. Briefly, 96-well polystyrene EIA microtiter flat bottom plates (Greiner Bio-One, Frickenhausen, Germany) were coated with the corresponding purified protein at 0.5 μg/well. Purified proteins were diluted in carbonate/bicarbonate buffer at pH 9.6. Plates were blocked for 1 h with PBS-5% skimmed milk. Mice sera were diluted in PBS-1% skimmed milk and assayed in triplicate.

Table 1
Primers used in this study.

Name	Sequence[a]	Recombinant Protein (product size)	Location in SUAM sequence (aa)
SUAM-1 Fw	5′-GAATTCTTAACGTCAACACTCGCAC-3′	SUAM-1fr (711 pb)	44–278
SUAM-1 Rv	5′-AAGCTTGCCAAACAAAATGAATTAG-3′		
SUAM-2 Fw	5′-GAATTCACACAATCTGACGAGGT-3′	SUAM-2fr (474 pb)	188–343
SUAM-2 Rv	5′-AAGCTTGCAGAAGTTGGGGCATA-3′		
SUAM-3 Fw	5′-GAATTCGAAGTTGGGGCATAC-3′	SUAM-3fr (525 pb)	342–513
SUAM-3 Rv	5′-AAGCTTCAAGTGCTCCGGTCTAT-3′		
SUAM-4 Fw	5′-GAATTCCCAAGTGCTCCGGTCT-3′	SUAM-4fr (512 pb)	511–678
SUAM-4 Rv	5′-AAGCTTGAAAAAGTTGCAAAAGAAA-3′		
SUAM-5 Fw	5′-GAATTCCTAGCCTTTAACTCTCA-3′	SUAM-5fr (551 pb)	691–871
SUAM-5 Rv	5′-AAGCTTGCATTTCCTACAGTTGATGAA-3′		

[a] Enzyme restriction sites are underlined; GAATTC: *EcoR* I, AAGCTT: *Hind* III.

Antibody binding was evaluated by incubation with anti-mouse IgG conjugated to peroxidase (Jackson, West Grove, PA, USA), and further incubation with hydrogen peroxide and tetramethylbenzidine. Optical density was measured at 450 nm using a microplate reader (Emax Microplate Reader, Molecular Devices, Sunnyvale, CA). Serum titers were determined also by ELISA. Briefly, the purified proteins (SUAM fractions or SUAM*wt*) were coated on microtiter plates and reacted with several dilutions of SUAM fractions or SUAM*wt* antibodies.

2.7. Western blot

The SUAM recombinant fractions and SUAM*wt* were equilibrated to 2 mg/ml, subjected to electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked with PBS-5% skimmed milk in PBS Tween 20 (PBST, 0.5%, v/v) and probed with primary antibodies (anti-SUAM fractions or SUAM*wt*) in PBST containing 0.1% skimmed milk. After three washings with PBST, membranes were incubated with anti-mouse IgG conjugated to peroxidase (Jackson, West Grove, PA, USA) diluted 1/8000 in 0.1% skimmed milk. The color reaction was developed using 4-chloro-1-naphthol (Bio-Rad) as substrate.

2.8. Mammary epithelial cells

A bovine mammary epithelial cell line (MAC-T) was used [18]. MAC-T cells were grown in 24-well cell culture plates at 37 °C in 5% CO_2:95% balanced air (v/v) using cell growth media (CGM) as described previously [5].

2.9. Inhibition adherence-internalization assay

Inhibition adherence-internalization assay was performed as described by Almeida et al. (2006) with modifications. The SU42 *S. uberis* strain was pre-incubated with 1/100 dilution of SUAM-1fr, -2fr, -3fr and -4fr antibodies for 1 h at 37 °C. As negative control SU42 *S. uberis* strain was incubated with 1/100 dilution of control mice serum. After incubation, bacterial suspension was washed three times in PBS, and cocultured with of MAC-T cells monolayers. After 2 h of incubation, wells were washed three times with PBS (pH 7.4), to eliminate the non-adhered or internalized bacteria, treated with 0.25% trypsin (Laboratorio MicroVet SRL, Argentina), and further lysed with 0.025% (v/v) Triton X-100. MAC-T cell lysates were 10-fold serially diluted, plated in triplicate on blood agar and incubated overnight at 37 °C. Each sample was run for triplicate and the assay was repeated 3 times. The results were expressed as percentage of adhesion-internalization, considering as 100% the number of bacteria recovered from SU42 strain opsonized with control serum and incubated for 2 h in MAC-T cells.

2.10. Statistical analysis

Data were analyzed by Mann Whitney, ANOVA or Kruskal–Wallis test, depending on the assay, followed by multiple comparison tests when significant differences between means or medians were found. Differences were considered significant at $P < 0.05$. Statistical analysis was performed using the software Graph- Pad Prism version 5.00 for Windows (GraphPad Software).

3. Results

3.1. Bioinformatic analysis and expression of SUAM recombinant fractions

To optimize the production of recombinant proteins it is desirable to reduce their size to less than 1000 bp [8]. This situation led to the need to select one or more fractions of SUAM, since its nucleotide sequence is 2715 bp. Through the use of two epitope prediction softwares, ABCpred and AAPPred, the SUAM aminoacidic sequence was analyzed looking for antigenic and immunogenic regions. A homogeneous distribution throughout the sequence of possible antigenic peptides was found. But the most outstanding epitopes were found since the amino acid 48 until 856. Therefore, the SUAM protein was cloned in 5 fractions that comprised most of the coding sequence. Following amplification of the *sua* gene by PCR from genomic DNA of SU05 *S. uberis* strain; the products were cloned into pET32a expression vector. The fractions obtained were confirmed by SDS PAGE following affinity purification (Fig. 1) and named: SUAM-1fr (aa44-278), SUAM-2fr (aa188-343), SUAM-3fr (aa342-513) and SUAM-4fr (aa511-678). The SUAM-5fr (aa691-871) was not able to be expressed correctly (Table 1).

3.2. Evaluation of SUAM-1fr, -2fr, -3fr and -4fr fractions immunogenicity and antibodies specificity

3.2.1. IgG antibody response

Specific IgG levels generated against the different SUAM fractions and SUAM*wt* was measured by indirect ELISA against the corresponding protein. The levels of antibodies generated by the groups immunized with recombinant proteins and SUAM*wt* were significantly higher than the control group ($P < 0.0001$; Kruskal-Wallis test) (Fig. 2A). After that, the quantification of specific IgG was assessed by ELISA (Fig. 2B). Mean titer values of $1{,}67 \times 10^6$ were determined for SUAM-1fr, $5{,}48 \times 10^6$ for SUAM-2fr, $1{,}92 \times 10^6$ for SUAM-3fr and $4{,}5 \times 10^6$ for SUAM-4fr.

3.2.2. Specificity of antibodies generated by recombinant fractions of SUAM against native SUAM

To determine the specificity of the antibodies generated by the different SUAM fractions sera from mice immunized with

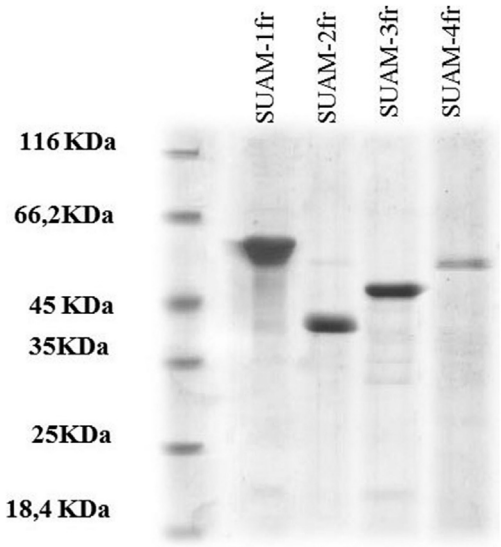

Fig. 1. SUAM recombinant fractions expressed and purified. SDS-PAGE of affinity purified recombinant fractions of SUAM. SUAM-1fr (Mw: 47,5 KDa = 26,6 of KDa SUAM-1fr + 20,9 KDa of His-Tag), SUAM-2fr (Mw: 37,9 KDa = 17 KDa of SUAM-2fr + 20,9 KDa His-Tag), SUAM-3fr (Mw: 40,6 KDa = 19,7 KDa of SUAM-3fr + 20,9 KDa His-Tag), SUAM-4fr (Mw: 42,9 KDa = 22 KDa of SUAM-4fr + 20,9 KDa His-Tag).

recombinant fractions were incubated with SUAM*wt* by a Western blot assay. The sera from animals immunized with the different fractions recognized a band of approximately 112 KDa in the lane corresponding to SUAM*wt*. The recognition of anti-SUAM-1fr antibodies to the protein SUAM*wt* was remarkable. Furthermore, each pooled sera recognized their corresponding protein. Finally, none of the control groups recognized recombinant fractions or SUAM*wt* (Fig. 3. A).

In addition, the ability of antibodies anti-SUAM-1fr; -2fr; -3fr and -4fr to recognize SUAM*wt* was evaluated by indirect ELISA (Fig. 3B). The anti-SUAM-1fr antibodies showed the highest recognition for SUAM*wt*. The anti-SUAM-1fr antibodies showed the highest recognition for SUAM*wt*. This result correlated with that observed in the Western blot assay. The anti-SUAM-2fr, SUAM-3fr and SUAM-4fr antibodies generated similar mean O.D. (0.42 ± 0.037), (0.69 ± 0.035) and (0.78 ± 0.055) respectively, and significantly lower than SUAM-1fr (2.18 ± 0.032). However, the Western blot showed that anti-SUAM-3fr antibodies recognized other proteins from *S. uberis*, indicating that part of the reading observed in this assay was due to non-specific recognition.

3.2.3. Specificity of anti-native SUAM antibodies

We assessed whether the antibodies anti-SUAM*wt* were able to recognize the different SUAM recombinant fractions designed in this work. This test allowed us to analyze which SUAM epitopes were antigenic experimentally. An indirect ELISA using as antigen each recombinant fraction of SUAM and the SUAM*wt* as positive control was performed (Fig. 4). IgG antibodies in mice immunized with SUAM fractions showed specificity towards SUAM*wt* protein. The recognition of SUAM-1fr by anti-SUAM*wt* was similar to recognition of SUAM*wt* (positive control); no significant differences between them was observed ($P > 0.05$; Man-Whitney Test). Meanwhile, recognition by anti-SUAM*wt* of the remaining recombinant SUAM fractions was significantly lower than the observed against SUAM-1fr and SUAM*wt* ($P < 0.05$; Test Kruskall-Wallis).

3.3. Effect of serum from mice immunized with recombinant fractions of SUAM on adherence-internalization of S. uberis into bovine mammary epithelial cells

Since SUAM is involved in the internalization process of *S. uberis* into mammary epithelial cells, the ability of anti-SUAMfrs antibodies to inhibit this mechanism was evaluated. Results presented in Fig. 5 show that the antibodies generated by SUAM-1fr decreased significantly the adhesion in relation to control group ($P < 0.05$) whereas antibodies generated against SUAM-2fr, SUAM-3fr and SUAM-4fr reduce adhesion partially but the difference was not significant in relation to control group.

4. Discussion

Although the use of the whole antigen is a sound practice for the rational design of vaccines, it does not necessarily ensures obtaining an effective immunogen [19]. Therefore, inclusion of fractional antigens may be useful if an appropriate functional region is found and if response to this fraction can be enhanced by use of new generation adjuvants. Furthermore, the use of one functional fraction has the advantage, over the use of the whole molecule, to focus the immune response towards a particular region. It has been reported for several pathogens as group B *Streptococcus*, *Clostridium difficile*, *Trichomonas vaginalis*, *Plasmodium falciparum*, and *Trypanosoma cruzi*, that large antigens have not protective

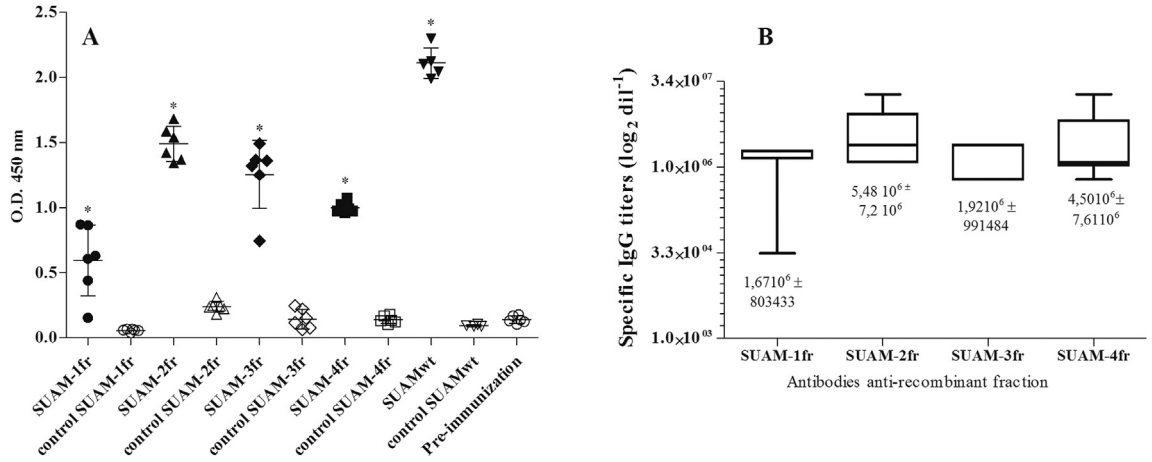

Fig. 2. IgG levels determined by ELISA assays. (A) Specific IgG for each recombinant protein from immunized animals confronted against their corresponding protein, expressed as O.D.450 obtained for sera diluted 1:100. The mean and standard deviation for each group are shown. (*) $P < 0.0001$; Mann Whitney test. The value of pre-immune sera represents the mean of all tests performed. (B) The mean, the maximum and minimum titers of each group are represented. $p > 0.05$; Kruskal Wallis test, Dunn post-test.

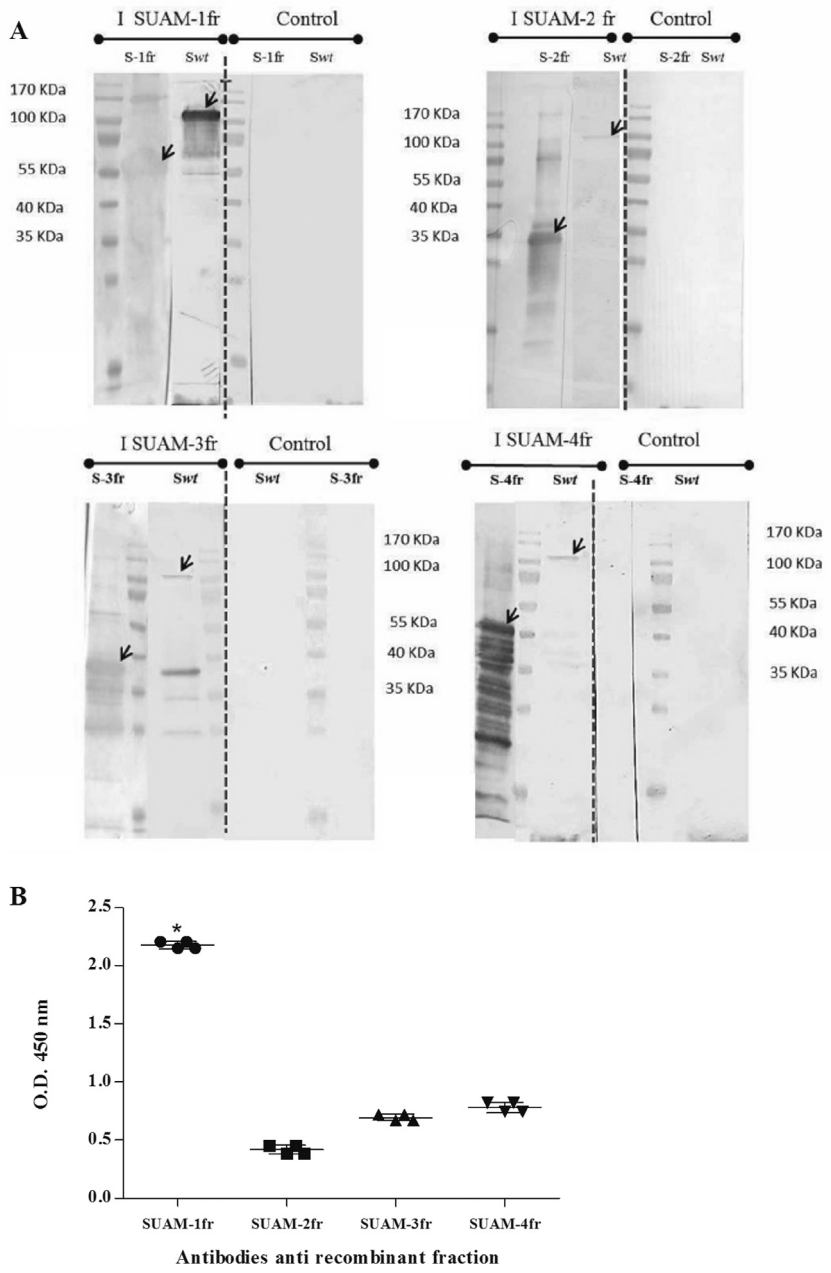

Fig. 3. Antibodies specificity generated by the different fractions of SUAM. A. Western blot; sera from the immunized animals (I) recognized their corresponding SUAM recombinant fraction: SUAM-1fr (S-1fr), SUAM-2fr (S-2fr), SUAM-3fr (S-3fr), SUAM-4fr (S-4fr) and SUAM*wt* (S*wt*). Sera from control animals did not recognize any of the tested proteins. B. ELISA against SUAM*wt*; the graphic shows the mean $O.D._{450}$ reading and standard deviation obtained for each group. The recognition of anti-SUAM-1fr antibodies to the protein SUAM*wt* is remarkable. The recognition of the antibodies generated by SUAM-2fr, -3fr and -4fr was similar among them, and significantly lower than SUAM-1fr (*) $P < 0.05$; Test Kruskal Wallis.

immunodominant epitopes, "distracting" the immune system of protective epitopes as an evasion strategy [20]. In the case of SUAM, the whole molecule was previously cloned and used to immunize cattle evaluating its efficiency as an immunogen with promising results [21,22]. Prado and coworkers found that the anti-phagocytic activity of anti recombinant SUAM antibodies were similar to those generated by a peptide of SUAM [22]. These results, combined with the difficulty to produce a large protein as SUAM at industrial scale, drove us to search for a protein fraction that preserves the potential of the complete protein.

In the rational design of vaccines it is essential to include antigens with conserved sequences [19]. Previously, we and others have determined that SUAM is not only present in most *S. uberis* isolates from Argentina but is also an antigen with highly conserved sequences regardless the geographical origin and epidemiological distribution [7,23]. Within these sequences, immunogenic regions have to be selected. The use of bioinformatic tools allows the identification of protein antigenic sites, either for the development of vaccines or immunodiagnostic test and antibodies production. In order to find a region containing the most immunogenic sites in the SUAM sequence, two bioinformatic softwares for identifying B epitopes were used: ABCpred and AAPPred. These softwares were previously characterized as those which showed better correlation among likely epitopes and true epitopes [24]. This allows predicting antigenic regions reducing the time and cost necessary for identifying these sequences. In this work, the potential epitopes were

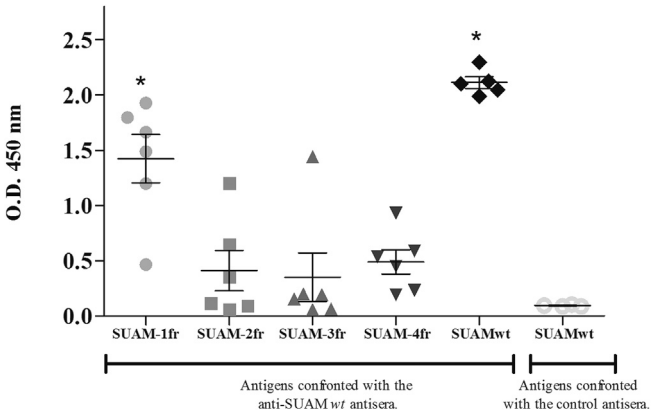

Fig. 4. Anti-Native SUAM antibodies specificity against the SUAM recombinant fractions. An indirect ELISA was performed confronting anti-SUAM*wt* protein to the four fractions of SUAM and SUAM*wt*. The recognition of anti-SUAM*wt* antibodies against SUAM-1fr was highlighted. The recognition of the antibodies generated by SUAM*wt* (positive control) was significantly higher than those generated by SUAM-2fr, -3fr, -4fr and control group (negative control) (*) $P < 0.05$, Man-Whitney.

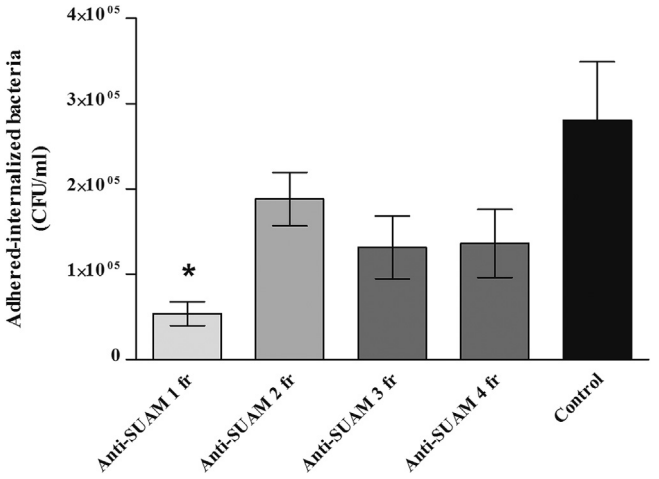

Fig. 5. Inhibition of the *S. uberis* SU42 strain of adherence-internalization in mammary epithelial cells (MAC-T) by serum generated by recombinant SUAM fractions in mice. Data are presented as colony-forming unit per ml (CFU/ml) and bars represent mean with standard error of the mean (SEM) of nine independent observations. Adhered-internalized bacteria for control group was 2.8×10^5 (100%); for anti-SUAM1fr was 5.3×10^4 (19%); for anti-SUAM2fr was 1.9×10^5 (67%); for anti-SUAM3fr was 1.3×10^5 (47%); for anti SUAM4fr was 1.4×10^5 (48%). No significant differences were observed with respect to the control group.

found homogeneously distributed throughout the sequence, without identifying a unique region rich in antigenic peptides on the amino acid sequence of SUAM. These results are consistent with previous observations in a study based on epitope prediction of SUAM, in which five overlapping regions along the SUAM sequence were found to have the most hydrophilic valleys and the highest peaks for B-turns [25]. However, in our study, a more exhaustive analysis was performed, since the software takes into account various parameters, which give greater sensitivity to the predictors [24]. Based on the results obtained with the predictors on the SUAM molecule, 4 regions which covered most of the molecule coding sequence were cloned to experimentally select antigenic regions.

The immune response elicited in mice with the different SUAM fractions was assessed. The antibodies generated by these immunogenic fractions could recognize native SUAM (SUAM*wt*) demonstrating their specificity. Furthermore, it was shown that antibodies generated in mice immunized with the SUAM*wt* were specific against all SUAM fractions despite the presence of TRX, indicating there was no structural impediment caused by this fusion protein. By Western blot and ELISA analysis we observed that anti-SUAM*wt* antibodies had similar recognition ability both to SUAM-1fr and to SUAM*wt*. It was also observed that antibodies generated by SUAM-1fr showed a significantly superior recognition of SUAM*wt* compared with those generated by the other SUAM fractions. Notably, SUAM-1fr was not the most immunogenic fraction as mice immunized with SUAM-2fr and SUAM-4fr showed a trend to induce higher antibody titers than those immunized with SUAM-1fr and SUAM-3fr, although differences were not statistically significant. Taken together, these results indicate remarkable properties of SUAM-1fr over the other fractions, which would allow to select it as a potential vaccine component against *S. uberis*. In addition, these findings are consistent with the postulate that whole protein antigens are not necessarily essential for immunity induction and protective epitopes should be enough to induce immune responses and provide protection against pathogens [26].

We could also demonstrate that the antibodies generated by antigens of a given strain were capable of recognizing proteins from a heterologous strain, since recombinant fractions were cloned from the genome of the SU05 strain and SUAM*wt* was extracted from the SU42 strain. This work strategy was designed since previous reports considered that a possible cause of the low effectiveness of a killed *S. uberis* vaccine was lack of protection against heterologous strains [6]. Lang et al. [27] considered that *S. uberis* genetic diversity could be responsible for this lack of protection. Indeed, bacterins include multiple virulence factors that may vary among strains, and these pools of antigens may guide the immune response against the more variable antigens. By focusing the immune response to a single virulence factor, SUAM, shown to have a highly conserved sequence [7,23], it is expected that potential levels of protection afforded by antibodies against this factor remain high against different isolates.

Previously, other studies have used Lf-binding proteins as targets for vaccine candidates such as LBPB and LBPA from *Moraxella catarrhalis* [28] and LBPA and LBPB from *Neisseria meningitidis* [29]. The interaction between SUAM, bovine Lf, and a putative Lf receptor on the bovine mammary epithelial cell surface serve as a bridging molecule for internalization of *S. uberis* into mammary epithelial cells [30,31]. Blocking this binding can reduce the ability of *S. uberis* to establish in the mammary gland. In this regard, Chen et al. [32] showed that deletion of the *sua* gene reduced the ability of *S. uberis* adherence and internalization in mammary epithelial cells. In the present work it was observed that only antibodies against SUAM-1fr and not the others fraction were able to decrease significantly adhesion-internalization to MAC-T cells. It has been reported that the adherence inhibition by antibodies against SUAM cannot be complete since *S. uberis* has other mechanisms mediating attachment to mammary epithelial cells [33].

The four fractions induced antibodies production; being the lowest IgG titer observed against SUAM-1fr. However, antibodies generated against SUAM-1fr showed the highest recognition of SUAM*wt* protein and those generated by SUAM*wt* presented a significantly higher recognizing ability of SUAM-1fr compared with the three other fractions. On the other hand, the SUAM-1fr include the amino acid sequence MTTADQSPKLQGEEACA expressed by Prado et al. as pepSUAM [22] that also inhibited adherence to and internalization of *S. uberis* into bovine mammary epithelial cells confirming the pathogenic role of this SUAM region. The SUAM-1fr is a 234 aa protein long, which homology to the same region of 20 isolates from Argentina is 95% (data not shown). The larger size of SUAM-1fr with respect to pepSUAM increases the epitope numbers. Therefore, we obtain a conserved fraction among *S. uberis* isolates with epitopes that elicited antibodies with a strong affinity for

SUAM*wt*. These properties determine that this fraction exhibits the best conditions to be selected as a potential subunit vaccine component against *S. uberis* mastitis.

5. Conclusion

In conclusion, results from this investigation showed that: (1) we created four recombinant fractions of SUAM based on bioinformatic analysis (2) the SUAM fractions induced specific antibodies in mice; (3) anti-SUAM*wt* antibodies had similar recognition ability both to SUAM-1fr and to SUAM*wt*; (4) anti-SUAM-1fr showed a significantly superior recognition of SUAM*wt* compared with those generated by the other SUAM fractions and (5) SUAM-1fr but not the other fractions serum antibodies inhibited adherence of *S. uberis* into bovine mammary epithelial cells. This information not only aids in the search of vaccine candidate antigens, but also increases the understanding of putative mechanism of action of this virulence factor. However, taking in mind that other molecules may mediated adhesion-internalization of *S. uberis* other subunits should be considered to be incorporated in a vaccine formulation against this infection.

Conflicts of interest

none.

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References

[1] C. Vissio, D.A. Agüero, C.G. Raspanti, L.M. Odierno, A.J. Larriestra, Productive and economic daily losses due to mastitis and its control expenditures in dairy farms in Córdoba, Argentina, Arch. Med. Vet. 47 (2015) 7–14.
[2] S. De Vliegher, L.K. Fox, S. Piepers, S. McDougall, H.W. Barkema, Invited review: mastitis in dairy heifers: nature of the disease, potential impact, prevention, and control, J. Dairy Sci. 95 (2012) 1025–1040.
[3] V.L. Douglas, S.G. Fenwick, D.U. Pfeiffer, N.B. Williamson, C.W. Holmes, Genomic typing of *Streptococcus uberis* isolates from cases of mastitis, in New Zealand dairy cows, using pulsed-field gel electrophoresis, Vet. Microbiol. 75 (2000) 27–41.
[4] J.A. Leigh, Immunisation of dairy cattle with recombinant *Streptococcus uberis GapC* or a chimeric CAMP antigen confers protection against heterologous bacterial challenge. M.C. Fontaine et al. [Vaccine 20 (2002) 2278-2286], Vaccine 20 (2002) 3047–3048.
[5] R.A. Almeida, D.A. Luther, H.M. Park, S.P. Oliver, Identification, isolation, and partial characterization of a novel *Streptococcus uberis* adhesion molecule (SUAM), Vet. Microbiol. 115 (2006) 183–191.
[6] J.M. Finch, A. Winter, A.W. Walton, J.A. Leigh, Further studies on the efficacy of a live vaccine against mastitis caused by *Streptococcus uberis*, Vaccine 15 (1997) 1138–1143.
[7] M.S. Perrig, M.B. Ambroggio, F.R. Buzzola, I.S. Marcipar, L.F. Calvinho, C.M. Veaute, M.S. Barbagelata, Genotyping and study of the *pauA* and *sua* genes of *Streptococcus uberis* isolates from bovine mastitis, Rev. Argent. Microbiol. 47 (2015) 282–294.
[8] T. Makino, G. Skretas, G. Georgiou, Strain engineering for improved expression of recombinant proteins in bacteria, Microb. cell factories (2011) 10.
[9] L. Odierno, L. Calvinho, P. Traverssa, M. Lasagno, C. Bogni, E. Reinoso, Conventional identification of *Streptococcus uberis* isolated from bovine mastitis in Argentinean dairy herds, J. Dairy Sci. 89 (2006) 3886–3890.
[10] B.M. Jayarao, J.J. Dore Jr., S.P. Oliver, Restriction fragment length polymorphism analysis of 16S ribosomal DNA of Streptococcus and Enterococcus species of bovine origin, J. Clin. Microbiol. 30 (1992) 2235–2240.
[11] D.A. Luther, R.A. Almeida, S.P. Oliver, Elucidation of the DNA sequence of *Streptococcus uberis* adhesion molecule gene (*sua*) and detection of *sua* in strains of *Streptococcus uberis* isolated from geographically diverse locations, Vet. Microbiol. 128 (2008) 304–312.
[12] S.P. Oliver, Frequency of isolation of environmental mastitis-causing pathogens and incidence of new intramammary infection during the nonlactating period, Am. J. Vet. Res. 49 (1988) 1789–1793.
[13] S. Saha, G.P. Raghava, Prediction of continuous B-cell epitopes in an antigen using recurrent neural network, Proteins 65 (2006) 40–48.
[14] J. Chen, H. Liu, J. Yang, K.C. Chou, Prediction of linear B-cell epitopes using amino acid pair antigenicity scale, Amino Acids 33 (2007) 423–428.
[15] U.K. Laemmli, F. Beguin, G. Gujer-Kellenberger, A factor preventing the major head protein of bacteriophage T4 from random aggregation, J. Mol. Biol. 47 (1970) 69–85.
[16] U. Neumann, The Protein Protocol Handbook. Totowa, NJ, 1996.
[17] National, Research and Council, Guide for the Care and Use of Laboratory Animals (NIH Guide, Revised), National Academy Press, Washington, D.C., 1996.
[18] H.T. Huynh, G. Robitaille, J.D. Turner, Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation, Exp. Cell Res. 197 (1991) 191–199.
[19] G.J. Nabel, Designing tomorrow's vaccines, N. Engl. J. Med. (2013) 551–560.
[20] A. Covacci, S. Censini, M. Bugnoli, R. Petracca, D. Burroni, G. Macchia, A. Massone, E. Papini, Z. Xiang, N. Figura, et al., Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 5791–5795.
[21] R.A. Almeida, O. Kerro-Dego, M.E. Prado, S.I. Headrick, M.J. Lewis, L.J. Siebert, G.M. Pighetti, S.P. Oliver, Protective effect of anti-SUAM antibodies on *Streptococcus uberis* mastitis, Vet. Res. 46 (2015) 133.
[22] M.E. Prado, R.A. Almeida, C. Ozen, D.A. Luther, M.J. Lewis, S.J. Headrick, S.P. Oliver, Vaccination of dairy cows with recombinant *Streptococcus uberis* adhesion molecule induces antibodies that reduce adherence to and internalization of *S. uberis* into bovine mammary epithelial cells, Vet. Immunol. Immunopathol. 141 (2011) 201–208.
[23] Y. Yuan, O.K. Dego, X. Chen, E. Abadin, S. Chan, L. Jory, S. Kovacevic, R.A. Almeida, S.P. Oliver, Short communication: conservation of *Streptococcus uberis* adhesion molecule and the *sua* gene in strains of *Streptococcus uberis* isolated from geographically diverse areas, J. Dairy Sci. 97 (2014) 7668–7673.
[24] J.G. Costa, P.L. Faccendini, S.J. Sferco, C.M. Lagier, I.S. Marcipar, Evaluation and comparison of the ability of online available prediction programs to predict true linear B-cell epitopes, Protein Pept. Lett. 20 (2013) 724–730.
[25] R.A. Almeida, D.A. Luther, D. Patel, S.P. Oliver, Predicted antigenic regions of *Streptococcus uberis* adhesion molecule (SUAM) are involved in adherence to and internalization into mammary epithelial cells, Veterinary Microbiol. 148 (2011) 323–328.
[26] S. Thomas, B.A. Luxon, Vaccines based on structure-based design provide protection against infectious diseases, Expert Rev. Vaccines 12 (2013) 1301–1311.
[27] P. Lang, T. Lefebure, W. Wang, R.N. Zadoks, Y. Schukken, M.J. Stanhope, Gene content differences across strains of *Streptococcus uberis* identified using oligonucleotide microarray comparative genomic hybridization, Infect. Genet. Evol. 9 (2009) 179–188.
[28] J.C. McMichael, Vaccines for *Moraxella catarrhalis*, Vaccine 19 (Suppl 1) (2000) S101–S107.
[29] A. Pettersson, J. Kortekaas, V.E. Weynants, P. Voet, J.T. Poolman, M.P. Bos, J. Tommassen, Vaccine potential of the *Neisseria meningitidis* lactoferrin-binding proteins LbpA and LbpB, Vaccine 24 (2006) 3545–3557.
[30] D. Patel, R.A. Almeida, J.R. Dunlap, S.P. Oliver, Bovine lactoferrin serves as a molecular bridge for internalization of *Streptococcus uberis* into bovine mammary epithelial cells, Vet. Microbiol. 137 (2009) 297–301.
[31] J.J. Rejman, J.D. Turner, S.P. Oliver, Characterization of lactoferrin binding to the MAC-T bovine mammary epithelial cell line using a biotin-avidin technique, Int. J. Biochem. 26 (1994) 201–206.
[32] X. Chen, O.K. Dego, R.A. Almeida, T.E. Fuller, D.A. Luther, S.P. Oliver, Deletion of *sua* gene reduces the ability of *Streptococcus uberis* to adhere to and internalize into bovine mammary epithelial cells, Vet. Microbiol. 147 (2011) 426–434.
[33] R.A. Almeida, W. Fang, S.P. Oliver, Adherence and internalization of *Streptococcus uberis* to bovine mammary epithelial cells are mediated by host cell proteoglycans, FEMS Microbiol. Lett. 177 (1999) 313–317.