

## Induced Immune Response of *Escherichia coli* BL21 Expressing Recombinant MSP1a and MSP1b Proteins of *Anaplasma marginale*

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### ABSTRACT

This work aims to evaluate the potential of immunization with *E. coli* BL21 expressing the recombinant rMSP1a and rMSP1b proteins of *Anaplasma marginale*. *E. coli* BL21 was transformed with recombinant plasmids pET102/msp1 $\alpha$  and pET101/msp1 $\beta$ , and rMSP1a and rMSP1b were expressed after induction by IPTG. BALB/c mice were vaccinated with formalized BL21/rMSP1a and BL21/rMSP1b, and the production in mice sera of whole IgG was determined by ELISA. The mice immunized with BL21/rMSP1a showed a better humoral response for whole IgG when compared to the mice immunized with BL21/rMSP1b; these mice exhibited a small response after the second vaccination. Sera of mice immunized with BL21/rMSP1a reacted via western blot with BL21 and rMSP1a, with molecular masses varying from 70 to 105 kDa. Sera of mice immunized with BL21/rMSP1b reacted with BL21 and rMSP1b with a molecular mass of 100 kDa. These results demonstrate that BL21 containing rMSP1a and rMSP1b in the outer membrane were able to produce an immune response in mice, reinforcing its use in vaccine models against bovine anaplasmosis.

**Key words:** *Anaplasma marginale*, MSP1a, MSP1b, vaccine, mice, cattle

### INTRODUCTION

*Anaplasma marginale*, an obligate intraerythrocytic pathogen belonging to the Anaplasmataceae family of the Rickettsiales order (Dumler et al., 2001), is the causative agent of bovine anaplasmosis worldwide, especially in tropical and subtropical regions, causing severe

losses in bovine livestock (Richey and Palmer, 1990).

Immunization with purified outer membranes induces protection against acute *A. marginale* infection and disease (Tebele et al., 1991). Some well-characterized outer membrane proteins, designated major surface proteins (MSPs), MSP1a, MSP1b, MSP2, MSP3, MSP4, MSP5, and recently VirB9 and VirB10, have been evaluated as

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potential candidates for antigens in vaccine production, diagnostic evaluation, and determination of intermolecular relationships (Palmer and McElwain, 1995; Lopez et al., 2007; Vidotto et al., 1994, 2008).

The proteins MSP1a (105 kDa) and MSP1b (100 kDa) form the MSP1 complex, and both proteins have been identified as putative adhesins for bovine erythrocytes (McGarey et al., 1994; Palmer et al., 1987; McGarey and Allred, 1994). The protein MSP1a is an *A. marginale* adhesin for both bovine erythrocytes and tick cells, while MSP1b is an adhesin for bovine erythrocytes only (de la Fuente et al., 2001).

MSP1a is encoded by a single *msp1a* gene in all *A. marginale* strains, and MSP1b by a multigene family (*msp1β1* and *msp1β2*), which manifests a small variation in MSP1b1 and MSP1b2 sequences during the multiplication phases in cattle and tick cells (Oberle et al., 1988). MSP1a is composed of a highly conserved C-terminal region and an N-terminal region consisting of 28 or 29 amino acid repeats that vary in number and sequences and which are responsible for variable molecular mass among *A. marginale* strains (Allred et al., 1990; Oberle et al., 1993). Despite variation in the N-terminal regions, all strains contain a conserved neutralization-sensitive epitope defined by monoclonal antibodies (Palmer et al., 1987; Allred et al., 1990; Kano et al., 2002). The protein complex MSP1 induces intense proliferation of mononuclear cells of peripheral blood and CD4<sup>+</sup> T-cells in cattle inoculated with homologous and heterologous isolates of *A. marginale* (Palmer et al., 1989; Brown et al., 2001). The proliferation of mononuclear cells is related to the participation of conserved T-cell epitopes, localized on the C extremity of MSP1a, which stimulates elevated levels of interferon  $\gamma$ . A weak cellular immune response has been seen against MSP1b, while an intense humoral response has been observed with MSP1a and MSP1b (Brown et al., 2001, 2002).

Vaccination can be an economic alternative for the control of bovine anaplasmosis; however, the current commercialized *A. centrale* live vaccines present limitations in protecting animals against heterologous isolates of *A. marginale* and frequently cause isoerythrolysis in calves due to colostral antibody transfer (Kuttler et al., 1984). Furthermore, live vaccines can potentially transmit blood-borne pathogens (such as viruses, bacteria, and protozoa) among animals in cattle herds

(Palmer et al., 1989; Kocan et al., 2000).

The aim of this work was to evaluate the immune response in mice immunized with recombinant *E. coli* expressing *A. marginale* MSP1a and MSP1b.

## MATERIALS AND METHODS

### Production of r-MSP1a and r-MSP1b on *E. coli*

Recombinant *E. coli* strains were obtained as previously described (Tamekuni et al., 2009). The *E. coli* BL21 (D3) strain was transformed with the recombinant plasmids pET102/*msp1a* and pET101/*msp1β*, and the recombinant strains were grown to an optical density of OD<sub>600 nm</sub> 0.8. IPTG (isopropyl-1- $\beta$ -D-thiogalactopyranoside) (Invitrogen Life Technologies, USA) was then added to 1 mM and incubated for 4 h. An aliquot of culture was removed, the cells were collected by centrifugation and the expression of proteins analyzed on SDS-PAGE 10%.

To obtain rMSPs purification, BL21/pET102/*msp1a* and pET101/*msp1β* strain was grown with IPTG for 4 h at 37°C. The cells were collected by centrifugation and incubated with lysis buffer, and rMSP1a and rMSP1b were purified using Ni-NTA columns (Qiagen Biotecnologia Brasil Ltda). The rMSP1a and rMSP1b strains were dialyzed and measured using the Bradford method and analyzed on SDS-PAGE 10%.

### Immunization of BALB/c mice

To assess the acquired humoral immune response against whole bacteria expressing rMSP1a and rMSP1b, 4- to 5-week-old mice were immunized with formalin-inactivated recombinant *E. coli*. Formalin-killed bacteria were obtained by growing in LB with IPTG for 4 h at 37 °C, titering to determine CFUs, resuspending in 1% formalin in 1X PBS and subsequently incubating at 37 °C for 24 h. The killed bacteria were washed with sterile PBS (pH 7.2) and resuspended in PBS for a final concentration of 1x10<sup>9</sup> CFU.

Six groups of seven female BALB/c mice were immunized with BL21/rMSP1a and BL21/rMSP1b to produce immune sera. The negative control group 1 (G1) received 100  $\mu$ L of PBS; group 2 (G2) was immunized with 100  $\mu$ g of protein from initial bodies of *A. marginale* PR1 isolate, obtained as previously described (Vidotto et al., 1994). Other groups were immunized as follows:

group 3 (G3) with  $10^9$  CFU of *E. coli* BL21/rMSP1a; group 4 (G4) with  $10^9$  CFU of *E. coli* BL21/rMSP1b; group 5 (G5) with  $10^9$  CFU of *E. coli* BL21/rMSP1a and *E. coli* BL21/rMSP1b; and group 6 (G6) with  $10^9$  CFU of *E. coli* BL21.

Vaccinations were carried out by subcutaneous injection at 0, 7 and 14 days. The recombinant bacteria and initial bodies of *A. marginale* were emulsified with complete Freund's adjuvant (Sigma-Aldrich, Brasil) in the first vaccination and with incomplete Freund's adjuvant (Sigma-Aldrich, Brasil) in subsequent vaccinations. After each inoculation, the animals were bled, the sera were separated and the titer and specificity of antibodies were analyzed by enzyme-immunoassay (ELISA) and western blot. Serum control was obtained from non-immunized animals.

#### Evaluation of antibody response by indirect ELISA

Optimal dilutions were established using checkerboard titrations with dilutions of sera, antigen and conjugates. The anti-*A. marginale* antibody response at 0, 7, 21 and 35 days was evaluated using *A. marginale* antigens (initial bodies) to coat wells in a 96-well microtiter plate (NUNC MaxiSorp™, California, USA) at 0.5 µg/well. After overnight incubation at 4 °C, serum samples from each group of immunized mice were diluted to 1:50 in PBST buffer and pH adjusted to 7.4 with 5 % skimmed powder milk. Then, 100 µL of the diluted sample were added in duplicate to each well and incubated for 45 min at 37 °C. After three washes, anti-mouse IgG conjugated with horseradish peroxidase conjugate (1:10.000) was added and incubated for 45 min at 37 °C. The substrate for enzyme detection was OPD (o-phenylenediamine) 0.4 mg/mL, H<sub>2</sub>O<sub>2</sub> 0.04 % in substrate buffer (0.1 M acid citric, 0.2 M Na<sub>2</sub>HPO<sub>4</sub>). The reaction was then blocked with 50 µL of 1 M HCl and the plates read at 490 nm. The absorbance values were estimated and the optical density (OD) value was obtained. The corrected ODs for G3, G4, and G5 were calculated using the OD mean of G6 to subtract the background of BL21, as follows:  $OD_{corr} = (OD_{sample} (G3, G4, \text{ or } G5) - OD_{average} \text{ of } G6 \text{ for each day } (0, 7, 21 \text{ or } 35))$ .

#### SDS PAGE and western blot

The purified rMSP1a and rMSP1b proteins were suspended in electrophoresis sample buffer (0.025 M Tris HCl, 2% SDS, 15 % glycerol, 2.5% 2-mercaptoethanol, pH 6.8), boiled for 5 min and electrophoresed in SDS-PAGE-10%. Mass molecular standard was used (Multimarck, Invitrogen). Gels were either stained with Coomassie blue or set up for western blot. The proteins were transferred to a 0.45-µm nitrocellulose membrane (Amersham Biosciences do Brasil Ltda), as described previously (Towbin and Gordon, 1984). The membranes were blocked in blocking buffer (PBS with 0.1% Tween 20 and 5% non-fat powdered milk) for 1 h at room temperature under agitation. Membranes were washed in PBST (PBS with 0.1% Tween 20) and incubated for 1 h with a pool of sera from each group, obtained from mice bled at day 35, and a pool of pre-immune sera at 1:100 dilution. The membranes were washed, and the proteins were detected by means of enhanced chemiluminescence (ECL) western blot system (Amersham Biosciences do Brasil Ltda). Protein molecular weight markers (Rainbow™ colored, Amersham Biosciences do Brasil Ltda) were used as standards.

#### Bioethics in animal experimentation

The animals were kept according to the National Institute of Health standards for the use of laboratory animals. The experiments were approved by the Ethics Committee in Animal Experimentation of the Universidade Estadual de Londrina (CEEA 47/05, process 26957/05).

## RESULTS

The whole IgG production in mice during the trial is shown at Fig 1, representing sera from BALB/c mice at 0, 7, 21, and 35 days after initial immunization. All pre-immunized sera and negative control group sera (G1) reacted below  $OD = 0.092$ . Mouse sera from the group immunized with *A. marginale* (G2) exhibited seroconversion ( $OD_{mean} = 0.305$ , day 7) after the first immunization, and this rate increased

throughout the experiment ( $OD_{\text{mean}} = 0.463$ , day 21, and  $OD_{\text{mean}} = 0.615$ , day 35). In G3 and G5, high antibody responses were obtained after the second immunization ( $OD_{\text{corr}}$  G3 = 0.309, 0.339, Western blot results using a pool of mouse sera, rMSP1a and rMSP1b strains of *A. marginale* and *E. coli* BL21 are shown in Fig 2. The pre-immunized and post-immunized sera of mice from G1 (PBS) (Fig 2) only reacted to an unspecific band of protein smaller than 100 kDa. Sera from mice immunized with *A. marginale* initial bodies (G2) reacted to all native MSPs (approximately 100, 76, 38, 31 kDa proteins) (Fig 2). Sera from mice immunized with BL21/rMSP1a (G3) reacted to 100-70 kDa proteins (rMSP1a), while mice immunized with BL21/rMSP1b (G4) reacted to rMSP1b (100 kDa proteins). Mice sera from G5, which received both recombinant bacteria, reacted to rMSP1a and rMSP1b strains of *A. marginale* and to *E. coli* BL21 proteins, as expected. Mouse sera from G6, which received only *E. coli* BL21, showed weak reactivity to proteins from this strain (approximately 100 kDa proteins)

## DISCUSSION

The MSP1 complex appears to be essential for bovine erythrocyte *A. marginale* infection and also important for development of a subunit vaccine. Immunization with MSP1 complex confers protection to susceptible cattle against homologous and heterologous *A. marginale* challenges (Palmer et al., 1989; Brown et al., 2002). However, unlike results obtained with native MSP1a/b complexes, immunization with recombinant MSP1a, MSP1b, or the combination of these two proteins has not induced significant protection (Palmer and McElwain, 1995).

In a previous study, we cloned *mSP1a* and *mSP1b* genes from the PR1 *A. marginale* strain and the recombinant *E. coli* strains BL21/pET102-*mSP1a* and BL21/pET101-*mSP1b* expressing rMSP1a and rMSP1b on the outer membrane and visualized antibodies produced against rMSP1a and rMSP1b in an IFA (Tamekuni et al., 2009). Also, these recombinant *E. coli* strains agglutinated bovine erythrocytes, and this agglutination showed the presence of the antibodies anti-rMSP1a and rMSP1b, confirming the adhesive functions of rMSP1a and rMSP1b (Tamekuni et al., 2009) as

and  $OD_{\text{corr}}$  G5 = 0.231, 0.258, mean from days 21 and 35, respectively), while G4 showed seroconversion only after the third immunization ( $OD_{\text{corr}}$  G4 = 0.272, mean from day 35). previously described (McGarey et al., 1994; Palmer et al., 1987; McGarey and Allred, 1994)

In this study, the mice immunized with recombinant *E. coli* BL21/pET102-*mSP1a* and BL21/pET101-*mSP1b* showed specific antibody responses to rMSP1a and rMSP1b as demonstrated by ELISA (Fig. 1). Also, sera from all mice receiving these bacteria reacted with rMSP1a proteins (70 to 105 kDa) and rMSP1b proteins (100 kDa) shown in western blot assay (Fig. 2). Thus demonstrates that recombinant *E. coli* expressing rMSP1a and rMSP1b can induce the production of specific antibodies.

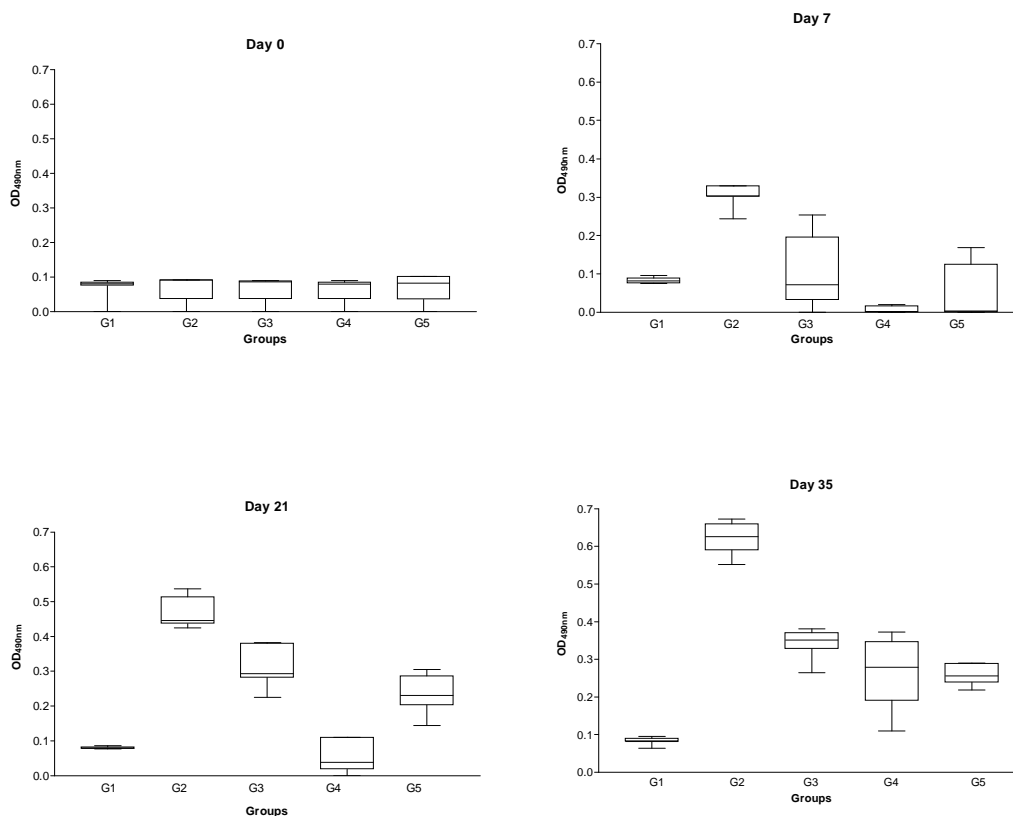
In addition to these data, Kano et al. (2008) recently observed that mice immunized with the association of recombinant plasmids (pcDNA*mSP1a*, pcDNA-*mSP1b* and pcDNA-*mSP5*) exhibited an intense lymph proliferation when compared to mice immunized with the plasmids alone. Also, the sera from immunized mice reacted with recombinant proteins (rMSP1a, rMSP1b and rMSP5), indicating humoral and cellular response. Our results presented above and the data obtained by Kano et al. (2008) reinforce previous information on immunization with native MSP1 heteromer, which induced strong IgG responses to both proteins. In contrast, only MSP1a stimulated strong CD4+ T-cell responses and produced a weak cellular immune response against MSP1b (Brown et al., 2001, 2002). Recently, constructs of CD4+ T-cell epitopes from MSP1a linked to MSP1b1 induced higher IgG titers against MSP1b1 (Macmillan et al., 2008), and this high MSP1b-specific antibody titer in the group immunized with the linked construct was likely due to a higher MSP1b1-to-MSP1a ratio than that in the native MSP1 complex. In the native complex, MSP1a is much more abundant than MSP1b1 (Macmillan et al., 2006).

Protective immunity against *A. marginale* requires stimulation of humoral and cellular mechanisms. Antibodies against the MSP1 facilitate phagocytosis, eliminating *A. marginale* (Cantor et al., 1993), and they inhibit the invasion of erythrocytes by *A. marginale* (McGarey and Allred, 1994).

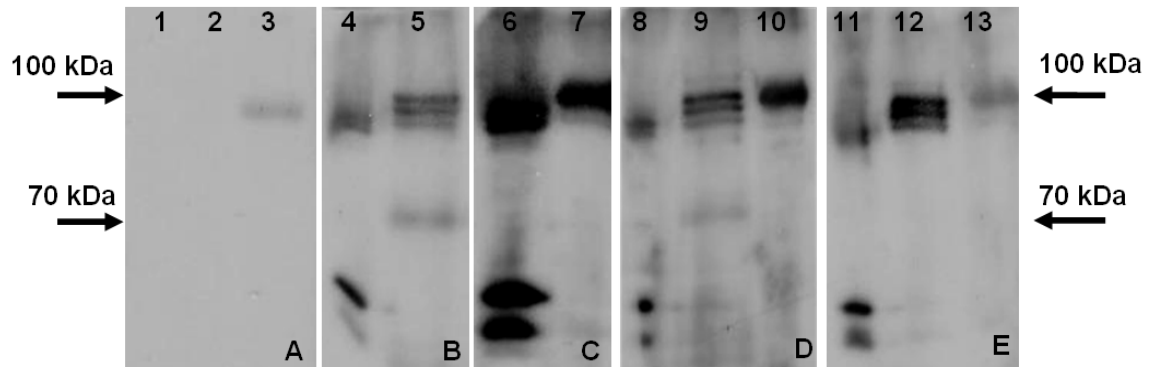
Recently, it was verified that the

ISCOMATRIX/rMSPs vaccine, containing rMSP1a, rMSP1b, rMSP4 and rMSP5 aggregated to ISCOM induced the production of specific antibodies to each of the rMSPs in mice, suggesting that a subunit vaccine containing rMSPs could be efficient against an infection of *A. marginale* (Kawasaki et al., 2007). Therefore, a vaccine with BL21/rMSP1a and BL21/rMSP1b or with outer membrane preparations of these

recombinant bacteria could be more easily prepared, considering the difficulty of purifying rMSPs in Ni columns on a large scale. In conclusion, our results demonstrate that BL21 containing rMSP1a and rMSP1b in the outer membrane were able to produce immune responses in mice, reinforcing its use in vaccine models against bovine anaplasmosis.



**Figure 1** - IgG response (ELISA) of mice immunized with recombinant *Escherichia coli* BL21, as follows: G1 - negative control (PBS); G2 - positive control (*Anaplasma marginale* initial bodies); G3 - BL21/rMSP1a; G4 - BL21/rMSP1b; G5 - BL21/rMSP1a + BL21/rMSP1b. The plates of the G1 and G2 were adsorbed with *A. marginale* initial bodies; G3 with rMSP1a; G4 with rMSP1b; and G5 with rMSP1a + rMSP1b.



**Figure 2** - Western blot with rMSP1a and rMSP1b and sera of mice groups immunized with BL21 and recombinant BL21. *Escherichia coli* BL21 – lines 1, 4, 6, 8, and 11; rMSP1a – lines 2, 5, 9, and 12; rMSP1b – lines 3, 7, 10, and 13. Panel A - Pre-immune pool sera; B - Group 3 pool sera (BL21/rMSP1a); C - Group 4 pool sera (BL21/rMSP1b); D - Group 5 sera (BL21/rMSP1a + BL21/rMSP1b); E - Group 2 pool sera (initial bodies of *Anaplasma marginale*).

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## RESUMO

Esse trabalho avaliou o potencial de imunização de *Escherichia coli* BL21 expressando as proteínas recombinantes rMSP1a e rMSP1b de *Anaplasma marginale*. A *E. coli* BL21 foi transformada com os plasmídios recombinantes pET102/*mSP1a* e pET101/*mSP1b* e as proteínas rMSP1a e rMSP1b foram expressas após indução com IPTG. Camundongos BALB/c foram vacinados com BL21/rMSP1a e BL21/rMSP1b formolisadas, e a produção de IgG total foi determinada pelo teste de ELISA nos soros dos camundongos imunizados. Os camundongos imunizados com a BL21/rMSP1a mostraram uma melhor resposta humoral para IgG total, comparada à resposta apresentada pelos camundongos imunizados com BL21/rMSP1b; estes camundongos exibiram uma menor resposta após a segunda vacinação. Soros

de camundongos imunizados BL21/rMSP1a reagiram pelo western blot com BL21 e rMSP1a, com massa molecular variando de 70 a 105 kDa. Soro de camundongos imunizados com BL21/rMSP1b reagiram com BL21 e rMSP1b com massa molecular de 100 kDa. Esses resultados demonstram que BL21 contendo rMSP1a e rMSP1b na membrana externa foram capazes de produzir resposta imune em camundongos, reforçando o seu uso em modelos de vacina contra a anaplasmoze bovina.

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