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Bovine immunoprotection against *Rhipicephalus (Boophilus) microplus* with recombinant Bm86-Campo Grande antigen

Imunoproteção de bovinos contra *Rhipicephalus (Boophilus) microplus* com antígeno recombinante Bm86-Campo Grande

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Abstract

The southern cattle fever tick, *Rhipicephalus* (*Boophilus*) *microplus*, is no doubt the most economically important ectoparasite of cattle globally. The inappropriate use of chemical acaricides has driven the evolution of resistance in populations of *R.* (*B.*) *microplus*. Anti-tick vaccines represent a technology that can be combined with acaricides in integrated control programs to mitigate the impact of *R.* (*B.*) *microplus*. The recombinant form of Bm86 antigen from the Campo Grande (rBm86-CG) strain of *R.* (*B.*) *microplus* was produced using the *Pichia pastoris* expression system to test its ability to immunoprotect cattle against tick infestation. Secretion of rBm86-CG by *P. pastoris* through the bioprocess reported here simplified purification of the antigen. A specific humoral immune response was detected by ELISA in vaccinated cattle. Immunoblot results revealed that polyclonal antibodies from vaccinated cattle recognized a protein in larval extracts with a molecular weight corresponding to Bm86. The rBm86-CG antigen showed 31% efficacy against the Campo Grande strain of *R.* (*B.*) *microplus* infesting vaccinated cattle. The rBm86-CG is an antigen that could be used in a polyvalent vaccine as part of an integrated program for the control of *R.* (*B.*) *microplus* in the region that includes Mato Grosso do Sul.

Keywords: Tick, *Rhipicephalus (Boophilus) microplus*, anti-tick vaccine, recombinant Bm86, *Pichia pastoris*.

Resumo

O carrapato *Rhipicephalus* (*Boophilus*) *microplus* é, sem dúvidas, o ectoparasito economicamente mais importante para o gado a nível mundial. A utilização inadequada de acaricidas tem impulsionado a evolução da resistência em populações de *R.* (*B.*) *microplus*. Vacinas contra o carrapato representam uma tecnologia que pode ser combinada com acaricidas em programas de controle integrado para diminuir o impacto de *R.* (*B.*) *microplus*. A forma recombinante da Bm86 da cepa Campo Grande (rBm86-CG) de *R.* (*B.*) *microplus* foi produzido utilizando o sistema de expressão em *Pichia pastoris* para testar sua capacidade de imunoproteção ao gado contra a infestação de carrapatos. A secreção de rBm86-CG em *P. pastoris* pelo bioprocesso, simplificou a purificação do antígeno. A resposta imune humoral específica foi detectada por ELISA em soros de bovinos vacinados. Resultados de "imunoblot" revelaram que anticorpos policlonais de bovinos vacinados reconheceram uma proteína em extratos de larvas com um peso molecular correspondente à Bm86. O antígeno rBm86-CG mostrou eficácia de 31% contra a amostra CG de *R.* (*B.*) *microplus* utilizada para infestar os bovinos vacinados. Pelos resultados obtidos, concluímos que a rBm86-CG é um antígeno que pode ser usado em uma vacina polivalente, como parte de um programa integrado para o controle de *R.* (*B.*) *microplus* no estado do Mato Grosso do Sul, Brasil.

Palavras-chave: Carrapato, *Rhipicephalus* (*Boophilus*) *microplus*, vacina, Bm86 recombinante, *Pichia pastoris*.

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Introduction

Rhipicephalus (*B.*) *microplus*, commonly known as the southern cattle fever tick, is arguably the most economically important ectoparasite of livestock in tropical and subtropical regions of the world. Chemical acaricides are readily available to ranchers for the control of *R.* (*B.*) *microplus* infesting cattle. The utility of chemical acaricides has been hampered by inappropriate use, which has driven the evolution of acaricide resistance among *R.* (*B.*) *microplus* populations (FURLONG, 2004). Vaccines provide an alternative tool for tick control (WILLADSEN; JONGEJAN, 1999). Integrated tick control programs that include vaccines can reduce the use of chemical acaricides, lower the cost of animal production, mitigate environmental contamination impacts, and decrease the risk of chemical residues in animal-derived products (DE LA FUENTE et al., 1998; GARCÍA-GARCÍA et al., 2000; REDONDO et al., 1999).

TickGard^{PLUS} (Intervet Australia Pty. Ltd., 91-105 Harpin Street, Bendigo East, Victoria) and Gavac^{TM} (Heber Biotec S.A., Havana, Cuba) are two Bm86-based vaccine products that were developed in the 1990s in Australia and Cuba, respectively (DE LA FUENTE et al., 2007). Bm86 is a glycoprotein isolated from *R.* (*B.*) *microplus* that appears to be located predominantly in tick gut digest cell membranes (GOUGH; KEMP, 1993). The R. (B.) microplus *bm86* gene is expressed in eggs a few days after oviposition, unfed and blood-fed larvae, nymphs, and male and female adults (NIJHOF et al., 2009). Additionally, Bm86 has been showed to be expressed in the ovaries of partially engorged adult females and silencing experiments revealed that Bm86 plays a role during the feeding period and blood digestion in *R.* (*B.*) *microplus* females fed on cattle acutely infected with *Babesia bovis* (BASTOS et al., 2010). There is a direct correlation between polyclonal anti-Bm86 antibody production and protection against *R.* (*B.*) *microplus* infestation in immunized cattle. Anti-Bm86 antibodies apparently bind to the surface of epithelial cells in the tick's intestine and disrupt endocytosis thereby causing cell lysis and reducing reproductive efficiency in engorged females (RIDING et al., 1994; WILLADSEN, 1997). The role of Bm86 in endocytosis was questioned by Nijhof et al. (2009) because its level of expression did not increase during blood uptake.

A pen trial conducted in Mato Grosso do Sul, Brazil, with TickGard^{PLUS} and Gavac^{TM} using the Campo Grande strain of *R.* (*B.*) *microplus* yielded 46.4% and 49.2% efficacy, respectively (ANDREOTTI, 2006). These results varied from those previously reported for both the anti-R. (B.) microplus vaccine products in other parts of the world. The level of efficacy reported for vaccines containing a recombinant form of Bm86 varies according to, among other factors, the expression system used for vaccine production, characteristics of the tick population targeted, and host factors (DE LA FUENTE et al., 1999; PATARROYO et al., 2002; RAND et al., 1989; RODRÍGUEZ et al., 1995; SITTE et al., 2002; ANDREOTTI et al., 2008; PARIZI et al., 2009). It has been postulated that sequence variation in the *bm86* locus is associated with low susceptibility of certain *R.* (*B.*) *microplus* populations infesting cattle immunized with commercial Bm86-based vaccines (GARCÍA-GARCÍA et al., 1999, 2000).

The degree to which sequence variations correlates with the level of Bm86-based vaccine efficacy remains to be fully understood. Based on polymorphism analysis of the *bm86* gene in South American strains of *R.* (*B.*) *microplus*, Sossai et al. (2005) suggested that geographic isolation is not the sole determinant of genomic variation. It has been suggested that diversity greater than 3% in amino acid sequence between the recombinant antigen and native Bm86 protein in target tick populations results in vaccination inefficiencies (GARCÍA-GARCÍA et al., 1999). The similarity between deduced protein sequences for Bm86 from the Campo Grande strain and the recombinant antigen used in the commercial vaccines was 96.5% (ANDREOTTI et al., 2008). Thus, using the recombinant Bm86-Campo Grande (rBm86-CG) protein as antigen for immunization could yield greater vaccine efficacy against *R.* (*B.*) *microplus* infesting cattle in Mato Grosso do Sul.

Of the 205 million head of cattle in the national herd reported for 2009, 13.9% was raised in Mato Grosso do Sul (IBGE, 2011). The evolution of resistance to commercially available chemical acaricides is a constant challenge for producers in the state and it is a major driver for research and development of novel strategies to control *R.* (*B.*) *microplus* so cattle can be raised sustainably (ANDREOTTI et al., 2011). We produced the recombinant version of Bm86-CG using the *Pichia pastoris* expression system to test the hypothesis that recombinant Bm86 from a local strain would enhance protection against *R.* (*B.*) *microplus* infesting immunized cattle in Mato Grosso do Sul state. Here, we report the results of a pen immunization trial with the recombinant Bm86-CG antigen.

Materials and Methods

1. Ticks

Rhipicephalus (*B.*) *microplus* used in the study were obtained from a laboratory colony maintained at EMBRAPA Beef Cattle. These ticks were collected originally from infested cattle in Campo Grande - MS, Brazil. Tick larvae were fed on cattle and collected as adults after repletion to allow for oviposition and hatching in humidity chambers at 28 °C and 95% relative humidity. Larvae were used for infestation 18 days after hatching.

2. DNA manipulations

The entire 1825-bp DNA fragment corresponding to Bm86-CG (GenBank accession no: EU352677.1) (ANDREOTTI et al., 2008) was amplified by PCR using primers BmCG-EcoRI-F1 (CGGAATTCTCATCCATTTGCTC) and BmCG-KpnI-R2 (TGGAAAGTCAAGTGCGGTACCCC) in a Mastercycler Personal thermal cycler (Eppendorf, Germany) using Platinum® *Taq* DNA Polymerase High Fidelity and cloned into the pPICZαA vector (Invitrogen). Insertion was confirmed by subjecting plasmid to PCR with 5'AOX1 and 3'AOX1 primers that flank the multiple cloning site in this plasmid. The resulting plasmid was termed pPICZαA-BmCG.

Pichia pastoris was transformed by electroporation (MicroPulser, Bio-rad, USA) according to manufacturer's instructions (INTROGEN, 2009). Briefly, an 80 µL aliquot of electrocompetent *P. pastoris* X33 strain was transformed using 10 µg of pPICZαA-BmCG linearized with *Sac*I, by applying one 20 kV pulse during 5 ms. The recombinant cells were selected on YPDS plates containing Zeocin™ (1% yeast extract; 2% peptone; 2% dextrose; 1 M sorbitol; 2% agar; 100 µg/mL Zeocin).

Genomic DNA was extracted from *Pichia* clones using the glass bead disruption method. A 1 mL pellet of culture was resuspended with 150 µL of STES (2.42% Tris base; 2.92% sodium chloride; 0.1% SDS; 0.372% EDTA; pH 7.6) in a 1.5 mL microtube. Fifty µL of glass beads (425-600 µm) and 150 µL of phenol:chloroform:isoamyl alcohol (12:12:1 v/v) was added and the mixture vortexed for 1 minute and centrifuged at 13,000 g for 5 minutes. The supernatant was precipitated with potassium acetate, the pellet recovered and rinsed with 70% ethanol, and the air-dried DNA resuspended in sterile ultrapure water. DNA was quantified using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA) and stored at –20 °C.

3. Production of polyclonal antibodies and collection of murine serum samples

Polyclonal sera against Gavac™ and TickGard^{PLUS}, *R.* (*B.*) *microplus* larval extract, and rBm86-CG were produced using BALB/c mice. To obtain the larval extract, 8 g of larvae were frozen at –20 °C, macerated using a mortar and pestle, and 150 mM Tris-HCl at pH 7.4 added to a final volume of 8 mL. The eluted extract was sonicated with two 10 seconds pulses at 60% amplitude and another four 10 seconds pulses at 20%. The final content was syringe-filtered (0.22 µm) and stored at –20 °C. The larval extract was quantified and 3 mg of total protein diluted in 15 mL of 150-mM Tris-HCl at pH 7.4 and emulsified in 15 mL of Freund's complete adjuvant, which yielded 30 mL of larval extract emulsion for immunization. The rBm86-CG vaccine formulation was prepared with 500 µg of rBm86-CG protein resuspended in 5 mL of 150 mM Tris-HCl at pH 7.4, and emulsified with 5 mL of complete Freund's adjuvant. Twenty-four female BALB/c mice were separated into four groups of six animals. Each group received one of four vaccines at a concentration of 50 $\mu g.mL^{-1}$ (Gavac™, TickGard^{PLUS}, or rBm86-CG), or 1,000 $\mu g.mL^{-1}$ (larval extract formulation), for each dose of 100 uL for all doses inoculated. Three intramuscular doses were applied at 21-day intervals. Serum samples were collected 21 days after the mice received the last immunization.

4. Screening of P. pastoris transformants by dot blotting

Each clone was subcultured in 5 mL of BMGY medium (1% yeast extract; 2% peptone; 100 mM potassium phosphate at pH 6.0; 1.34% YNB; 4×10^{-5}% biotin; 1% glycerol) and incubated in an orbital shaker set at 220 rpm and 30 °C along with negative controls. After 24 hours of incubation, cultures were centrifuged at 1500 g for 5 minutes, the supernatant was discarded, and the cells resuspended in 5 mL of BMMY (1% yeast extract; 2% peptone; 100 mM potassium phosphate at pH 6.0; 1.34% YNB; 4×10^{-5}% biotin; 0.5% methanol). The cultures were returned to the shaker for another 4 days. Each culture was supplemented with methanol every 24 hours to a final volume of 0.5%. The cultures were eventually centrifuged and the supernatants treated with 1 mM PMSF for collection in 2 mL microtubes that were stored at –20 °C.

A PVDF membrane was washed 10 s in methanol and then 5 min in sterile ultrapure water. A 5 µL aliquot of each selected clone's supernatant was applied to the pretreated PVDF membrane and dried in an oven at 30 °C. Five µL aliquots of Gavac™ and Tick Gard^{PLUS} were used as positive controls on the membranes. The membrane was blocked using phosphate buffered saline (PBS) containing 5% nonfat dry milk with stirring for 1 hour at 30 °C, and subsequently washed twice (5 minutes each time) with PBS-T (PBS plus 0.1% Tween 80, pH 7.4). Each membrane was then stirred for 1 hour at 30 °C in the presence of polyclonal antibodies from the mice vaccinated with Gavac™ or TickGard^{PLUS} diluted 1:150 in PBS. The membranes were then subjected to three additional washes with PBS-T followed by incubation with peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Sigma-Aldrich, USA) diluted 1:1000 in PBS-T. The process was completed by three additional washes with PBS-T and incubation of the membranes in 5 mL of developing solution (0.5% diaminobenzidine; 0.075% urea) until coloration was observed, which was followed by washing in sterile ultrapure water. Photos were taken of the fully processed membranes.

5. Screening for Mut phenotype in P. pastoris transformed strains

Phenotypically, recombinant clones can be either Mut^+ or Mut^S. The Mut^S (methanol utilization slow) phenotype results from partial loss of activity of alcohol oxidase caused by a mutation in the AOX1 (alcohol oxidase 1) gene. Despite this mutation, the AOX2 gene is still functional. The result is slow growth in methanol by comparison with growth rates observed in Mut^+ strains. Both classes can be produced by the X33 strain. For genotypic analysis of the phenotype under investigation, DNA extracted from each clone and DNA from GS115 (Mut^+) and GS115/albumin (Mut^s) were subjected to PCR using two pairs of primers (3'AOX1 with 5'AOX1, and 3'AOX1 with α-Factor) followed by molecular characterization according to conditions set forth by the manufacturer. PCR products from genomic DNA for each clone (Mut^+ and Mut^S) were sequenced with primers designed for the P_{AOX1} promoter. These sequences and Bm86-CG sequence data were compared using ClustalW software for sequence alignment.

6. Production of rBm86-CG

For rBm86-CG expression, one colony each from Mut^+ and Mut^s clones was plated and isolated, inoculated into a 500 mL Erlenmeyer flask containing 25 mL of BMGY medium, and incubated overnight in an orbital shaker (220 rpm) at 30 °C until reaching $OD_{600\ nm} = 4$, which took 16-18 hours. The cultures

were subsequently centrifuged at 1500 g for 5 minutes at room temperature and the supernatants discarded. Precipitates of the Mut^+ clone and the negative X33 were resuspended with ~200 mL of BMMY medium to an $OD_{600\ nm}$ = 1.0. Each culture was supplemented with methanol every 24 hours to a final volume of 1%, with a total induction period of 96 hours. Precipitates of the Mut^S clone and a GS115/Albumin clone were resuspended in 250 mL of BMGY medium and incubated overnight. These cultures were centrifuged again, resuspended in 200 mL of BMMY medium, and incubated. Induction was maintained by supplementation with methanol every 24 hours to a final volume of 0.5%, for 96 hours. After this induction period, the cultures were centrifuged at 3,300 g at 4 °C for 5 minutes, and the supernatants separated, treated with 1 mM PMSF, and frozen at –20 °C.

7. Purification and quantification of rBm86-CG

Supernatants from the material expressed by Mut^+ and Mut^S clones were quantified by the standard curve method with bovine serum albumin (BSA) on SDS-PAGE. Fifty µL aliquots of a BSA solution (0.5 $\mu g.\mu L^{-1}$) were used as quantification standards in a 7.5% polyacrylamide gel. Bands formed on the gel were quantified by visual inspection and through the use of TotalLab 2.0 software (Amersham/Biosciences, United Kingdom), which served to estimate the apparent molecular mass of bands.

Twenty-five mL of the Mut^+ supernatant was incubated for 1 hour with methanol 1:1 (v/v) to precipitate proteins by centrifugation (5000 g) for 30 minutes at 4 °C. Proteins pellets were eluted by adding denaturing binding buffer (8 M urea, 20 mM Na_3PO_4, pH 7.8, 500 mM NaCl). This solution was loaded onto a Ni^{2+} charged Ni-NTA (Qiagen, Hilden, Germany) affinity column equilibrated with denaturing binding buffer. The column was first washed with denaturing binding buffer followed by five washes with denaturing wash buffer (pH 6.0). The rBm86-CG protein was eluted in denaturing elution buffer (pH 4.0). The purification process was monitored by 10% SDS-PAGE. Eluted fractions of high purity were pooled and dialyzed against PBS.

8. rBm86-CG electrophoresis and Western blotting

Fifty µL of supernatant from each of two selected clones were mixed with 10 µL of SDS-PAGE buffer separately. These samples were incubated for 6 minutes at 95 °C, left to cool down and pipetted (20 µL) on a 7.5% polyacrylamide gel. Electrophoresis was run for 1.5 hours at 30 mA in a 2D electrophoresis system. Electrophoresis material was used for Western blotting analysis with 10 µL of Mut^+ supernatant per well. Proteins were blotted to a PVDF membrane, pretreated with methanol for 10 seconds and sterile ultrapure water for 5 minutes, with a transfer system immersed in buffer (0.3% Tris base; 1.44% glycine; 15% methanol; pH 8.4) at 100 mA for 2 hours. The membrane was washed with PBS, left to dry in a stove at 25 °C for 1 hour, stained with Ponceau S solution (0.5% Ponceau S; 1% glacial acetic acid), and cut into strips. These strips were stirred and blocked with PBS containing 5% nonfat dry milk at 30 °C for 1 hour, washed twice with PBS-T for 5 minutes, and incubated with primary antibody at 1:150 in PBS at 30 °C for 1 hour. One strip was processed for each type of serum: anti-Gavac, anti-TickGard, anti-larval extract, and anti-rBm86-CG. Each strip was subsequently stirred, and washed twice for 5 minutes with PBS-T, and incubated with peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (1:1000) in PBS-T at 30 °C for 1 hour. This was followed by three additional washes, rinsing in sterile ultrapure water, and incubation in development solution (5 mL of Milli-Q water; 5 mg of diaminobenzidine; 0.75 mg of NH_4) until well-defined bands appeared on the PVDF membrane.

9. Pen trial

The rBm86-CG protein expressed by *P. pastoris* was adjuvated with Montanide ISA 61 VG (Seppic, Paris) to produce 2 mL doses each containing 100 µg of antigen. A controlled pen trial was conducted to evaluate the immunogenic and protective capacity of the rBm86-CG antigen. One-year old Holstein calves were acclimated prior to experimentation and received proper veterinary care during the study. Cattle were randomly assigned to two groups of six animals each. One group was immunized with the rBm86-CG antigen following the protocol described below. The other six animals served as the control group and were treated as per protocol except that they received 2 mL injections of adjuvant alone each time. The protocol of Andreotti (2006) was slightly modified so each animal was injected intramuscularly at 0, 2 and 4 weeks. Serum samples were taken from each animal before immunization and weekly thereafter. Twenty-one days after the last injection the animals were challenged with 15,000 *R.* (*B.*) *microplus*-CG larvae delivered along their back in three applications during a week. Tick collections were performed daily once the engorged females started detaching spontaneously from the control animals. Tick samples were brought to the laboratory, weighed, and incubated at 29 °C and 85% relative humidity until egg-laying was complete. Egg masses were weighed and incubated to determine hatch rate, i.e. fertility.

10. Bovine serum collection and analysis

Blood samples were obtained from cattle before immunization and weekly thereafter. Sera obtained from the blood samples was frozen for subsequent ELISA analysis. Serum samples were pooled by treatment group for each collection date prior to testing. Microtiter plates were coated with 1 mg/mL of rBm86-CG antigen in 20 mM carbonate buffer (pH 9.6) overnight at 4 °C. The plates were blocked with 2% serum bovine albumin in PBST (200 mL) and then washed five times with Na_3PO_4 buffer (PBST, pH 7.4, 0.1% Tween 20). This was followed by incubation with test bovine serum diluted to 1:100 in PBST (100 mL) for 45 minutes at 37 °C and another washing cycle. Then, 50 µL of rabbit anti-bovine IgG peroxidase conjugate (Sigma, St. Louis, MO), diluted 1:20,000, were added and the plate incubated for 30 minutes at room temperature followed by washing. The process was completed by adding 50 µL of the chromogenic substrate O-phenylenediamine (1.0 mM), and the reaction stopped after

15 minutes with 100 µL of NaOH (0.2 M). A microplate reader was used to assess the results with absorbance set at 490 nm.

11. Statistical and efficacy analyses

Vaccination effects on tick biology and efficacy were determined as described by García-García et al. (1999) and Andreotti (2006). Briefly, reduction rates associated with Bm86-CG immunization relative to the unvaccinated group were determined for adult female ticks (% DT), egg-laying capacity (% DO), and fertility (% DF). Vaccine efficacy was calculated as 100 × [1 – (CRT × CRO × CRF)], where CRT, CRO, and CRF are respectively, the reduction in the number of adult female ticks, egg-laying capacity, and fertility. The Mann–Whitney nonparametric test was used to compare biological data and to assess vaccine efficacy. Mean antibody levels were determined for each group and compared using ANOVA as described previously (ANDREOTTI, 2006).

Results

1. Production and characterization of P. pastoris strains expressing rBm86-CG

A recombinant plasmid was designed by inserting the Bm86-CG protein gene sequence in the multiple cloning site of the pPICZαA plasmid, between the *Eco*R I and *Kpn* I restriction enzyme sites in the multiple cloning polylinker (Figure 1). The sequence of the insert was confirmed by sequencing and alignment with Bm86-CG gene (GenBank accession no: EU352677.1) with 100% identity. This sequence was cloned taking into consideration the expression cassette controlled by the AOX1 gene promoter. The multiple cloning site was maintained to precede the Bm86-CG sequence, which allows future cloning of other sequences of interest.

2. rBm86-CG protein expression by P. pastoris

The Mut$^+$ and Mut^s clones were selected and induced for expression. Total protein yield in the supernatant, which included rBm86-CG expressed by the Mut$^+$ and Mut^s clones, was 870 mg.L^{-1}. Analysis of the supernatants on 7.5% SDS-PAGE for protein expression revealed bands of approximately 80 kDa. The expected molecular weight for the rBm86-CG protein expressed in this system was of 79.1 kDa, which corresponded to the SDS-PAGE band observed (Figure 2).

3. rBm86-CG protein recovery and purification

Following electrophoresis of culture supernatants, recoverable levels of rBm86-CG were 62.5 and 32 mg.L^{-1} for Mut$^+$ and Mut^s, respectively. Nickel column purification followed by SDS PAGE revealed a protein with an estimated molecular weight of 80 kDa (Figure 3).

4. rBm86-CG protein characterization

The mouse was used to evaluate immune cross-reactivity between rBm86-CG and the native and commercial recombinant forms of the protein. Western blot analysis of the rBm86-CG

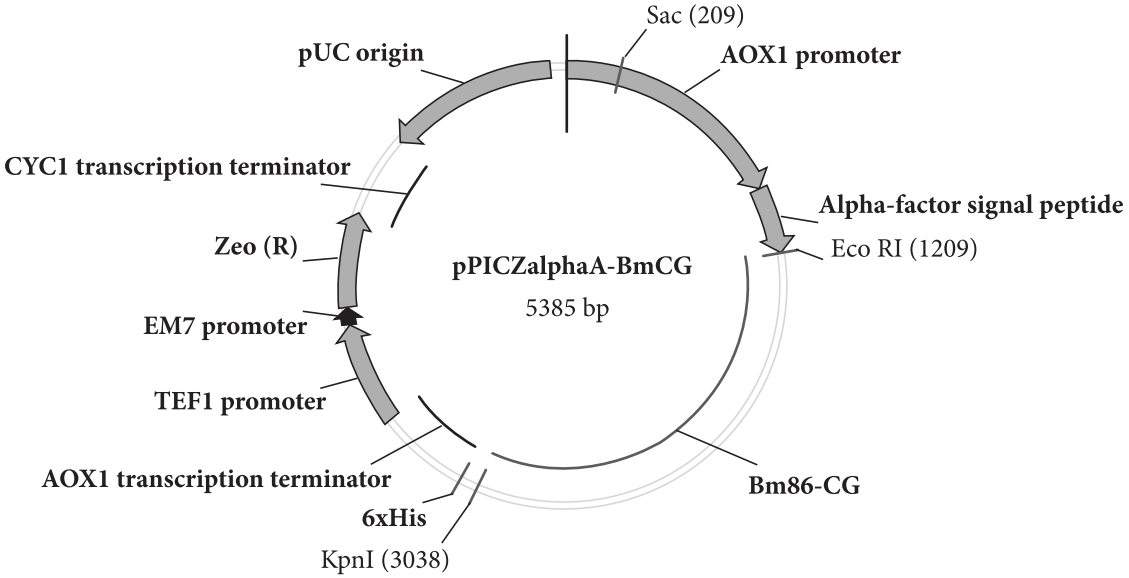

Figure 1. Map of plasmid vector pPICZαA-Bm86-CG used to transform *P. pastoris* expression system. The map was created with Vector NTI 8 software; 5'AOX1 promoter: promoter strongly induced by methanol; *Sac* I: enzyme used to linearize the plasmid before transformation in *P. pastoris*; Alpha-factor: sequence corresponding of secreted peptide of rBm86-CG; *Eco*R I: recognition sequencing of the enzyme used for cloning of 5' region; Bm86-CG: *bm86-CG* sequence inserted in plasmid; *Kpn* I: recognition sequence of the enzyme used for cloning the 3' region; 3'AOX1 Termination: sequence that determines the end of the transcript; Zeo(R): Zeocin™ resistance gene allowing selection of transformants in *Pichia*; pUC origin: Replication origin for *Escherichia coli*.

protein performed with serum samples from mice immunized with Gavac™, TickGard^{PLUS}, *R.* (*B.*) *microplus* larval extract, and rBmCG revealed affinity for a protein of approximately 80 kDa. These results indicated that the rBmCG protein contained epitopes with the potential to protect cattle against *R.* (*B.*) *microplus* infestation.

5. Seroconversion of cattle vaccinated with rBm86-CG

Unlike the control animals, vaccinated cattle developed a significant antibody response against rBm86-CG (Figure 4) ($p < 0.05$). Antibody levels peaked 21 days after the third immunization. The bovine humoral immune response to the rBm86-CG antigen showed a negative correlation when compared to engorged female tick number production. A decrease in the level of rBm86-CG antibodies was noted in immunized cattle one week after infestation, which was equivalent to that observed the week before ticks were placed on the same animals (Figure 4). Kinetics of the antibody response between cattle vaccinated with rBm86-CG and animals immunized with Gavac™ (RODRÍGUEZ et al., 1994) and TickGard^{PLUS} (WILLADSEN et al., 1989) were similar**.**

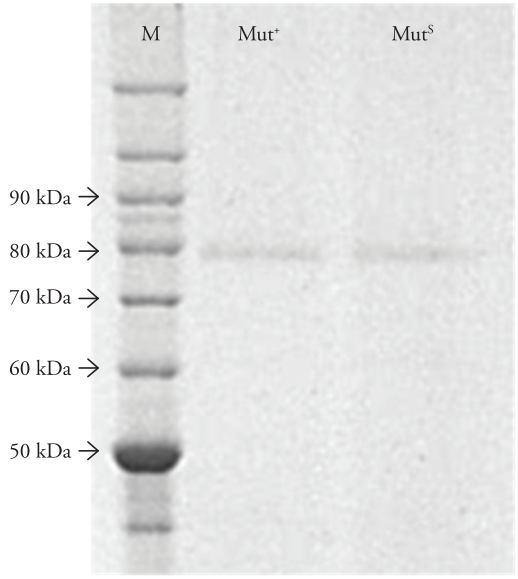

Figure 2. Expression of rBm86-CG by *P. pastoris*. SDS-PAGE 7.5% analysis of the fraction obtained by nickel chromatography process of the culture supernatant for clones Mut$^+$ and Mut^S. M: BenchMark™ Protein Ladder standards for SDS-polyacrylamide gel electrophoresis ranging in molecular weight from 10 to 220 kDa. Mut$^+$ and Mut^S: 10 µg per well of rBm86-CG expressed in Mut$^+$ and Mut^S clones.

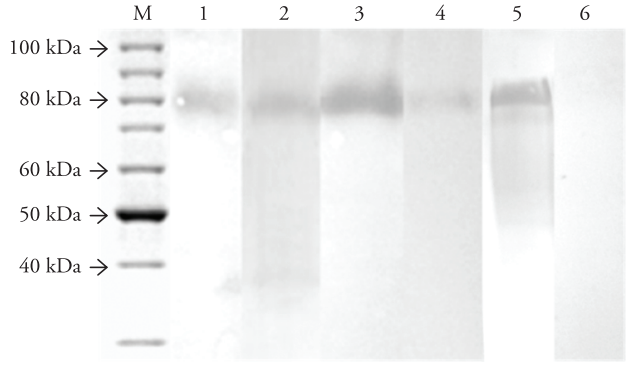

Figure 3. Western blot of rBm86-CG expressed in clone Mut$^+$. Lanes 1: anti-Gavac™ serum; 2: anti-TickGard^{PLUS} serum; 3: anti-larval extract serum; 4: anti-rBm86-CG serum; 5: larval extract as antigen recognized by bovine serum immunized with rBm86-CG; 6: Negative control. M: BenchMark™ Protein Ladder standards for SDS-polyacrylamide gel electrophoresis ranging in molecular weight from 10 to 220 kDa.

6. Immunoprotection

Daily collections of engorged ticks dropping from cattle were terminated 15 days after tick detachment commenced. The dynamics of detachment and egg production showed that a significant peak in the number of ticks dropping and weight of egg masses occurred on the fourth day ($p < 0.05$; data not shown). Immunization of cattle with rBm86-CG reduced the number of adult female ticks on them by 28%. No decrease was observed in egg-laying capacity. Fertility was reduced 8% (Table 1). Vaccination of cattle with the rBm86-CG antigen afforded 31% efficacy against *R.* (*B.*) *microplus* infestation.

Discussion

The recombinant form of Bm86 from the CG strain of *R.* (*B.*) *microplus* was produced using the *P. pastoris* expression system to test its ability to immunoprotect cattle against tick infestation. Secretion of rBm86-CG by *P. pastoris* through the bioprocess reported here simplified purification of the antigen.

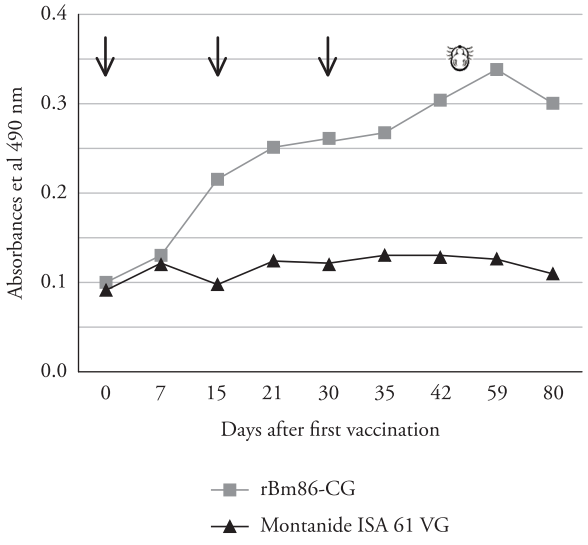

Figure 4. Antibody response of cattle vaccinated with rBm86-CG antigen. Control animals were injected adjuvant Montanide ISA 61 VG alone. Antibody titers of immunized cattle are depicted as the $OD_{490\ nm}$ value of the 1:100 dilution of serum samples that were pooled for each day of testing. Arrows indicate the day cattle were vaccinated. Tick icon indicates day 51 after the first vaccination, which is when cattle were infested.

Table 1. Effects on females, their progeny, and efficacy of vaccination with rBm86-CG antigen against *R.* (*B.*) *microplus* CG strain infesting cattle.

	% Reduction[a] (vaccinated/control)			
Antigen	DT	DO	DF	Eficacy (%)[b]
rBmCG	28	–5	8	31
	(3,305/4,594)	(0.138/0.145)	(88.66/96.63)	

[a]Percent reduction was calculated in relation to the control unvaccinated group: DT, adult female ticks; DO, egg laying capacity; DF, fertility. [b]Efficacy (%) = 100 [l –(CRT × CR0 × CRF)]; where CRT: reduction in the number of adult female ticks, CRO: reduction in the egg laying capacity, CRF: reduction in fertility.

This process enhances recovery and purity of recombinant Bm86 antigens expressed in the yeast system for vaccine production (CANALES et al., 2008). The specific humoral immune response in vaccinated cattle detected by ELISA confirmed the immunogenicity of rBm86-CG. Immunoblot results revealed that polyclonal antibodies from vaccinated cattle recognized a protein in larval extracts with a molecular weight corresponding to Bm86, which indicated conformational resemblance between rBm86-CG and the native protein form.

Immunoprotection of cattle vaccinated with the experimental formulation of rBm86-CG yielded a level of efficacy 18% lower than that reported with the Bm86-based vaccine Gavac™ against the *R.* (*B.*) *microplus* CG strain (ANDREOTTI, 2006). Bioinformatic analyses by Andreotti et al. (2008) revealed that the similarity of the deduced amino acid sequence between Bm86 from *R.* (*B.*) *microplus* CG and the Bm86 antigen in Gavac™ was 96.3%. The 31% efficacy attained with rBm86-CG was considerably greater than the efficacy of Bm86 against a strain of *R.* (*B.*) *microplus* from Argentina (GARCÍA-GARCÍA et al., 2000). A link between vaccine efficacy and sequence variability has been questioned because there are factors other than primary amino acid sequence of the antigen that influence the efficacy of a vaccine (WILLADSEN, 2008). The DNA clone used to produce rBm86-CG included sequences coding for 608 of the 650 amino acids in the full *bm86* open reading frame. Ten of the missing amino acids are located in the N-terminus and the rest in the C-terminus of the deduced protein sequence. The missing amino acids do not appear to contain predicted structural features of high immunogenic potential (ANDREOTTI et al., 2008). Aspects related to vaccine formulation or host factor polymorphisms may have caused the lesser than expected efficacy obtained in cattle immunized with the rBm86-CG antigen.

It is possible that our experimental vaccine formulation lacked a component that could have enhanced the immunogenicity of rBm86-CG. Advances in vaccinology have enabled the refinement of adjuvants and other components of antigen formulations. Changes in the supply chain required the substitution of Montanide 888 (from animal source) with Montanide 888 VG (from vegetal source) in Gavac™ (SEGURA et al., 2009). Two initial vaccinations with Gavac^{plus} elicited a high anti-Bm86 antibody response in naïve cattle, which caused similar effects in *R.* (*B.*) *microplus* as those observed with the use of three doses (VARGAS et al., 2010). The immunogenicity of the rBm86-CG antigen could be enhanced by using other adjuvants. Some bacterial cell wall components have been shown to be highly immunogenic. Monophosphoryl lipid A, a lipopolysaccharide derivative, was approved for use in combination with aluminum hydroxide in a commercial human papillomavirus vaccine (MCKEE et al., 2010).

Variation in the immune response between animals can impair the development of recombinant and peptide vaccines. Major histocompatibility complex (MHC) polymorphisms have been shown to affect the magnitude of the host immune response to vaccination (FELLAY et al., 2011). A single amino acid deletion in the antigen recognition site of an MHC class II molecule affected the response to TickGard (SITTE et al., 2002). The observation by Andreotti et al. (2008), who suggesting that structural portions of rBm86-CG may bind to MHC class I molecules, must still be tested experimentally. Consideration of the immunogenetic background of cattle requires attention during discovery research efforts to develop the next generation of commercial anti-*R.* (*B.*) *microplus* vaccines with an efficacy profile that is consistent across breeds.

The efficacy obtained with Bm86-based vaccines against *R.* (*B.*) *microplus* infesting cattle can range between 10 and 89% (WILLADSEN, 2008). Because of their high efficacy against *R. annulatus*, the use of Bm86-based vaccines is being explored for eradication purposes (MILLER et al., 2012). Scientific and technological advancements offer the opportunity to develop new vaccines against cattle ticks. Existing technology based on the Bm86 paradigm provides the foundation to develop a multi-antigen vaccine with an improved profile yielding a high level of efficacy against *R.* (*B.*) *microplus* and *R. annulatus*. The positive impact of tick vaccines for cattle has been documented (DE LA FUENTE et al., 2007). A second generation of vaccine anti-*R.* (*B.*) *microplus* must meet strict criteria targeting investment opportunities for R & D required by international animal health company expecting to commercialize the technology globally (MEAT & LIVESTOCK AUSTRALIA LIMITED, 2008). The rBm86-CG is an antigen that could be used in a polyvalent vaccine as part of an integrated program for the control of *R.* (*B.*) *microplus* in the region that includes Mato Grosso do Sul state.

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