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Rhipicephalus (*Boophilus*) *microplus*: expressão e caracterização da Bm86-CG em *Pichia pastoris*

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Abstract

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is responsible for great economic losses. It is mainly controlled chemically, with limitations regarding development of resistance to the chemicals. Vaccines may help control this parasite, thereby reducing tick pesticide use. In this light, we performed subcloning of the gene of the protein Bm86-GC, the homologue protein that currently forms the basis of vaccines (Gavac™ and TickGard^{PLUS}) that have been developed against cattle ticks. The subcloning was done in the pPIC9 expression vector, for transformation in the yeast *Pichia pastoris*. This protein was characterized by expression of the recombinant Mut$^+$ strain, which expressed greater quantities of protein. The expressed protein (rBm86-CG) was recognized in the Western-blot assay using anti-Gavac, anti-TickGard, anti-larval extract and anti-rBm86-CG polyclonal sera. The serum produced in cattle vaccinated with the antigen CG rBm86 presented high antibody titers and recognized the native protein. The rBm86-GC has potential relevance as an immunogen for vaccine formulation against cattle ticks.

Keywords: *Ixodes*, ticks, bovines, rBm86-CG, immunization.

Resumo

O carrapato-do-boi *Rhipicephalus* (*Boophilus*) *microplus* é responsável por grandes perdas econômicas. Seu controle é principalmente químico e apresenta limitações quanto ao desenvolvimento de resistência aos princípios ativos. As vacinas podem auxiliar no controle deste parasita diminuindo as aplicações de carrapaticidas. Considerando isso, foi realizada a subclonagem do gene da proteína Bm86-CG, proteína homologa a que atualmente é a base das vacinas desenvolvidas (Gavac™ e TickGard^{PLUS}) contra o carrapato-do-boi, no vetor de expressão pPIC9, para ser transformado em levedura, *Pichia pastoris*. Esta proteína foi caracterizada pela expressão da cepa recombinante Mut$^+$ que expressou maior quantidade de proteína. A proteína expressa, rBm86-CG, foi reconhecida no ensaio de Western-blot pelos soros policlonais anti-Gavac, anti-TickGard, anti-Extrato de larva e anti-rBm86-CG. O soro produzido em bovinos vacinados com o antígeno rBm86-CG apresentou altos títulos de anticorpo e reconheceu a proteína nativa. A rBm86-CG possui potencial relevância como imunógeno para formulação vacinal contra o carrapato de bovinos.

Palavras-chave: *Ixodes*, carrapatos, bovinos, rBm86-CG, imunização.

Introduction

Considerable economic losses are currently caused by *Rhipicephalus* (*Boophilus*) *microplus*, a cattle ectoparasite occurring in tropical and subtropical areas (WILLADSEN; JONGEJAN, 1999). Chemical control, albeit predominant, has been hampered by poor utilization of active agents, thus leading to development of resistance and requiring the use of higher concentrations of active compounds and more frequent application, year by year (FURLONG, 2004). Vaccines are an alternative for tick infestation control. In association with tick pesticides, they can reduce the use of these agents, thereby lowering the costs of animal production, levels of environmental contamination and presence of residues in animal-derived products (GARCÍA-GARCÍA et al., 2000).

The protein Bm86 is one of the antigens most used in vaccines against *R.* (*B.*) *microplus*, and it yields satisfactory results in tick control (DE LA FUENTE et al., 1998; 1999; PATARROYO et al., 2002; WILLADSEN; KEMP, 1988; WILLADSEN; MCKENNA, 1991; WILLADSEN et al., 1989). It is a glycoprotein predominantly located in the membrane of tick gut cells (GOUGH; KEMP, 1993).

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When this protein is used as a vaccine antigen, the antibodies bind to the surface of epithelial cells in the tick intestine. This causes cell lysis and reduces reproductive efficiency among engorged females (WILLADSEN, 1997), since these cells are probably implicated in the mechanism of endocytosis (RIDING et al., 1994).

In the state of Mato Grosso do Sul, Brazil, a previous study on two vaccine formulations based on Bm86, namely, TickGard^{PLUS} (Intervet Australia Pty. Ltd., 91-105 Harpin Street, Bendigo East, Victoria, Australia) and Gavac^{TM} (Heber Biotec S.A., Havana, Cuba), yielded effectiveness rates of 46.4 and 49.2%, respectively, regarding protection against this cattle tick (ANDREOTTI, 2006). However, these levels were lower than those achieved in other regions around the world (DE LA FUENTE et al., 1999; PATARROYO et al., 2002; RAND et al., 1989; RICHARDSON et al., 1993).

In a recent investigation, Andreotti et al. (2008) compared the predicted amino acid sequences of Bm86-CG (Gen Bank accession no. ACA57829) with those of Bm86 (Gen Bank accession no. M29321.1) and Bm95 (Gen Bank accession no. AF150891) and found identity levels of 96.5 and 96.3%, respectively. The study suggested that two differences in the Bm86-CG sequence in hydrophobic regions may have great potential for influencing the dynamics of antibody recognition. This might account for the low effectiveness of Gavac^{TM} for protecting cattle raised in the Campo Grande region, against locally isolated infestation by *R.* (*B.*) *microplus* (ANDREOTTI, 2006).

A number of vaccines based on recombinant Bm86 have been formulated (CANALES et al., 2009; DE LA FUENTE et al., 1999; PATARROYO et al., 2002; RAND et al., 1989; RODRIGUEZ et al., 1995). However, these have varying effectiveness levels against cattle tick strains from different geographic areas. This feature is possibly related to natural allele variations in the Bm86 gene (DE LA FUENTE; KOCAN, 2003). Differences in amino acid sequence in Bm86 variations of more than 3.4% would be sufficient to make vaccination ineffective against different strains of the tick (GARCÍA-GARCÍA et al., 1999).

The purpose of the present study was to produce a regional antigen containing the partial sequence of Bm86-CG$_{21\text{-}603}$, using a eukaryotic expression system based on *Pichia pastoris*. This system was selected because Bm86-CG is a glycoprotein and because *P. pastoris* is capable of effecting glycosylation and correctly folding the recombinant protein. These factors may be decisive in determining the composition of the three-dimensional structure of this protein, and therefore the effectiveness of antibody production.

Material and Methods

1. Bm86-CG sequence

Bm86-CG (GenBank accession no. EU352677.1) was used as a template. Briefly, this sequence was amplified from total cDNA of larval *R.* (*B.*) *microplus* and cloned into plasmid pMOS blue (GE Healthcare, United Kingdom) (ANDREOTTI et al., 2008). The 1825-bp DNA fragment corresponding to Bm86-CG was amplified from plasmid pMOS blue by means of PCR using the primers BmCG-EcoRI-F1 (CGGAATTCTCATCCATTTGCTC) and BmCG-NotI-R2 (GCGGCCGCAGCACTTGACTT) (both synthesized by Invitrogen, Australia) in a Mastercycler personal thermal cycler (Eppendorf, Germany).

2. Construction of plasmid pPIC9BmCG

The vector pPIC9 (Invitrogen, Australia) was selected for subcloning the gene to be expressed by *P. pastoris*. The vector was transformed into chemically competent top 10F' *E. coli* cells using the heat-shock method (INVITROGEN, 2009) to promote propagation. Because the plasmid included a gene that confers ampicillin resistance to successfully transformed *Escherichia coli* cells, these were inoculated into 10 mL of LB medium containing 100 μg.mL^{-1} of ampicillin and incubated overnight in an orbital shaker at 37 °C. The pPIC9 vector was extracted from the cells using a NucleoSpin plasmid kit (Macherey-Nagel, Germany) and then eluted in water.

The purified PCR product and the pPIC9 vector were initially incubated with the EcoRI enzyme (Promega, USA) and subsequently with the NotI enzyme (Promega, USA), in accordance with to the manufacturer's instructions, separately. The resulting material was purified using the NucleoSpin Extract II kit (Macherey-Nagel, Germany) and finally eluted in Milli-Q water. Amounts of digested PCR product (34 ng) and digested pPIC9 (50 ng) were incubated in a system containing the T4 DNA-ligase enzyme (Promega, USA), in accordance with the manufacturer's instructions, and were incubated overnight at 4 °C. The ligation was subjected to agarose gel electrophoresis (0.8%) and was quantified by means of UV spectrophotometry. A 10 μL aliquot of the ligation system was transformed into chemically competent top 10F' *E. coli* using the heat-shock method (WILEY, 2003). The transformed cells were seeded in petri dishes containing LB agar amended with 100 μg.mL^{-1} of ampicillin and incubated for two days at 37 °C. Ten colonies were selected for subculturing in 50 mL Falcon tubes containing 10 mL of LB medium with 100 μg.mL^{-1} of ampicillin, and these were incubated overnight in an orbital shaker at 120 rpm at 37 °C. From each sample, 500 μL aliquots were collected to be centrifuged and resuspended in 500 μL of LB medium with 15% glycerol for storage at –80 °C. From the remaining 9.5 mL, the recombinant plasmid was extracted using a NucleoSpin plasmid kit (Macherey-Nagel, Germany). Insertion was confirmed by subjecting each plasmid to PCR with 5'AOX1 and 3'AOX1 primers, which flanked the multiple cloning region in this plasmid. The resulting plasmid was named pPIC9BmCG (Figure 1).

3. Cloning into Pichia pastoris

The *P. pastoris* transformation involved electroporation in a MicroPulser apparatus (Bio-rad, USA), in accordance with the manufacturer's instructions, in the *Pichia* expression kit (INVITROGEN, 2009). Briefly, an 80 μL aliquot of electrocompetent *P. pastoris* GS115 strain was transformed using 10 μg of pPIC9BmCG linearized with *Sac*I (Invitrogen, Australia), by applying 2.0 kV pulses at 5 ms intervals. The recombinant

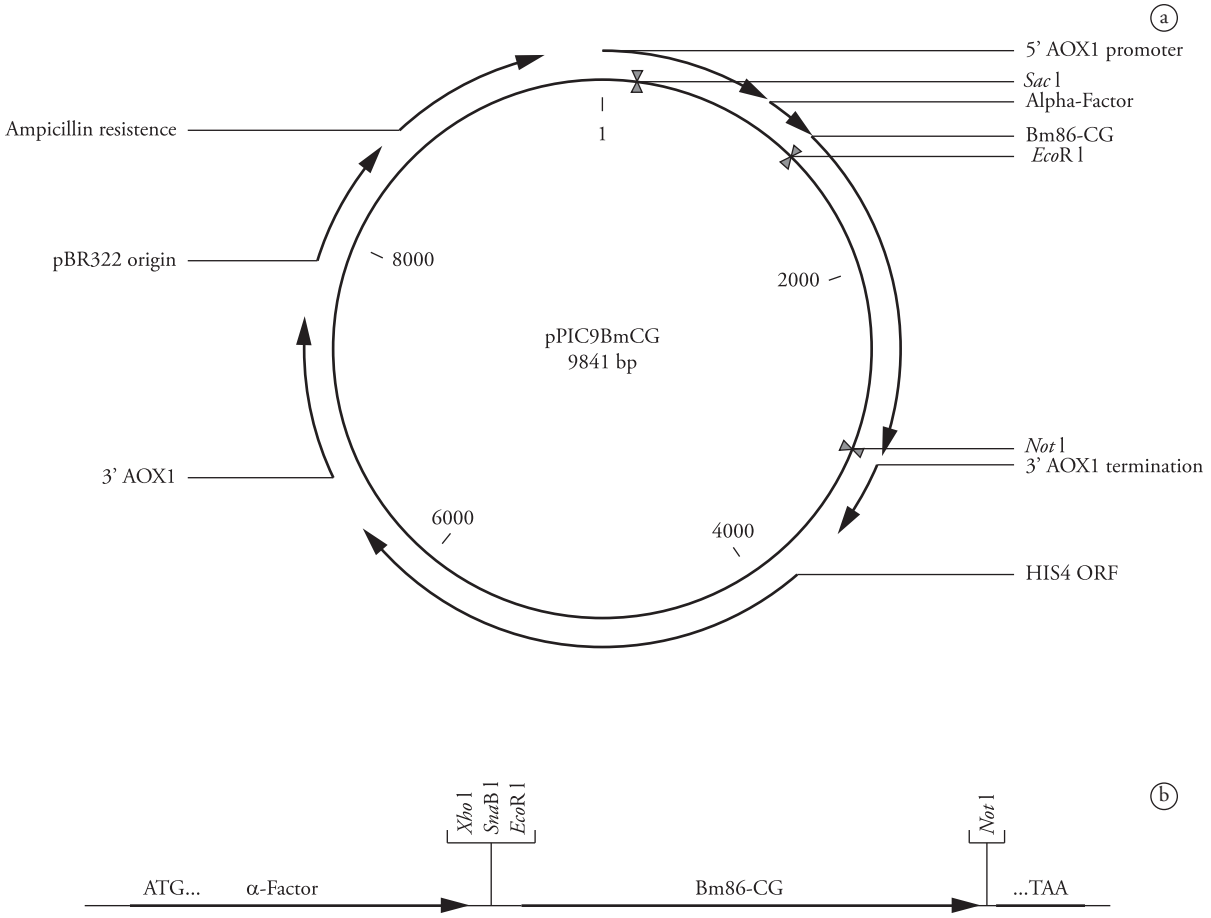

Figure 1. a) pPIC9BmCG represented using the CLC Viewer 6.0.2 software; 5´AOX1 promoter: promoter strongly induced by methanol; Sac I: enzyme used to linearize the plasmid before transformation in *P. pastoris*; 5´AOX1 primer: annealing site of primer 5´AOX1; Alpha-factor: sequence corresponding to secreted peptide of rBm86-CG; *Eco*R I: recognition sequencing of the enzyme used for cloning of 5´ region; Bm86-CG: gene sequencing of Bm86-CG inserted in the plasmid; *Not* I: recognition sequence of the enzyme used for cloning the 3' region; 3´AOX1 termination: sequence that determines the end of the transcript; HIS4 ORF: autotrophic selection factor; pBR322 origin: replication origin for *E. coli*; Ampicillin resistance: selection factor that confers resistance to ampicillin for *E. coli*. b) Schematic representation of mRNA transcribed by pPIC9BmCG. ATG: codon start; α-factor: corresponding sequence of peptide signal secretion of rBm86-CG; *Xho* I, *Sna*B I and *Eco*R I: multiple cloning site; Bm86-CG: gene sequence of Bm86-CG inserted in the plasmid; *Not* I: recognition sequence of the enzyme used for cloning of 3´ region; TAA: stop codon.

cells were selected on plates containing histidine-free MD agar (1.34% YNB; 4 × 10^{-5}% biotin; 2% dextrose; 0.15% agar) and were incubated for 7 days at 30 °C. The clones were seeded in 10 mL of YPD (1% yeast extract; 2% peptone; 2% dextrose) and incubated for 24 hours at 220 rpm at 30 °C. A 2 mL volume was centrifuged at 1,500 x g for 5 minutes, the supernatant was discarded and the cells were resuspended in 1 mL of YPDG (1% yeast extract; 2% peptone; 2% dextrose; 15% glycerol) and frozen at –80 °C. The remaining 8 mL were used for genomic DNA extraction.

4. Extraction of genomic DNA from clones

Genomic DNA was extracted using the glass bead method. A 1 mL pellet of the culture was resuspended in 150 µL of STES (2.42% Tris base; 2.92% sodium chloride; 0.1% SDS; 0.372% EDTA; pH 7.6), and 50 µL of glass beads (425-600 µm; Sigma-Aldrich, USA) and 150 µL of phenol: chloroform: isoamyl alcohol (12:12:1 v/v) were added to the resuspension, in a 1.5 mL microtube. The system was vortexed for 1 minute and centrifuged at 13,000 x g for 5 minutes. The supernatant was collected and precipitated with 3M potassium acetate at –20 °C for 1 hour. The tube was centrifuged at 13,000 x g for 20 minutes. The pellet was washed with 100 µL of 70% ethanol and left to dry at room temperature, after which it was eluted in 45 µL of sterile Milli-Q water and left overnight at 4 °C. DNA was quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) and stored at –20 °C.

5. Production of polyclonal sera

Polyclonal sera against Gavac™ and TickGard^{PLUS} vaccines, *R.* (*B.*) *microplus* larval extract and Bm86-CG recombinant protein (rBm86-CG) were produced using BALB/c mice. To obtain the

larval extract, 8 g of larvae were frozen at –20 °C and macerated using a mortar and pestle. To this, 150 mM Tris-HCl at pH 7.4 was then added to make up 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 were diluted in 15 mL of 150 mM Tris-HCl at pH 7.4 and emulsified in 15 mL of Freund's complete adjuvant (Sigma-Aldrich, USA). This yielded 30 mL of larval extract emulsion to be used as vaccine. The anti-rBm86-CG vaccine 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 (Sigma-Aldrich, USA). Twenty-four female BALB/c mice were used, which were separated into four groups of six animals. Each group received one of four vaccines at a concentration of 50 $\mu g.mL^{-1}.dose^{-1}$ (Gavac™, TickGard^{PLUS} or rBm86-CG) or 100 $\mu g.mL^{-1}.dose^{-1}$ (larval extract vaccine). Three intramuscular doses were applied at 21-day intervals. Sera were collected 21 days after the last dose.

Inoculation of mice with rBm86-CG resulted in production of antibodies that reacted with both the native version of the recombinant and the native versions of the antigen, thus showing antigenicity.

6. Screening for recombinants by dot blot

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 at 220 rpm at 30 °C, as were the GS115/albumin strain and one non-recombinant GS115 strain. After 24 hours of incubation, the cultures were centrifuged at 1,500 x g for 5 minutes, the supernatant was discarded, the cells were 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), and the cultures were returned to the shaker for another four days. To each culture, 0.5% absolute methanol was added every 24 hours. Finally, the cultures were centrifuged and the supernatants were treated with 1 mM PMSF and collected into 2 mL microtubes and frozen at –20 °C.

The PVDF membrane was washed for 10 seconds in methanol and for 5 minutes in sterile Milli-Q water. A 5 µL aliquot of the supernatant from each culture and, as positive controls, 5 µL each of Gavac™ and Tick Gard^{PLUS}, were pipetted onto a PVDF membrane and dried in an oven at 30 °C. The membrane was blocked with phosphate-buffered saline (PBS) containing 5% nonfat dried milk under stirring for 1 hour at 30 °C and subsequently washed twice (5 minutes each time) with PBS-T (PBS containing 0.1% Tween 80; pH 7.4). Next, the membrane was incubated under stirring for 1 hour at 30 °C with mouse-produced primary polyclonal antibodies, from animals vaccinated with Gavac™ or TickGard^{PLUS}, diluted 1:150 in PBS. This was followed by three washes with PBS-T, and by incubation under stirring for 1 hour at 30 °C with peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Sigma-Aldrich, USA), diluted 1:1000 in PBS-T. Finally, the material was washed another three times (5 minutes each) with PBS-T and incubated in 5 mL of developing solution (0.5% diaminobenzidine; 0.075% urea) until the appearance of coloration on the membrane, which was washed in sterile Milli-Q water and photodocumented.

7. Screening for Mut^{+} and Mut^{S}

Recombinant cells can be generated into two phenotypic classes: Mut^{+} and Mut^{S}. The Mut^{S} (methanol utilization slow) phenotype results from loss of activity of the alcohol oxidase caused by a mutation in the AOX1 (alcohol oxidase 1) gene.

8. Sequencing

The PCR products from genomic DNA of clone B (Mut^{+}) were sequenced with primers designed for the pAOX1 promoter, in an ABI 3100 automated sequencer using BigDye Terminator chemistry, in accordance with the manufacturer's instructions. The sequencing results were compared with the Bm86-CG using ClustalW software for sequence alignment.

9. Production of Bm86-CG

To determine the rBm86-CG expression, one colony from clone B (Mut^{+}) was isolated on plates containing solid MD medium. Colonies of the strains GS115/albumin and non-recombinant GS115 (Invitrogen, Australia) were also isolated as positive and negative controls respectively. The colonies were inoculated in a 250 mL Erlenmeyer flask containing 25 mL of BMGY medium, and incubated overnight in an orbital shaker at 220 rpm at 30 °C until reaching $OD_{600\ nm} = 4$, after approximately 16-18 hours. The cultures were subsequently centrifuged at 1,500 x g for 5 minutes at room temperature and the supernatants were discarded. The precipitates of clone B and GS115 were resuspended in 2 L of Erlenmeyer's solution with BMMY medium until reaching $OD_{600\ nm} = 1.0$, at an approximate volume of 200 mL. To each culture, 1% absolute methanol was added every 24 hours, for an induction period of 96 hours. The induction was maintained by addition of 1% methanol every 24 hours, for a period of 96 hours. After induction, the cultures were centrifuged at 3,300 x g at 4 °C for 5 minutes and the supernatants were separated, treated with 1 mM PMSF and frozen at –20 °C until quantification time.

10. Quantification of rBm86-CG

The supernatants from the material expressed by clones B and D were quantified by means of the standard curve method with bovine serum albumin (BSA) on 7.5% SDS-PAGE. BSA was previously prepared as a 1 $\mu g.\mu L^{-1}$ solution. 25 µL of this solution were added to 10 µL of SDS-PAGE sample buffer and 15 µL of sterile Milli-Q water, yielding 50 µL of 0.5 $\mu g.\mu L^{-1}$ BSA solution. Aliquots of 1, 2, 4, 6, and 8 µL of this solution were applied on 7.5% polyacrylamide gel, in quantities of 0.5, 1, 2, 3, and 4 $\mu g.\mu L^{-1}$, respectively, along with the samples to be quantified. The bands formed on the gel were quantified by visual analysis and also

using the TotalLab 2.0 software (Amersham Biosciences, United Kingdom), which also served to estimate the apparent molecular mass of bands.

11. SDS-PAGE and Western blot for rBm86CG

50 µL of both supernatants of clone B, clone D, GS115 and GS115/albumin were mixed with 10 µL of denaturing buffer 6x SDS-PAGE, separately. The samples were incubated for 6 minutes at 95 °C, left to cool down and pipetted (20 µL) on 7.5% polyacrylamide gel. Electrophoresis was run for 1.5 hour at 30 mA in a 2D electrophoresis system (Amersham Biosciences, United Kingdom). Western blot analysis was performed on material from a 7.5% polyacrylamide gel run with 10 µL of clone B supernatant. The protein was transferred from the gel to a Polyvinylidene fluoride (PVDF) membrane (pretreated with methanol for 10 seconds and sterile Milli-Q water for 5 minutes) using a transfer system (Amersham Biosciences, United Kingdom) immersed in transfer 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 an stove at 25 °C for 1 hour, stained with Ponceau S solution (0.5% Ponceau S; 1% glacial acetic acid), and cut into strips. The strips were blocked with PBS containing 5% nonfat dried milk at 30 °C for 1 hour under stirring, washed twice with PBS-T for 5 minutes and incubated with primary antibody at 1:150 in PBS at 30 °C for 1 hour under stirring (one strip for each type of serum: anti-Gavac, anti-TickGard, anti-larval extract and anti-rBm86-CG). They were subsequently washed twice for 5 minutes with PBS-T and separately incubated with peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Sigma-Aldrich, USA), 1:1000 in PBS-T at 30 °C for 1 hour under stirring. The strips were washed another three times for 5 minutes with PBS-T, rinsed in sterile Milli-Q water and developed (5 mL of Milli-Q water; 5 mg of DAB; 0.75 mg of NH_4) until well-defined bands (5-10 seconds) appeared on the PVDF membrane.

As a negative control, the supernatant from non-recombinant *P. pastoris* GS115 was used as the antigen in Western blotting with serum of mice immunized against Gavac vaccine.

12. Antigen preparation and animal vaccinations

The vaccine was prepared with rBm86-CG expressed in yeast, as reported above, by mixing the emulsified antigen with equal volumes of Montanide (ISA 61 VG; Seppic, Paris, France) using an ultra-homogenizer. Each vaccine consisted of 1 mL containing 100 µg of rBm86-CG combined with 1 mL of adjuvant.

A total of 12 month old dairy steers (82% cross-bred Friesian), without previous exposure to *R.* (*B.*) *microplus*, were used in this study. Each animal was randomly allocated to an immunized or a control group of six cattle each. The vaccinated animals were immunized with rBm86-CG and the negative control group was injected with phosphate-buffered saline (PBS). The animals were housed in pens and were fed concentrates daily, and provided with hay and water throughout the study. Blood samples were collected weekly. The cattle were inoculated intramuscularly three times at two-week intervals.

13. Enzyme-linked immunosorbent serological assay (ELISA)

Sera from each group were collected weekly and antibody levels were determined by means of ELISA with rBm86-CG. Microtiter plates were coated with 100 ng of rBm86-CG per well in 100 mM carbonate buffer (pH 9.8), washed three times at each step with PBS containing 0.05% (v/v) Tween-20, blocked with 0.5% skimmed milk powder diluted in PBS, and the antisera used as the primary antibody was diluted 1:500. Horseradish peroxidase-conjugated rabbit anti-bovine IgG (SIGMA, Steinheim, Germany) was diluted 1:5000 to serve as a secondary antibody, and antigen-antibody complexes were detected with 4.10^{-4}% o-phenylenediamine (OPD; SIGMA, Steinheim, Germany) and 0.1% H_2O_2, before optical densities at 490 nm were measured. Additional controls without antigen, primary antibody, secondary antibody or substrate were read to ensure that the colorimetric reaction was because of the formation of antigen-antibody complexes and not because of non-specific reactions.

Anti-rBm86-CG IgG titers were determined by means of ELISA as described above. Titers from individual animals were expressed as the maximum dilution having an $OD_{492\ nm}$ higher than two times the average from a seronegative bovine.

To analyze the antibody response results from vaccination experiments in the pen trial, mean antibody levels were compared using an ANOVA test ($p < 0.01$). All analyses were carried out using the SAS software v. 9.1.

Results

1. Construction of pPIC9BmCG

Recombinant plasmid was built by inserting the Bm86-CG protein gene sequence into the multiple cloning site of the pPIC9 plasmid, between the *Eco*R I and *Not* I restriction enzymes (Figure 1). The sequence of the insert was confirmed by sequencing and alignment with Bm86-CG (GenBank accession no. EU352677.1), with 100% identity. The sequence was cloned taking into account the expression cassette controlled by the AOX1 gene promoter. The multiple cloning was maintained so as to precede the Bm86-CG sequence, thus allowing other sequences and subsequent cloning (e.g. a sequence modulating the immune response). The absence of a purification system in this plasmid precluded performing affinity chromatography to purify the protein.

2. AOX1 gene mutation analysis

The plasmid cloned into *P. pastoris* was previously linearized with the *Sac*I enzyme to allow for two types of integration into the yeast genome, thus resulting in two possible phenotypes: Mut^s, with AOX1 gene mutation; and Mut^+, with the conserved gene. Clonal variation requires testing of recombinants for both phenotypes, since their capabilities to express the recombinant protein cannot be compared beforehand. In the present study,

the clone for phenotype Mut$^+$ expressed recombinant protein in greater amounts than did the clone for Mut^s.

3. rBm86-CG protein expression

Clone B (Mut$^+$) was selected and induced to expression. Analysis of the supernatants on 7.5% SDS-PAGE for protein expression revealed bands of approximately 80 kDa (Figure 2). The expected molecular weight of 69.1 kDa for the rBm86-CG protein expressed in this system was calculated using CLC Viewer 6.0.2 software. The difference can be explained by the fact that rBm86-CG has a glycosylation site in its amino acid sequence, with a resultant larger molecular weight on SDS-PAGE.

4. Expression quantification

Direct quantification from culture supernatants revealed rBm86-CG production levels of 62.5 and 32 mg.L^{-1} with regard to Mut$^+$ and Mut^s, respectively.

5. Western blotting

Western blot analysis on the rBm86-CG protein was carried out using four primary sera from mice: anti-Gavac™, anti-TickGard^{PLUS}, anti-*R.* (*B.*) *microplus* larval extract and anti-rBm86-CG. It revealed affinity for a protein of approximately 80 kDa (Figure 3). Western blot using the larval extract as an antigen and anti-rBm86-CG as a bovine antibody revealed that immunized bovine with r-BmCG recognized native antigen with the expected protein size (Figure 3).

6. Enzyme-linked immunosorbent serological assay

Before the first immunization, serum samples were assayed by means of ELISA for anti-rBm86-CG antibodies and were found to be seronegative. Specific antibody levels in sera from immunized animals increased one week after the first immunization, while sera from controls remained negative (Figure 4). Antibody levels increased one week after the second inoculation, in all the immunized animals. A better response was observed in week 8, which occurred after a small decline. The trends of specific antibody levels increased after every inoculation. However, sera from rBm86-CG-immunized cattle demonstrated the highest level of antibodies for rBm86-CG throughout the study, and no significant titers to rBm86-CG were observed among the control group sera.

Following the immunization using the rBm86-CG-based vaccine (in weeks 0, 2 and 4), all the experimental animals developed a strong and specific humoral immune response characterized by high anti-rBm86-CG IgG titers. Specific antibody titers were in all cases over 1:640. However, in the eighth week, specific antibody titers were over 1:2560.

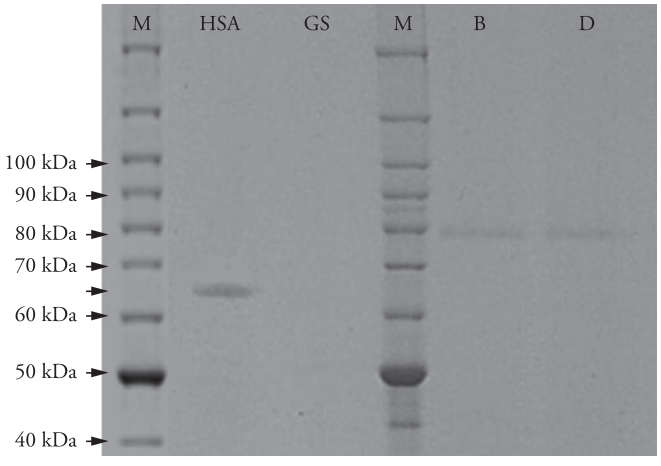

Figure 2. Analysis on recombinant proteins using SDS-PAGE: 7.5% of supernatants from clone B (Mut$^+$) and clone D (Mut^s). HSA: supernatant GS115/albumin; GS: supernatant GS115; M: BenchMark™ Protein Ladder standard for SDS-polyacrylamide gel electrophoresis ranging in molecular weight from 10 to 220 kDa. B: supernatant of the expression of clone B; D: supernatant of the expression of clone D.

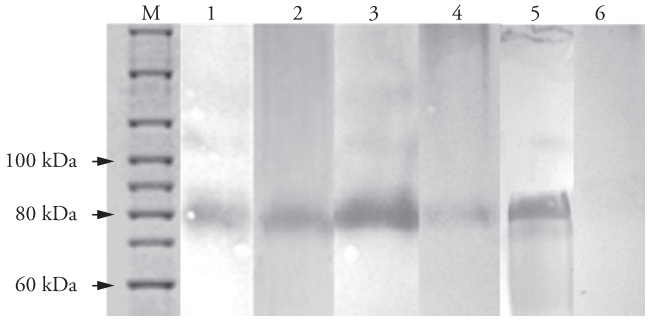

Figure 3. Western blot gel of rBm86-CG expressed by clone B. 1: anti-Gavac serum; 2: anti-TickGard serum; 3: anti-larval extract serum; 4: anti-rBm86-CG serum; 5: larval extract as antigen recognized with bovine serum immunized with rBm86-CG; 6: Negative control. M: BenchMark™ Protein Ladder standard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

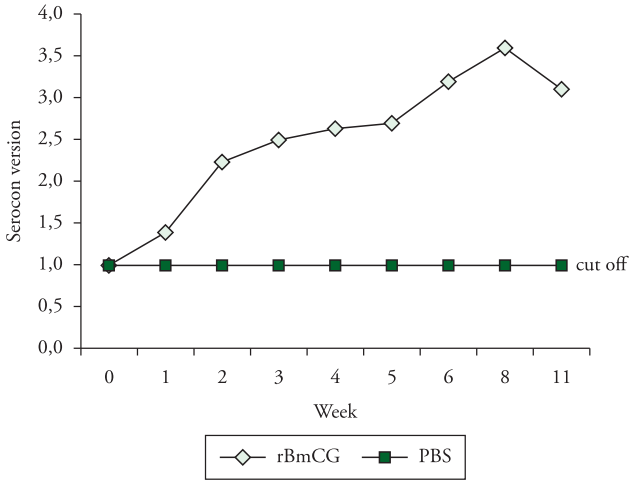

Figure 4. Recognition of rBm86-CG by antisera from cattle immunized with antigens. Seroconversion from ELISA tests of weekly antisera samples collected from two groups of six cattle immunized at 0, 2 and 4 weeks with rBm86-CG (dark line) and phosphate-buffered saline (light line).

Discussion

The system of expression in *P. pastoris* selected for this investigation is currently efficient for producing heterologous proteins, requires fewer procedures, offers lowering production costs and is also superior to prokaryotic systems in relation to its capacity to express proteins in their correct three-dimensional structure.

A pPIC9BmCG plasmid was successfully constructed and proved capable of effectively expressing the recombinant rBm86-CG protein. Since latter is soluble in the culture medium, it can easily be obtained by precipitation from the supernatant, which will facilitate the purification process and thus lead to a reduction in the production cost.

In addition to being simpler than purification from cell membranes or cytoplasm, the method preserves the antigenic and immunogenic properties of the expressed protein, which is important for producing an effective immunogen.

SDS-PAGE showed a well-defined majority band of approximately 80 kDa, corresponding to rBm86-CG, which should weigh 69.1 kDa (as calculated using the CLC Viewer 6.0.2 software). However, this value does not take into account eukaryotic protein modification such as glycosylation, disulfide bond formations and proteolytic processing.

Recognition of rBm86-CG by the anti-Gavac™ serum revealed that the system was capable of expressing a recombinant protein that had similar antigenic properties and was recognizable by antibodies generated by this vaccine. Considering that the Bm86 antigen contained in Gavac™ was also expressed in *P. pastoris*, sera produced by the TickGard^{PLUS} vaccine served as controls. Since their antigen was produced in *E. coli*, they failed to produce antibodies against *P. pastoris*, thus confirming that the protein revealed on Western blotting is not a product of the yeast. Western blot analysis demonstrated that Bm86-CG expressed in *P. pastoris* was recognized by antibodies, since it was recognized by antibodies generated by both the TickGard and the Gavac vaccine. One particularly important finding is that rBm86-CG was recognized by the antibodies against the larval extract vaccine, which contains native Bm86-CG. This finding confirms that a recombinant protein immunogenically similar to native Bm86-CG was expressed, thus suggesting that the recombinant form is a potential antigen for use in cattle vaccines. Recognition of rBm86-CG by anti-rBm86-CG sera shows that the protein was capable of inducing an immune response. Analysis of the membrane revealed that there were no responses to other possible proteins, since the vaccine was prepared with the complete expressed supernatant.

The expression system selected for this investigation allowed rBm86-CG to be produced in vitro at a concentration of 62.5 mg.L^{-1}, which is an improvement in comparison with another study, in which the expression levels ranged between 1.0 and 6.0 mg.L^{-1}, thus representing 1.5 to 13.2% of the total proteins in the supernatant. Nevertheless, in this study, when rBm86-CG was subjected to fermentation, the expression level rose initially to 150 mg.L^{-1}, thus representing at least a 50-fold increase (CANALES et al., 2008). The highest published protein production in *P. pastoris* was 1.5 g.L^{-1}, in a fermenter (RODRÍGUEZ et al., 1994)

The goal of using a eukaryotic expression system to produce a regional antigen with a potential for use in vaccines was thus achieved in the present investigation.

The anti-rBm86-CG antibody levels in cattle immunized with rBm86-CG were significantly higher than the levels in the cattle. No specific antibodies against the rBm86-CG immunogen were detected in pre-immunization serum ($p < 0.01$). These results indicated that rBm86-CG-based vaccine was immunogenic and induced production of antibodies that recognized rBm86-CG.

It has now been demonstrated that certain amounts of antibodies against rBm86 correlate with damage to ticks engorging on immunized cattle (DE LA FUENTE et al., 1998; VARGAS et al., 2010). Previous experiments have also suggested that a damaging effect mediated by specific antibodies can be expected in these ticks engorging on immunized cattle, with a titer cutoff of 1:640 (DE LA FUENTE et al., 1998).

The antibody responses in vaccinated animals indicated that this phase of the study was successful, and that vaccines formulated with rBm86-CG are capable of eliciting high antibody titers in cattle.

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