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Multi-antigenic vaccine against the cattle tick *Rhipicephalus* (*Boophilus*) *microplus*: A field evaluation

Luís F. Parizi[a,1], José Reck Jr.[a,b,h,1], Daiane P. Oldiges[a], Melina G. Guizzo[a], Adriana Seixas[a,c,h], Carlos Logullo[d,h], Pedro L. de Oliveira[e,h], Carlos Termignoni[a,f,h], João R. Martins[b,h], Itabajara da Silva Vaz Jr.[a,g,h,*]

[a] *Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, Prédio 43421, Campus do Vale, Caixa Postal 15005, Porto Alegre, RS 91501-970, Brazil*
[b] *Laboratório de Parasitologia, Instituto de Pesquisas Veterinárias Desidério Finamor, Fundação Estadual de Pesquisa Agropecuária, Estrada do Conde, 6000, Eldorado do Sul, RS 92990-000, Brazil*
[c] *Departamento de Ciências Básicas da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre, Rua Sarmento Leite, 245, Porto Alegre, RS 90050-170, Brazil*
[d] *Laboratório de Química e Função de Proteínas e Peptídeos and Unidade de Experimentação Animal, Universidade Estadual do Norte Fluminense "Darcy Ribeiro", Av. Alberto Lamego, 2000, Horto, Campos dos Goytacazes, RJ 28015-20, Brazil*
[e] *Instituto de Bioquímica Médica, Programa de Biotecnologia e Biologia Molecular, Universidade Federal do Rio de Janeiro, Cidade Universitária, Rio de Janeiro, RJ 21941-590, Brazil*
[f] *Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, R. Ramiro Barcelos, 2600, Porto Alegre, RS 90035-003, Brazil*
[g] *Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9090, Porto Alegre, RS 91540-000, Brazil*
[h] *Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular, Brazil*

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ABSTRACT

The tick *Rhipicephalus* (*Boophilus*) *microplus* is a blood-sucking ectoparasite of cattle that severely impairs livestock production. Studies on tick immunological control address mostly single-antigen vaccines. However, from the commercial standpoint, so far no single-antigen vaccine has afforded appropriate protection against all R. microplus populations. In this context, multi-antigen cocktails have emerged as a way to enhance vaccine efficacy. In this work, a multi-antigenic vaccine against *R. microplus* was analyzed under field conditions in naturally infested cattle. The vaccine was composed by three tick recombinant proteins from two tick species that in previous single-vaccination reports provided partial protection of confined cattle against R. microplus infestations: vitellin-degrading cysteine endopeptidase (VTDCE) and boophilus yolk pro-cathepsin (BYC) from *R. microplus*, and glutathione S-transferase from *Haemaphysalis longicornis* (GST-Hl). Increased antibody levels against three proteins were recorded after immunizations, with a distinct humoral immune response dynamics for each protein. Compared to the control group, a statistically significant lower number of semi-engorged female ticks were observed in vaccinated cattle after two inoculations. This reduction persisted for 3 months, ranging from 35.3 to 61.6%. Furthermore, cattle body weight gain was significantly higher in vaccinated animals when compared to control cattle. Compared to the single-antigen vaccines composed by VTDCE, BYC or GST-Hl, this three-antigen vaccine afforded higher protection levels against *R. microplus* infestations.

1. Introduction

The tick *Rhipicephalus* (*Boophilus*) *microplus* has a significant economic impact on cattle breeding industry worldwide, estimated at billions of dollars annually [1,2]. This parasite causes a variety of deleterious effects in cattle, mainly as result of bodyweight reduction, blood loss and the transmission of disease-causing agents [1,2]. The intensive use of acaricides in order to control tick infestation raises concerns as to the potential presence of pesticide residues in milk, meat, and the environment [3]. For these reasons, a tick vaccine, as an alternative control method, is a major economic issue [4,5].

It has been repeatedly demonstrated that the stimulation of bovine immune system by tick proteins vaccination induces a

* Corresponding author at: Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Prédio 43421, Campus do Vale, Caixa Postal 15005, Porto Alegre, RS 91501-970, Brazil. Tel.: +55 51 3308 6078; fax: +55 51 3308 7309.
E-mail addresses: luisfparizi@cbiot.ufrgs.br (L.F. Parizi), jose.reck@gmail.com (J. Reck Jr.), daioldiges@hotmail.com (D.P. Oldiges), meligarcia@cbiot.ufrgs.br (M.G. Guizzo), adriana@cbiot.ufrgs.br (A. Seixas), carloslogullo@yahoo.com.br (C. Logullo), pedro@bioqmed.ufrj.br (P.L. de Oliveira), ctermignoni@cbiot.ufrgs.br (C. Termignoni), joaorsm@terra.com.br (J.R. Martins), itabajara.vaz@ufrgs.br (I. da Silva Vaz Jr.).
[1] These authors contributed equally to the work.

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protective immune response against *R. microplus* [6]. In 1986, a protective protein from *R. microplus* named Bm86 was discovered, when this antigen became the first tick antigen to compose a commercial vaccine against an ectoparasite [7]. Although vaccine formulations based on Bm86 in most cases elicit protective immune responses against *R. microplus*, they vary considerably in terms of protection level depending, among other things, on the genetic variability of tick and bovine populations [8–13]. Therefore, the discovery of new tick antigens focusing on those displaying minimal genetic variability among *R. microplus* populations could improve vaccination efficacy and reduce variation in the protection level afforded by the Bm86-based vaccines. However, except for a few studies [14], data regarding cross-reactivity between tick proteins are scarce, although some tick antigens have been shown to induce cross-protective immunity against some tick species [14,15]. Another strategy to enhance anti-tick vaccine efficacy is to combine two or more antigens [16]. The initial proof of concept supporting this approach came from vaccination experiments, in which mixtures of antigens were more efficacious than single components, including Bm86 [4]. Some experimental studies used this approach against tick infestations [16–23]; however, in most cases, this strategy resulted in a statistical significant but slightly improvement in protection level.

Although tick infestation experiments using bovines in confined indoors can indicate vaccine efficacy, field trials are necessary to evaluate vaccine performance under real husbandry conditions [24]. However, most of the protocols used in experiments to evaluate bovine vaccination against ticks employ confined bovines, a more practical and cost-saving approach, compared to field experiments which demand laborious handling of cattle and the availability of a large area [16,25]. Our research group has been studying several *R. microplus* molecules in order to find antigens that could be used in an anti-tick vaccine. In previous studies, immunizations of cattle with native or recombinant forms of an aspartic protease named *Boophilus*Yolk pro-cathepsin (BYC) induced overall protections (measured by the reproductive potential, including reduction in number and weight of engorging ticks and in egg weight and hatchability) around 30% [26,27]. Also, immunization with a *R. microplus* cysteine endopeptidase (VTDCE), involved in vitellin digestion [28,29], elicited an immunoprotection of 21% in vaccinated cattle [30]. More recently, an overall protective efficacy of 57% against *R. microplus* was achieved using a recombinant *Haemaphysalis longicornis* GST (rGST-Hl) [31]. In this work, we evaluated a multi-antigenic vaccine composed by BYC, VTDCE and GST-Hl recombinant proteins against *R. microplus* infestation in cattle. Vaccine efficiency was evaluated under field conditions, based on semi-engorged female tick numbers and weight gain differences between vaccinated and control cattle groups.

2. Materials and methods

2.1. Expression of the recombinant proteins

rGST-Hl, rBYC, and rVTDCE were expressed and purified as previously described [32–34]. Briefly, rBYC and rGST-Hl were expressed in *Escherichia coli* strain AD494 (DE3) pLysS. Recombinant VTDCE was expressed in *E. coli* strain BL21 (DE3) Star. The insoluble forms of rBYC and rVTDCE were solubilized with 6 M guanidine hydrochloride (GuHCl) and purified using a nickel-chelating Sepharose column (GE Healthcare, Uppsala, Sweden). The soluble form of recombinant GST-Hl was purified through affinity chromatography using GSTrap FF column (GE Healthcare, Uppsala, Sweden). Protein concentrations were determined by the Bradford method [35] and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using bovine serum albumin as standard.

2.2. Animals

From September 2009 to January 2010 (spring to summer), a total of 38 Aberdeen Angus and Devon cattle heads (15 ± 1-month-old) the beginning of the trial were maintained under field conditions in a experimental farm (Estação FEPAGRO São Gabriel, São Gabriel, RS, Brazil; 30°20′S, 54°15′W). Cattle were allowed to graze freely on natural pastures, characterized by annual grass species, and supplemented with mineral salt, receiving water *ad libitum*. All animals were treated with levamisole (600 mg/100 kg body weight) three times (days 22, 43 and 64) to avoid endoparasite infestations along the vaccine trial, and managed under identical conditions in the same paddock during the whole trial. Cattle were managed in accordance with local institutional guidelines and all procedures were in accordance with international guidelines [36].

2.3. Immunization protocols

Vaccinated and control groups were formed by 18 and 20 animals, respectively. Antigens were administered subcutaneously. Each dose consisted of a mixture of recombinant proteins rBYC, rGST-Hl and rVTDCE (200 μg each, 0.5 mL) mixed with 0.5 mL of adjuvant (Montanide 888 and Marcol 52), emulsified according to the vortex method [37]. The control group received an emulsion of PBS (0.5 mL) plus adjuvant (0.5 mL). Both groups received three booster injections at 21-day intervals (days 22, 43, and 64).

2.4. Cattle sera collections and body weight

Blood samples (10 mL) were collected via caudal vein from pre-immunized and post-immunized cattle (days 1, 78 and 127), and used for sera recovery. Blood samples were centrifuged at $5000 \times g$ for 10 min and sera were stored at −20 °C. At days 1 and 127, all bovines were weighted.

2.5. SDS-PAGE and Western blot

SDS-PAGE and Western blot analysis were performed as previously described [31]. Purified recombinant proteins (1 μg protein/lane) were applied to SDS-PAGE (14% gel). For Western Blot, the nitrocellulose membranes were incubated with cattle sera (diluted 1:100) collected on days 1 and 78.

2.6. Antigen-specific IgG detection in sera by dot-blot

Levels of antigen-specific antibodies in the serum samples were assessed by dot-blot. Nitrocellulose membrane circles of 0.5 cm of diameter were coated with 1 μg of each antigen in PBS. The membranes were dried and incubated for 1 h at 37 °C with blotto [38], followed by a second incubation with cattle sera diluted in blotto (1:100) for 16 h at 37 °C. Washing times with blotto for 10 min ensued, and the peroxidase conjugated antibody diluted in blotto (1:5000) was added and incubated for 1 h at 37 °C. After three washes with PBS for 10 min, the membranes were incubated with 2.5 mg 3,3′-diaminobenzidine tetrahydrochloride, 10 μL H_2O_2, and 150 μL $CoCl_2$ in 5 mL of PBS. The recognition levels were quantified by gel scanning, and were analyzed using the software Image J [39].

2.7. Tick analysis

Along the vaccination trial, bovines were continuously exposed to tick infestation (since the beginning of the immunization process) because they were under natural conditions in a tick-infested pasture. Attached adult female ticks (sized between 4.5 mm and 8.0 mm) were counted on the left side of vaccinated and control groups, to follow the tick infestation rate [40]. Animals were

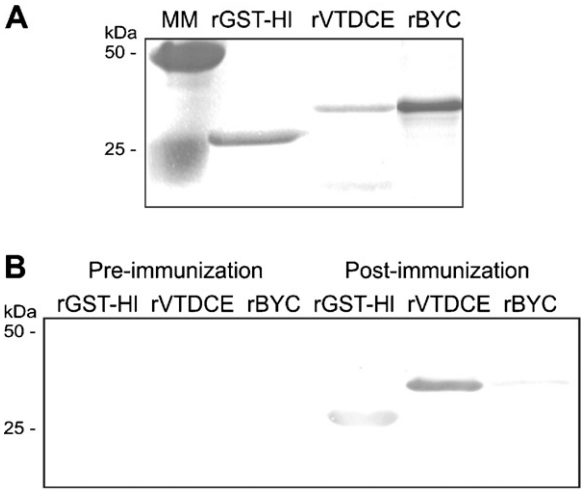

Fig. 1. Purity and antigenicity of recombinant GST-Hl, VTDCE and BYC proteins, analyzed by 14% SDS-PAGE stained with Coomassie blue G-250 (A) and Western blot (B). Western blot was probed with the pre-immunized and post-immunized sera from one bovine belonging to vaccinated group. Molecular mass standards are expressed as kDa.

immobilized and ticks were counted by the same investigator. All examinations were carried out at the same period of the day (morning/afternoon). Tick count was a blind-procedure: the investigator did not know which bovines belonged to control or vaccinated groups.

2.8. Statistical analysis

Statistical significance differences among the experimental groups concerning level of antigen-specific antibodies, tick count and cattle body weight gain was analyzed by Student's *t* test. Data were expressed as mean ± S.E.M. of each group. A p value of less than 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, USA) software.

3. Results

3.1. Production of recombinant proteins

The recombinant proteins BYC, GST-Hl and VTDCE were expressed in *E. coli* strains and purified by affinity chromatography. The purity of the three recombinant proteins was analyzed by a 14% SDS-PAGE (Fig. 1A). All preparations showed a major protein band for rBYC, rGST-Hl, and rVTDCE in the gel, and these bands matched the predicted molecular masses for respective proteins.

3.2. Development of humoral immune response in cattle

Dot blot analysis revealed an increased antibody recognition level of vaccinated bovine sera (collected at day 78) to the three recombinant proteins, compared to the vaccinated bovine pre-immune sera (day 1) (Fig. 2). Compared to day 1, the level of recognition from vaccinated cattle sera on day 78 for rGST-Hl, rVTDCE and rBYC increased by more than 6, 10, and 2 times, respectively. The level of recognition remained constant at the end of the experiment (day 127) for rGST-Hl, reducing by half for rVTDCE, and returning to pre-immunization level for rBYC. Also, the level of recognition measured from vaccinated cattle sera was approximately 8, 4, and 2.5 times higher for rGST-Hl, rVTDCE, and rBYC respectively, than those recorded from animals injected with placebo on day 78.

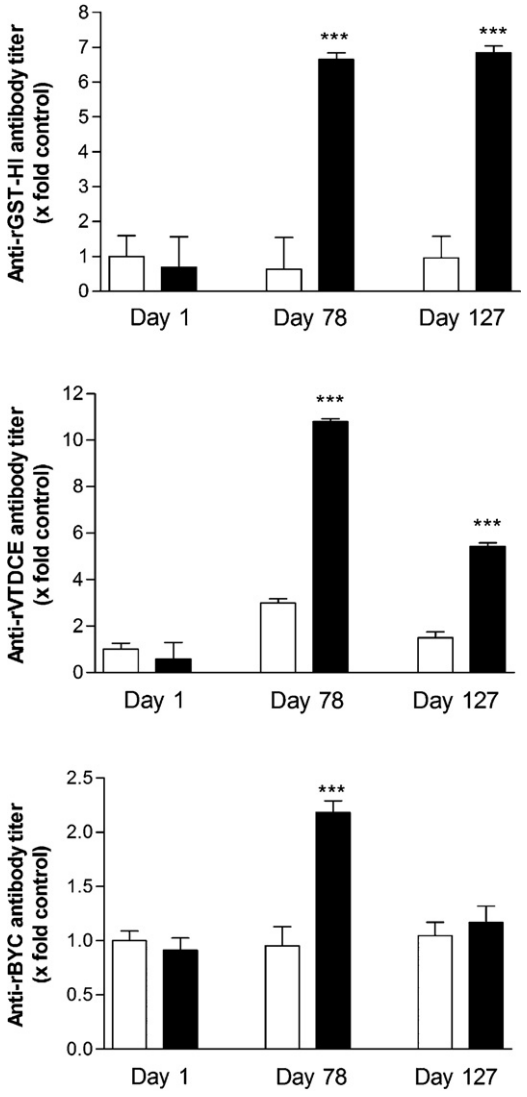

Fig. 2. Antigens-specific IgG levels from cattle sera analyzed by dot-blot. The levels of recognition of pre-immunized (day 1) and post-immunized (days 78 and 127) cattle sera from vaccinated and control groups were analyzed for recombinant proteins GST-Hl (A), VTDCE (B) and BYC (C). ***$p < 0.001$ (Student's *t*-test).

Western blot revealed that sera from one representative bovine of the vaccinated group recognize all recombinant proteins (Fig. 1B). The proteins rBYC, rGST-Hl and rVTDCE were not recognized by pre-immune serum of this animal.

3.3. Vaccination effect on tick infestation and body weight

The reduction in the number of ticks attached to bovines conferred by immunization with rBYC, rGST-Hl and rVTDCE is shown in Fig. 3 and Table 1. In the first three counts, tick number means from both groups were similar. From the fourth count on (days 36–127), means in the two groups were statistically different, except for day 57. During this period, bovines vaccinated with recombinant proteins showed statistical reductions that ranged from 35.3 to 61.6% (Table 1) in the number of semi-engorged ticks, as compared with the control group. Interestingly, even before the immunization period had ended it was already possible to detect a drop in tick infestation (Fig. 3, day 36). Also, there was an increase in cattle body weight in both groups between days 1 and 127, although the gain was statistically higher in the vaccinated group (Fig. 4). In

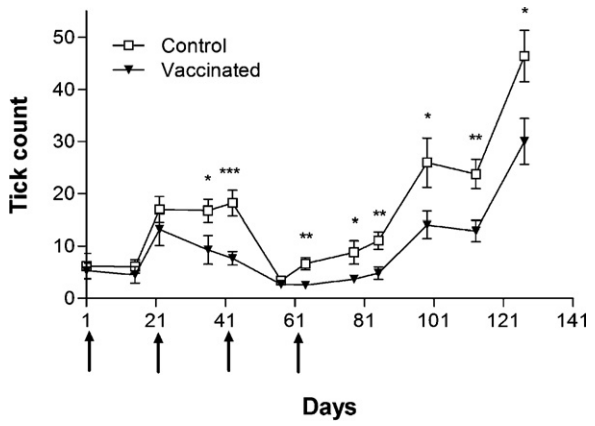

Fig. 3. Kinetics of the average numbers of semi-engorged ticks in the vaccinated and control groups. Arrows indicate the days of immunization. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's *t*-test).

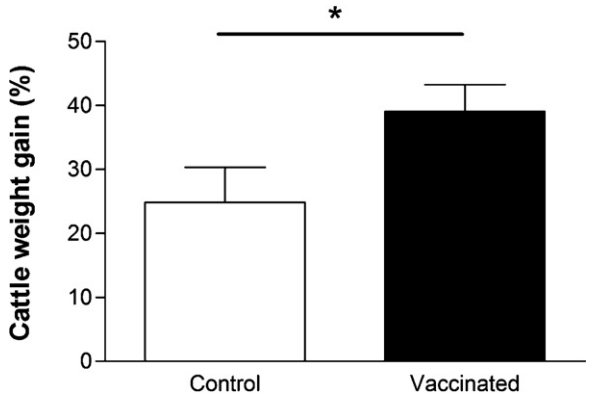

Fig. 4. Body weight gain in cattle along the vaccination trial. Cattle body weight gain in vaccinated group and control groups is expressed as percentage of mean body weight gain from day 1 to day 127. $^{*}p < 0.05$ (Student's *t*-test).

the vaccinated and control cattle groups, body weight gain was 39% and 25%, respectively.

4. Discussion

Tick vaccines derived from the gut antigen Bm86 have been extensively investigated in the quest for a suitable tick control method. This antigen was shown to be partially protective against *R. microplus* field infestations in Australia, Cuba, and in some regions of Argentina, Brazil, Mexico and other countries [12,13,24,41,42]. These and other studies provide proof of concept for anti-arthropod vaccines. Nevertheless, following the commercialization of Bm86-based vaccines, a considerable body of results challenged the initial optimism that Bm86 would be effective against all *R. microplus* populations [24,43,44]. Consequently, there is a need to enhance the efficacy of the available tick vaccines as well as to develop new ones against other tick species, especially of medical and veterinary importance. Several antigens are currently under field investigation [14,45,46], though so far no single antigen has been found to achieve the desired protection threshold against all tick populations under field conditions [14,45]. To increase the field performance of anti-tick vaccine candidates, it is theoretically possible to design a multi-component vaccine, a concept that has already been shown to work against other parasites [16,47,48]. Theoretically, vaccines composed of synergistic antigens could elicit more effective responses against ticks [16]. However, limited studies reporting comprehensive evaluation of the performance of tick antigens cocktails against tick infestation have been published [16–23].

Table 1
Effect of cattle vaccination on the number of semi-engorged *R. microplus* females.

Day	Tick count [a]		
	Control group	Vaccinated group	Difference (%) [b]
1	6.1 ± 2.4	5.3 ± 1.4	14.2
15	6.1 ± 1.3	4.5 ± 1.6	26.2
22	17.0 ± 2.4	13.2 ± 3.1	22.2
36	16.7 ± 2.3	9.3 ± 2.7	44.6*
43	18.2 ± 2.5	7.7 ± 1.3	57.9***
57	3.4 ± 0.6	2.7 ± 0.6	21.6
64	6.6 ± 1.2	2.6 ± 0.5	61.6**
78	8.8 ± 2.2	3.7 ± 0.6	58.3*
85	11.0 ± 1.7	4.8 ± 1.2	56.3**
99	25.9 ± 4.7	14.1 ± 2.6	45.8*
113	23.7 ± 2.8	12.9 ± 2.0	45.7**
127	46.4 ± 4.9	30.0 ± 4.4	35.3*

[a] Measured by counting the number of semi-engorged female ticks on the animals (average values ± S.D.).
[b] Difference (%) = 100 × [1 − (vaccine/control)].
* $p < 0.05$ (Student's *t*-test).
** $p < 0.01$ (Student's *t*-test).
*** $p < <0.001$ (Student's *t*-test).

In the present work demonstrated that these three recombinant proteins are immunogenic in cattle when administered simultaneously, although differences in immune response dynamics occur between antigens. In agreement with previous studies [27,30,31], we found that rGST-Hl elicited a more persistent humoral response than rBYC and rVTDCE. Immunization with the three recombinant proteins together induced a partial protective immune response in the experimental animals, evidenced by a decrease in the number of female ticks feeding on the vaccinated animals, in comparison with the control group. The number of females feeding on the hosts was statistically different between the two groups 14 days after the second immunization, and remained lower in the vaccinated group until the last day of the experiment (days 36–127). During days 43–85, vaccination conferred a statistically significant protection against tick infestation, ranging from 56.3 to 61.6%. However, the protection decreased to 35.3% two months after the last booster, along a decrease in antibody levels to rBYC and rVTDCE, suggesting the importance of these antibodies in protection rates obtained in previous counts.

The reduction in tick infestation following immunization with the three proteins is directly correlated with cattle body weight gain. Actually, body weight signals cattle fitness, a major productive parameter that is used as an indicator of vaccine effectiveness in field trials [1,41,42]. Under experimental conditions, body weight gain was significantly higher in vaccinated animals than in the control group. This effect seems to be a result of reduction in cattle damage by parasitism due to blood loss caused by the attaching ticks, and consequently, an improving in the overall health of the cattle.

In sum, the immune response generated by simultaneous vaccination with rGST-Hl, rBYC, and VTDCE affects tick physiology,

The proteins selected as antigens in this study play crucial physiological roles in ticks, such as vitellin mobilization (BYC and VTDCE) [28,29,49] and detoxification (GST) [50,51]. Indeed, previous studies demonstrated that these antigens, when administered in a mono vaccine, induce partial protective immune responses [27,30,31]. In these studies, the biological parameters evaluated to analyze tick control were the number of fully engorged ticks, egg laying capacity, and egg fertility, while the main parameter affected in ticks fed on vaccinated cattle was the number of fully engorged ticks, although the other parameters investigated were also affected, improving overall protection. These studies also demonstrated the immunogenicity of rGST-Hl, rBYC, and VTDCE and confirmed that specific IgG were elicited in vaccinated cattle for these proteins.

decreasing the number of females feeding in the host, resulting in an improved body weight gain of cattle. When compared to rGST-Hl, rBYC, or VTDCE single-antigenic vaccination in confined cattle, the multi-antigenic vaccine produced higher protection against R. microplus infestation. In spite of the differences between the vaccination protocols, these results demonstrate the possibility of developing a cattle multi-antigenic vaccine against *R. microplus* that seems to be more effective than a single antigenic vaccine against tick infestation under natural field conditions. More work is necessary to evaluate the economic benefits of a multi-antigen or a single-antigen vaccine to control ticks. However, the use of such vaccine, associated with existent and/or available control methods could result in a more efficient control of *R. microplus*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.08.078.

References

[1] Jonsson NN. The productivity effects of cattle tick (*Boophilus microplus*) infestation on cattle, with particular reference to *Bos indicus* cattle and their crosses. Vet Parasitol 2006;137:1–10.
[2] de la Fuente J, Estrada-Pena A, Venzal JM, Kocan KM, Overview Sonenshine DE. Ticks as vectors of pathogens that cause disease in humans and animals. Front Biosci 2008;13:6938–46.
[3] Henrioud AN. Towards sustainable parasite control practices in livestock production with emphasis in Latin America. Vet Parasitol 2011;180:2–11.
[4] Willadsen P. Anti-tick vaccines. Parasitology 2004;129:367–87.
[5] Seixas A, Oliveira P, Termignoni C, Logullo C, Masuda A, da Silva Vaz Jr I. *Rhipicephalus* (*Boophilus*) *microplus* embryo proteins as target for tick vaccine. Vet Immunol Immunopathol 2012;148:149–56.
[6] Akhtar M, Muhammad F, Lodhi LA, Hussain I, Anwar MI. Immunity against ticks––a review. Pak Vet J 2011;31:9–16.
[7] Willadsen P, Mckenna RV, Riding GA. Isolation from the cattle tick, *B. microplus*, of antigenic material capable of eliciting a protective immunological response in the bovine host. Int J Parasitol 1988;18:183–9.
[8] Andreotti R, Pedroso MS, Caetano AR, Martins NF. Comparison of predicted binders in *Rhipicephalus* (*Boophilus*) *microplus* intestine protein variants Bm86 Campo Grande strain, Bm86 and Bm95. Rev Bras Parasitol Vet 2008;17:93–8.
[9] Ben Said M, Galai Y, Canales M, Nijhof AM, Mhadhbi M, Jedidi M, et al. Hd86, the Bm86 tick protein ortholog in *Hyalomma scupense* (syn. *H. detritum*): expression in *Pichia pastoris* and analysis of nucleotides and amino acids sequences variations prior to vaccination trials. Vet Parasitol 2012;183:215–23.
[10] Freeman JM, Davey RB, Kappmeyer LS, Kammlah DM, Olafson PU. Bm86 midgut protein sequence variation in South Texas cattle fever ticks. Parasite Vectors 2010;3:101.
[11] Penichet M, Rodriguez M, Castellano O, Mandado S, Rojas Y, Rubiera R, et al. Detection of Bm86 antigen in different strains of *Boophilus microplus* and effectiveness of immunization with recombinant Bm86. Parasite Immunol 1994;16:493–500.
[12] Rodriguez M, Massard CL, da Fonseca AH, Ramos NF, Machado H, Labarta V, et al. Effect of vaccination with a recombinant Bm86 antigen preparation on natural infestations of *Boophilus microplus* in grazing dairy and beef pure and cross-bred cattle in Brazil. Vaccine 1995;13:1804–8.
[13] Rodríguez M, Penichet ML, Mouris AE, Labarta V, Luaces LL, Rubiera R, et al. Control of *Boophilus microplus* populations in grazing cattle vaccinated with a recombinant Bm86 antigen preparation. Vet Parasitol 1995;57:339–49.
[14] Parizi LF, Githaka NW, Logullo C, Konnai S, Masuda A, Ohashi K, da Silva Vaz Jr. Universal vaccine against ticks: cross-immunity insights. Vet J; http://dx.doi.org/10.1016/j.tvjl.2012.05.023, in press.
[15] Kumar B, Azhahianambi P, Ray DD, Chaudhuri P, de la Fuente J, Kumar R, et al. Comparative efficacy of rHaa86 and rBm86 against *Hyalomma anatolicum anatolicum* and *Rhipicephalus* (*Boophilus*) *microplus*. Parasite Immunol 2012;34:297–301.
[16] Willadsen P. Antigen cocktails: valid hypothesis or unsubstantiated hope. Parasitol Today 2008;24:164–7.
[17] Willadsen P, Smith D, Cobon G, McKenna RV. Comparative vaccination of cattle against *Boophilus microplus* with recombinant antigen Bm86 alone or in combination with recombinant Bm91. Parasite Immunol 1996;18:241–6.
[18] McKenna RV, Riding GA, Jarmey JM, Pearson RD, Willadsen P. Vaccination of cattle against the *Boophilus microplus* using a mucin-like membrane glycoprotein. Parasite Immunol 1998;20:325–36.
[19] Andreotti R, Gomes A, Malavazi-Piza KC, Sasaki SD, Sampaio CAM, Tanaka AS. BmTI antigens induce a bovine protective immune response against *Boophilus microplus tick*. Int Immunopharmacol 2002;2:557–63.
[20] Almazan C, Kocan KM, Blouin EE, de la Fuente J. Vaccination with recombinant tick antigens for the control of *Ixodes scapularis* adult infestations. Vaccine 2005;23:5294–8.
[21] Imamura S, Namangala B, Tajima T, Tembo ME, Yasuda J, Ohashi K, et al. Two serine protease inhibitors (serpins) that induce a bovine protective immune response against *Rhipicephalus appendiculatus* ticks. Vaccine 2006;24:2230–7.
[22] Imamura S, Konnai S, Junior IDV, Yamada S, Nakajima C, Ito Y, et al. Effects of anti-tick cocktail vaccine against *Rhipicephalus appendiculatus*. Jpn J Vet Res 2008;56:85–98.
[23] Almazan C, Moreno-Cantu O, Moreno-Cid JA, Galindo RC, Canales M, Villar M, et al. Control of tick infestations in cattle vaccinated with bacterial membranes containing surface-exposed tick protective antigens. Vaccine 2012;30:265–72.
[24] de la Fuente J, Rodriguez M, Montero C, Redondo M, Garcia-Garcia JC, Mendez L, et al. Vaccination against ticks (*Boophilus* spp.): the experience with the Bm86-based vaccine Gavac (TM). Genet Anal 1999;15:143–8.
[25] Vargas M, Montero C, Sánchez D, Pérez D, Valdés M, Alfonso A, et al. Two initial vaccinations with the Bm86-based Gavac^{plus} vaccine against *Rhipicephalus* (*Boophilus*) *microplus* induce similar reproductive suppression to three initial vaccinations under production conditions. BMC Vet Res 2010;6:43.
[26] da Silva Vaz Jr I, Logullo I, Sorgine M, Velloso FF, Rosa de Lima MF, Gonzales JC, et al. Immunization of bovines with an aspartic proteinase precursor isolated from *Boophilus microplus* eggs. Vet Immunol Immunopathol 1998;66(December):331–41.
[27] Leal AT, Seixas A, Pohl PC, Ferreira CAS, Logullo C, Oliveira PL, et al. Vaccination of bovines with recombinant *Boophilus* yolk pro-cathepsin. Vet Immunol Immunopathol 2006;114:341–5.
[28] Seixas A, Dos Santos PC, Velloso FF, da Silva Vaz Jr I, Masuda A, Horn F, et al. A *Boophilus microplus* vitellin-degrading cysteine endopeptidase. Parasitology 2003;126:155–63.
[29] Seixas A, Estrela AB, Ceolato JC, Pontes EG, Lara F, Gondim KC, et al. Localization and function of *Rhipicephalus* (*Boophilus*) *microplus* vitellin-degrading cysteine endopeptidase. Parasitology 2010;137:1819–31.
[30] Seixas A, Leal AT, Nascimento-Silva MC, Masuda A, Termignoni C, da Silva Vaz Jr I. Vaccine potential of a tick vitellin-degrading enzyme (VTDCE). Vet Immunol Immunopathol 2008;124:332–40.
[31] Parizi LF, Utiumi KU, Imamura S, Onuma M, Ohashi K, Masuda A, et al. Cross immunity with *Haemaphysalis longicornis* glutathione S-transferase reduces an experimental *Rhipicephalus* (*Boophilus*) *microplus* infestation. Exp Parasitol 2011;127:113–8.
[32] da Silva Vaz Jr I, Imamura S, Ohashi K, Onuma M. Cloning, expression and partial characterization of a *Haemaphysalis longicornis* and a *Rhipicephalus appendiculatus* glutathione S-transferase. Insect Mol Biol 2004;13:329–35.
[33] Leal AT, Pohl PC, Ferreira CAS, Nascimento-Silva MC, Sorgine MH, Logullo C, et al. Purification and antigenicity of two recombinant forms of *Boophilus microplus* yolk pro-cathepsin expressed in inclusion bodies. Protein Exp Purif 2006;45:107–14.
[34] Oldiges DP, Parizi LF, Zimmer KR, Lorenzini DM, Seixas A, Masuda A, et al. A *Rhipicephalus* (*Boophilus*) *microplus* cathepsin with dual activity: peptidase and antimicrobial. Int J Parasitol 2012;42:635–45.
[35] Bradford MM. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[36] Federation of Animal Science Societies (FASS)., 1999. Guide for the care and use of agricultural animals in agricultural research and teaching. 2nd ed. Savoy: FASS; 1999.
[37] Dallas BF, Lieping C. A simple and rapid vortex method for preparing antigen/adjuvant emulsions for immunization. J Immunol Methods 2003;276:239–42.
[38] Johnson DA, Gautsch JW, Sportsman JR, Elder JH. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal Tech 1984;1:3–8.
[39] Abramoff MD, Magalhaes PJ, Ram SJ. Image Processing with ImageJ. Biophoton Int 2004;11:36–42.
[40] Wharton RH, Utech KBW. The relation between engorgement and dropping of *Boophilus microplus* (Canestkini) (Ixodidae) to the assessment of tick numbers on cattle. J Aust Entamol Soc 1970;9:171–82.
[41] de la Fuente J, Rodríguez M, Redondo M, Montero C, García-García JC, Méndez L, et al. Field studies and cost-effectiveness of vaccination with Gavac against the cattle tick *Boophilus microplus*. Vaccine 1998;16:366–73.

[42] Jonsson NN, Matschoss AL, Pepper P, Green PE, Albrecht MS, Hunterford J, et al. Evaluation of TickGARD(PLUS), a novel vaccine against *Boophilus microplus*, in lactating Holstein–Friesian cows. Vet Parasitol 2000;88:275–85.
[43] García-García JC, Gonzalez IL, Gonzalez DM, Valdes M, Mendez L, Lamberti J, et al. Sequence variations in the *Boophilus microplus* Bm86 locus and implications for immunoprotection in cattle vaccinated with this antigen. Exp Appl Acarol 1999;23:883–95.
[44] Garcia-Garcia JC, Montero C, Redondo M, Vargas M, Canales M, Boue O, et al. Control of ticks resistant to immunization with Bm86 in cattle vaccinated with the recombinant antigen Bm95 isolated from the cattle tick, *Boophilus microplus*. Vaccine 2000;18:2275–87.
[45] de la Fuente J, Kocan KM. Strategies for development of vaccines for control of ixodid tick species. Parasite Immunol 2006;28:275–83.
[46] Parizi LF, Pohl PC, Masuda A, da Silva Vaz Jr I. New approaches toward anti-*Rhipicephalus* (*Boophilus*) *microplus* tick vaccine. Rev Bras Parasitol Vet 2009;18:1–7.
[47] Lightowlers MW, Rolfe R, Gauci CG. *Taenia saginata*: vaccination against cysticercosis in cattle with recombinant oncosphere antigens. Exp Parasitol 1996;84:330–8.
[48] Doolan DL, Hoffman SL. DNA-based vaccines against malaria: status and promise of the multi-stage malaria dna vaccine operation. Int J Parasitol 2001;31:753–62.
[49] Logullo C, Vaz IS, Sorgine MH, Paiva-Silva GO, Faria FS, Zingali RB, et al. Isolation of an aspartic proteinase precursor from the egg of a hard tick, *Boophilus microplus*. Parasitology 1998;116:525–32.
[50] Wei SH, Clark AG, Syvanen M. Identification and cloning of a key insecticide-metabolizing glutathione S-transferase (MdGST-6A) from a hyperinsecticide-resistant strain of the housefly *Musca domestica*. Insect Biochem Mol Biol 2001;31:1145–53.
[51] da Silva Vaz Jr I, Lermen TT, Michelon A, Ferreira CAS, de Freitas DRJ, Termignoni C, et al. Effect of acaricides on the activity of a *Boophilus microplus* glutathione S-transferase. Vet Parasitol 2004;119:237–45.