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Constituting a glutathione S-transferase-cocktail vaccine against tick infestation

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ABSTRACT

Cocktail vaccines are proposed as an attractive way to increase protection efficacy against specific tick species. Furthermore, such vaccines made with different tick antigens have the potential of cross-protecting against a broad range of tick species. However, there are still limitations to the selection of immunogen candidates. Acknowledging that glutathione S-transferases (GSTs) have been exploited as vaccines against ticks and other parasites, this study aimed to analyze a GST-cocktail vaccine as a potential broad-spectrum tick vaccine. To constitute the GST-cocktail vaccine, five tick species of economic importance for livestock industry were studied (*Rhipicephalus appendiculatus, Rhipicephalus decoloratus, Rhipicephalus microplus*, *Amblyomma variegatum*, and *Haemaphysalis longicornis*). Tick GST ORF sequences were cloned, and the recombinant GSTs were produced in Escherichia coli. rGSTs were purified and inoculated into rabbits, and the immunological response was characterized. The humoral response against rGST-Rd and rGST-Av showed a stronger cross-reactivity against heterologous rGSTs compared to rGST-Hl, rGST-Ra, and rGST-Rm. Therefore, rGST-Rd and rGST-Av were selected for constituting an experimental rGST-cocktail vaccine. Vaccination experiment in rabbits showed that rGST-cocktail caused 35% reduction in female numbers in a *Rhipicephalus sanguineus* infestation. This study brings forward an approach to selecting immunogens for cocktail vaccines, and the results highlight rGST–Rd and rGST-Av as potentially useful tools for the development of a broad-spectrum tick vaccine.

1. Introduction

Ticks are ubiquitous blood-sucking vectors, second only to mosquitoes in transmitting pathogens such as protozoa, viruses, bacteria, and helminths, both to humans and to animals [1]. For decades, the control of tick infestation has relied on acaricide use, yet the rising number of cases of acaricide-resistant ticks currently disputes the suitability of acaricides. Notably, concerns over potential contamination of milk and beef, and environmental pollution further undermine acaricide use against ticks and tick-borne diseases. In response, researchers suggest a number of alternative tick control methods [2,3], among which vaccination stands out as the most promising, ideal, and user-friendly approach [4]. Since the concept of anti-tick vaccination was introduced [5], several promising single-antigen tick vaccines have been identified [6], of which many have shown high protection efficacy against particular tick species. A few vaccines - for instance, Bm86 [7–9], and Subolesin [10,11] - have shown protection against more than one tick species. A recent review [12] shows the phylogenetic relationship between Subolesin sequences from different tick species, and discusses the broad protective potential of Subolesin vaccine against arthropod ectoparasite infestations and pathogen infection.

Broad-spectrum vaccines (*i.e.* vaccines that can protect against a wide range of species) represent an ideal alternative way to address multiple-species infestation, a persistent stumbling block toward development of the livestock industry in different parts of the world. For instance, in East Africa, three important tick species burden the industry: *Rhipicephalus appendiculatus* (the main vector of *Theileria* spp, a pathogen that causes East Coast Fever), *R. decoloratus* (the main vector of *Anaplasma* spp and *Babesia* spp, pathogens that cause anaplasmosis and babesiosis), and *Amblyomma variegatum* (the main vector of *Erilichia* ruminantium, a pathogen that causes heartwater) [13,14]. Additionally, broad-spectrum vaccines could be useful in areas affected by a single tick species, in cases of new tick species invasion as reported in West Africa [15,16]. Strikingly, however, there are still only a few potential broad-spectrum tick vaccines [17]. Therefore, Willadsen [18] proposes that combining at least two antigens (a cocktail vaccine)

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could increase the efficacy of the currently characterized experimental tick vaccines.

Different research groups have shown proof-of-concept studies applying potential cocktail vaccines against ticks [10,19–21], but the findings are yet to be replicated under field conditions. Questions remain about what should be considered when designing cocktail tick vaccines, and how they should be developed. For instance, they could be made up of proteins produced in various stages of tick development, inducing protection against a particular species or cross-protection against different tick species. Alternatively, vaccines could be a combination of antigens derived from ticks and tick-borne pathogens, inducing protection against haemoparasites.

Potential targets for developing broad-spectrum tick vaccines include glutathione S-transferases (GSTs), an enzyme superfamily shown to have a role in the metabolic detoxification of endobiotics (endogenous compounds) and xenobiotics (exogenous compounds) [22,23]. GST enzymes are ubiquitous in tick tissues, at different expression levels [30–32]. Despite reports suggesting that GST enzymes are involved in tick resistance to pyrethroids [24–28], it remains unclear whether they also play a role in tick resistance to other classes of acaricides. Emerging evidence shows that GSTs have a role in heame detoxification [29]. rGST-Hl antigen, from *Haemaphysalis longicornis,* was shown to induce cross-protection against two different tick species, *Rhipicephalus microplus* and *R. appendiculatus* [37,38]. The potential of GSTs has been also exploited in developing vaccines against parasitic worms [33–36]. These findings have led us to hypothesize that tick GST enzymes are potential candidates for constituting a cocktail vaccine for broad-spectrum protection, yet there is still no clear approach to select the cocktail antigen components.

The premise of this study was to constitute a GST-cocktail vaccine, toward the development of a broad-spectrum tick vaccine. To achieve this goal, GST open read frame (ORF) sequences from different tick species of economic interest (*A. variegatum, R. appendiculatus, R. decoloratus*, *R. microplus*, and *H. longicornis*) were cloned and expressed. rGST antigens were screened for potential use in the GST-cocktail vaccine. Finally, we used *R. sanguineus* infestation in rabbits, a usual laboratory model, to evaluate the immune protection of GST-cocktail as a potential broad-spectrum tick vaccine.

2. Materials and methods

2.1. Ethics statement

Animals used in the experiments were housed at Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS) facilities. This study was conducted according to the ethical and methodological norms prescribed by the International and National Directives and Norms by the Animal Experimentation Ethics Committee of UFRGS. Protocol (number 27559) was approved by the Comissão de Ética no Uso de Animais – CEUA – UFRGS.

2.2. Ticks and experimental animals

R. sanguineus ticks used in this study were previously collected from Rio de Janeiro, Brazil [39], and have since been maintained in our laboratory tick colony. *R. appendiculatus, A. variegatum*, and *R. decoloratus* ticks were collected from farms in Tororo, Uganda (latitude 0°44′59.99″N longitude 34°04′60.00″E), under approval of the UNCST and NaLIRRI Science and Ethics committee. All immunization experiments were performed on isolated, caged, three-month-old female New Zealand rabbits weighting approximately 2 kg.

2.3. RNA extraction and cDNA synthesis

Ticks were dissected using a pictorial guide [40], ovaries were extracted and suspended in TRIzol™ (Invitrogen). Total RNA from each ovary tissue was isolated according to the manufacturer's instructions, preserved in isopropanol and transported to Brazil. Total RNA (5 μg) was added to 0.5 μg/μl oligo $(dT)_{12\text{-}18}$ (Invitrogen), 1 μl dNTPs (10 mM), DEPC-treated water q.s. 13 μl, mixed and incubated at 65 °C for 5 min. Samples were then incubated on ice for 1 min, combined with 4 μl of First-strand buffer (5×), 2 μl DTT (0.1 M) and 1 μl SuperScript™ III (Invitrogen), and further incubated at 50 °C for 60 min, and then at 70 °C for 15 min. The cDNA samples were stored at −70 °C until further use.

2.4. Tick GST cDNA cloning

Partial sequences of *R. appendiculaus, A. variegatum,* and *R. decoloratus* GST ORFs were amplified using sets of previously designed primers [41]. The amplification reaction consisted of 1 U Taq DNA polymerase (Ludwig Biotec), 0.2 mM dNTPs, 3 mM $MgCl_2$, 1 X PCR buffer (Ludwig Biotec), and 0.5 mM primers. The reactions were performed in a 2720 thermocycler (Applied Bio-systems) as follows: 94 °C for 5 min, 94 °C (30 sec)/54 °C (30 sec)/72 °C (90 sec) for 34 cycles, and 72 °C for 5 min. Reaction products were resolved by electrophoresis on ethidium bromide-stained agarose gel 0.8%, purified using the GENECLEAN II kit (MP Biomedicals), and ligated into pGEM-T Vector (Promega), as per the manufacturer's instructions. The ligation products were transformed into *Escherichia coli* TOP10 cells (Invitrogen) using heat shock method [42], and recovered plasmids were sequenced. The generated sequences were blasted against NCBI (Genbank) hard tick sequences to identify the corresponding conserved GST sequences.

Based on tick GST conserved sequences retrieved from Genbank database, new primers were designed to amplify full-length ORF sequences. *A. variegatum* GST primers were based on *A. americanum* (EZ000199.1) and *A. variegatum* (BK007327.1) sequences. *R. decoloratus* GST primers were based on *R. annulatus* GST sequence (EF440186.1), and *R. appendiculatus* GST primers were based on *R. appendiculatus* GST sequence (AY298732). GST full-length ORF sequences were amplified using the reaction conditions described above, but with different, prime-specific annealing temperatures (Supplementary Table 1). The reaction products were resolved using electrophoresis, purified and ligated into pGEM-T Vector (Promega) as described above. Ligation products were transformed into *E. coli* TOP10 cells and the recovered plasmids submitted for sequencing. The generated sequences were assembled using Lasergene 7 software (DNASTAR), the consensus nucleotide sequence was translated into amino acid sequence using BioEdit software 7.2.6.1 [43], then blasted against Genbank database.

Additionally, GST full-length ORF sequences were amplified using the same conditions, but with a set of primers containing recognition sites for *Nde* I and *Xho* I restriction enzymes (Supplementary Table 1). The amplified products were digested with *Nde* I and *Xho* I, purified using GENECLEAN II kit (following manufacturer's instructions, MP Biomedicals), and cloned into expression plasmid pET-43.1a (Novagen). Competent XL1-Blue *E. coli* cells were transformed with the ligated plasmid using BIO-RAD Gene-Pulser Xcell™ Electroporation system (Bio-Rad), and cultured on Luria-Bertani (LB) agar plates containing 50 μg/mL ampicillin. Plasmids from selected transformant colonies were purified using miniprep protocol [42], and screened using restriction enzymes and PCR. The putative clones were subsequently submitted for DNA sequencing. Generated sequences were assembled using Lasergene 7 software (DNASTAR), the consensus was translated using BioEdit software 7.2.6.1, and nucleotide sequences blasted against the Genbank database to confirm GSTs identity.

2.5. Phylogenetic analyses

Deduced amino acid sequences from confirmed GST ORF sequences from *R. appendiculatus, A. vareigatum*, and *R. decoloratus*, as well as sequences retrieved from Genbank *(H. longicornis [AY298731], R. microplus [AF077609], R. sanguineus [KC514943] and R. microplus [AAL99403])* were aligned using ClustalW algorithm on BioEdit software 7.2.6.1.

Phylogenetic analyses were performed using Mega 7 software [44] based on *R. appendiculatus, A. variegatum*, and *R. decoloratus* GST deduced amino acid sequences, together with GST amino acid sequences from Genbank database: *Amblyomma americanum (EZ000199), Amblyomma maculatum (JO843100), Dermacentor variabilis (AY241958), Dermacentor variabilis (DQ224235), Dermacentor variabilis (EU551642), Haemaphysalis longicornis (AY298731), Ixodes pacificus (AY674232), Ixodes scapularis (XM 002401705), Rhipicepalus microplus (AF077609), Rhipicephalus annulatus (EF440186), Rhipicephalus appendiculatus (AY298732), Rhipicephalus microplus (KF784792), Rhipicephalus sanguineus (KC514943).* Phylogenetic tree for GST sequences was constructed using the neighbor-joining method [45] after 1000 bootstrap replicates [46].

2.6. Recombinant GST expression

Recombinant proteins used in this study were GST-Rd, GST-Av, GST-Ra (produced in the present work), and the previously characterized GST-Bm [32], and GST-Hl [41]. To express GST sequences, the confirmed pET-43a-GST plasmids (see Section 2.4 above) were inserted into the *E. coli* AD494 (DE3) pLysS expression host strain (Invitrogen) using heat shock transformation method [42]. Transformed cells were cultured for 12 h at 37 °C on Luria-Bertani (LB) agar plates containing 50 μg/ml ampicillin. One colony of the transformed cells was picked, cultured in LB broth for 2–4 h and induced for 6 h with 1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG). To harvest the produced proteins, culture broth was centrifuged for 10 min at 16,000*g* at 4 °C, cell pellet was washed twice in PBS pH 7.2, and stored at 4 °C.

2.7. rGST purification

Cell pellets were separately suspended in PBS and lysed using an ultrasonic homogenizer with 5 cycles of 30 pulses for 30 s (Pulse Sonics Vibra-cell VCX 500–700). The lysate was centrifuged at 16,000*g* for 10 min to separate soluble proteins from cell debris. GST proteins were next purified by affinity chromatography using GSTrap™ 4B column (GE Healthcare Bio-Sciences) [41]. Purification was performed in PBS pH 7.3 (washing and binding buffer) and 10 mM GSH in 50 mM Tris HCl pH 8.0 (elution buffer). Fractions containing the eluted protein were dialyzed in PBS pH 7.2 for 12 h at 4 °C. Next, protein purity was verified by 14% SDS -PAGE [47,48] under reduced conditions, and quantified using UV/visible spectrophotometer (Ultrospec 1000 Amersham Biosciences, Pharmacia Biotech).

2.8. rGST enzyme activity

Using a previously described colorimetric assay, the purified rGSTs were tested for activity against common substrates, 1 mM CDNB (1, 2-dichloro-4-nitrobenzene), and 1 mM DCBN (1, Dichloro-4-nitrobenzene), in the presence of 3 mM GSH (Glutathione) co-substrate, and 100 mM Tris pH 7.5 [41,49].

2.9. Screening for GST-cocktail vaccine constituents

To determine the immunogenicity of the rGSTs, five four-month-old rabbits were inoculated four times at intervals of 2 weeks with 200 μg of each individual rGST in 500 μl of adjuvant (Montanide 888 – Seppic and Marcol 52 – Exxon Mobil Corporation). On the first and seventh day after each inoculation, blood was collected and centrifuged at 16,000*g* for 5 min. Pre- and post-immune sera were stored at −20 °C.

Selection of the constituents for the GST-cocktail vaccine was based on the immunogenicity and serum cross-recognition of heterologous rGSTs. Using sera from rGST immunized rabbits, ELISA tests were performed to determine immunogenicity. Microtiter plates were coated with 0.1 μg/well of rGST in carbonate/bicarbonate buffer (500 mM pH 9.6) and incubated for 12 h at 4 °C. Plates were washed three times (5 min each), and incubated for 2 h with 200 μl PBS/0.05% Tween 20 pH 7.2. Plates were then incubated for 2 h at 37 °C with the different anti-rGST sera (diluted from 1:8000 to 1:128,000), followed by three washes in PBS pH 7.2 and 1 h incubation at 37 °C with 100 μl of anti-rabbit IgG-peroxidase conjugate (1:5000). Plates were again washed, and incubated for 15 min at room temperature with 100 μl of chromogen substrate (3.4 mg o-phenylenediamine, 5 ml H_2O_2 in 0.1 M citrate–phosphate buffer, pH 5.0). Fifty microliters of 12.5% H_2SO_4 were added to stop the reaction, and the optical density (OD) of the product was measured at 490 nm. Test serum was considered positive when the average OD reading of the test serum was more than two standard deviations greater than the average OD reading of the pre-inoculation serum. Multiple comparisons were performed by two-way analysis of variance (ANOVA) and *a posteriori* Tukey's test for pairwise comparisons.

Positive control reactions were set up using anti-rGST serum and the respective homologous rGST, while negative control reactions were set up using pre-inoculation serum and homologous or heterologous rGSTs. Serum cross-recognition was calculated as percentage value relative to the positive control, a reaction between the homologous rGST and the corresponding serum. All cross-recognition assays were performed in duplicates in at least two independent experiments.

Cross-recognition of heterologous rGSTs by anti-rGST sera was determined using Western blot [37] with slight modifications. rGST was processed by SDS-PAGE (14%) at a concentration of 0.5 μg protein/cm. Proteins on the gel were transferred onto nitrocellulose membrane (Bio-rad) in 12 mM carbonate buffer, pH 9.9 [50]. The 4-mm-wide strips were blocked with 5% blotto for 2 h at room temperature, incubated with sera (1:1000 in 5% blotto) for 2 h at room temperature, then with conjugate for 1 h at room temperature. Images were analyzed using ImageJ software 1.46r [51]. The same Western blot protocol was used to determine anti-rGST sera cross-recognition of GST in crude soluble protein extracts from *R. sanguineus* eggs, prepared as previously described [38].

2.10. GST-cocktail sera characterization

rGST-cocktail serum was tested for three parameters (immunogenicity, cross-recognition, and avidity) using ELISA and Western blot procedures described above with slight modifications. GST-cocktail immunogenicity analysis was performed using rabbit serum collected after the third inoculation before *R. sanguineus* infestation. GST-cocktail serum was tested against rGST-Av and rGST-Rd (rGST-cocktail constituents) at 1:8,000–1:128,000 dilutions. GST-cocktail serum cross-recognition tests were performed by ELISA and Western blot against rGST-Av, rGST-Rd, rGST-Rm, rGST-Ra, and rGST-Hl, at 1:64,000 dilution. ELISA results were analyzed using multiple comparisons by one-way ANOVA and *a posteriori* Dunnett's test for pairwise comparisons.

rGST-cocktail serum avidity was tested using rabbit sera collected after the 1st, 2nd, 3rd and 4th inoculations (before and during *R. sanguineus* infestation). ELISA plates were coated with one

rGST, washed three times, topped with 200 μl of rGST-cocktail sera (1:8,000) and incubated for 2 h at 37 °C. Plates were then washed, treated with 100 μl of denaturant (0, 4, or 6 M urea in PBS/0.05% Tween 20 pH 7.2) for 3 min at room temperature, then washed again. Plates were further incubated with 100 μl of anti-rabbit IgG-peroxidase conjugate for 1 h at 37 °C. After another wash step, chromogen was added and peroxidase activity measured as described above. rGST-cocktail serum avidity index (AI) was calculated as the optical density ratio of bound to unbound antibodies with or without urea treatment [52,53]. All analyses were performed in triplicates. Results were analyzed using multiple comparisons by two-way ANOVA and *a posteriori* Turkey's test for pairwise comparisons.

2.11. GST-cocktail vaccination against R. sanguineus

To determine the protection effect of the constituted GST-cocktail vaccine, two groups with three rabbits each (vaccination and control groups) were subcutaneously inoculated three times at intervals of two weeks. The vaccination group was inoculated with GST-cocktail vaccine, constituted by combining rGST-Av (100 μg) and rGST-Rd (100 μg) with the adjuvant (Montanide 888 -Marcol 52), whereas the control group was inoculated with PBS pH 7.2 combined with the same adjuvant. One week after the third immunization, rabbits were infested with a total of 30 female and 30 male adult ticks (15 male and 15 female ticks on each ear). Engorged female ticks were collected daily from each rabbit for 15 days, counted and weighed. The engorged ticks were kept in a humidity chamber at 28 °C for 30 days upon which the oviposition was determined. To determine the protection efficacy of GST-cocktail as vaccine against *R. sanguineus* infestation, the weight of engorged ticks, eggs and hatched larvae were analyzed and compared to the control group.

3. Results

3.1. GST ORF sequences

Electrophoresis separation of PCR products amplified from tissues from African ticks (*R. appendiculatus*, *R. decoloratus* and *A. variegatum)* using previously designed primers showed 300 bp fragments which were sequenced and confirmed as partial GST sequences. Alignment of GST partial sequences with GST sequences retrieved from Genbank also revealed conserved sequence regions. Therefore, new primers for amplifying the GST full-length ORF sequences from African ticks were designed based on the con-

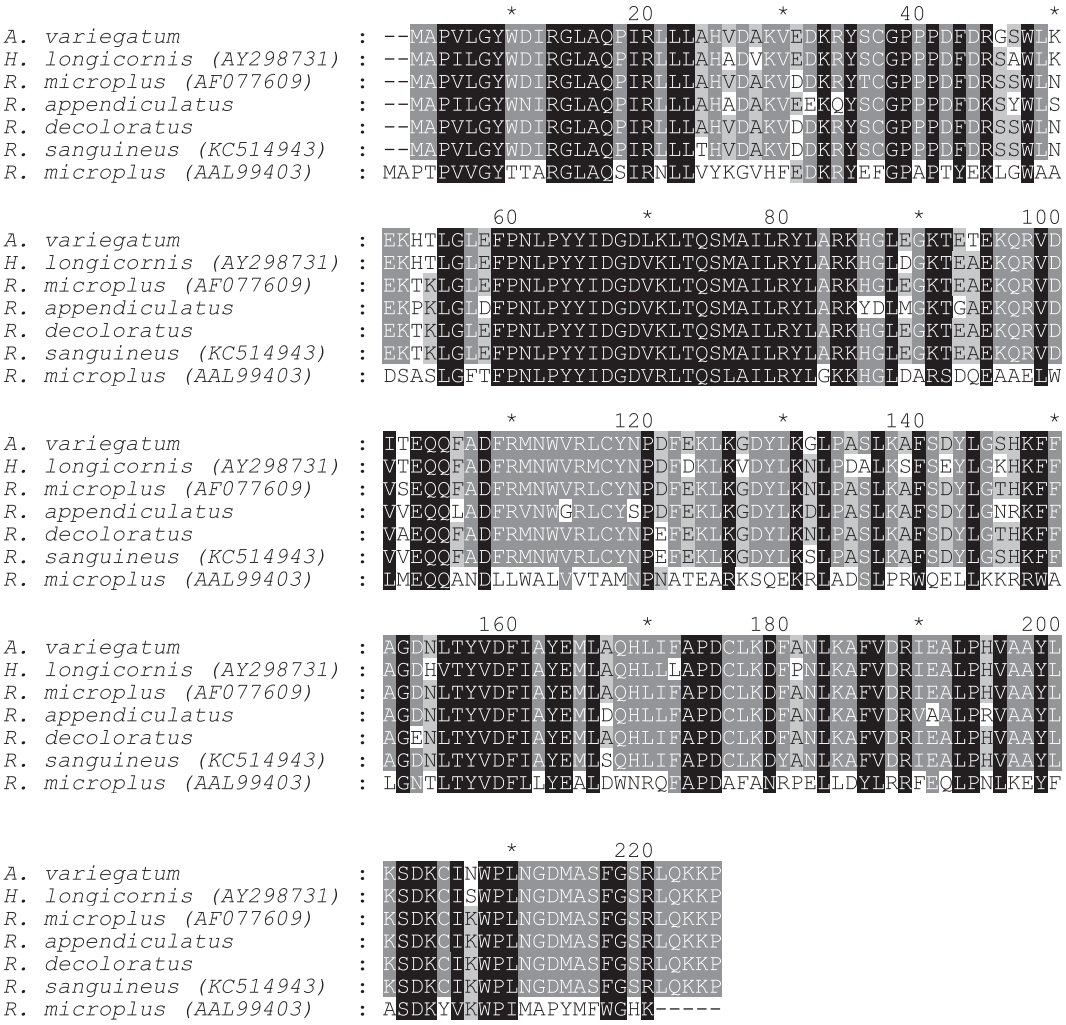

Fig. 1. Full-length GST amino acid sequence alignment. ClustalW alignment of sequences obtained in this study (*Rhipicephalus appendiculatus* MK133338, *Rhipicephalus decoloratus* MK133339, *Amblyomma variegatum* MK133337), and sequences retrieved from NCBI Genbank (*Rhipicephalus microplus* (AF077609 and AAL99403), *Rhipicephalus sanguineus* (KC514943), and *Haemaphysalis longicornis* (AY298731). Black and gray shades show conserved regions among tick GSTs. The unshaded regions show differences among tick GSTs.

served sequence regions. Electrophoresis of PCR products amplified using this second set of primers showed 600 bp fragments which were sequenced and compared to Genbank sequences, confirming their identity as class Mu GST full-length ORF sequences. Genbank accession numbers are MK133338, MK133339, and MK133337 for ORFs from *R. appendiculatus*, *R. decoloratus,* and *A. vareigatum*, respectively.

Among GST amino acid sequences from African ticks, interspecies pairwise similarities ranged between 91 and 96%. The similarity between African-tick GSTs and *R. microplus* GSTs is between 39 and 98% for an orthologue gene, and around 57% for a non-orthologue gene (Fig. 1). Phylogenetic analysis (Fig. 2) showed that the five GST sequences analyzed in this study are closely related to class Mu GST, rather than to class A GST.

3.2. rGST expression and enzyme activity

SDS-PAGE of the purified rGST-Av, rGST-Ra, and rGST-Rd showed 25-kDa protein bands (data not shown), corresponding to the size previously reported for tick GSTs [41]. Moreover, all rGSTs showed activity against 1, 2-dichloro-4-nitrobenzene (CDNB), but not against 1, Dichloro-4-nitrobenzene (DCBN) (data not shown).

3.3. Constituting the rGST-cocktail

Immunogenicity analyses of rGST-Av, rGST-Ra, rGST-Rd, rGST-Rm, and rGST-Hl using ELISA and Western blot indicated a humoral response was induced against rGSTs after rabbit immunization. The sera separately recognized the respective homologous rGST (titer 64,000); hence all rGSTs were immunogenic, as previously reported for rGST-Rm and rGST-Hl [32,41]. Additionally, rGST-induced sera showed cross-recognition of heterologous rGST proteins (Fig. 3). Sera produced against rGST-Rd and rGST-Av showed stronger cross-recognition of heterologous rGST compared to sera against rGST-Ra, rGST-Hl, or rGST-Rm. Moreover, Western blot analyses (Supplementary Fig. 1) confirmed that rGST sera recognized the homologous rGST and cross-recognized the heterologous rGST proteins. Additionally, Western blot indicated that sera against all the rGSTs cross-recognized *R. sanguineus* crude egg protein extracts (Supplementary Fig. 2). Sera against rGST-Rd and rGST-Av showed stronger cross-recognition of *R. sanguineus* crude egg protein extracts compared to serum against rGST-Ra, rGST-Hl, and rGST-Rm. Taken together, these results led to the selection of rGST-Rd and rGST-Av for constituting a rGST-cocktail vaccine.

3.4. GST-cocktail sera characteristics

Immunogenicity analyses of rGST-cocktail serum indicated recognition of the rGST-cocktail antigen constituents (rGST-Av and rGST-Rd) at a titer of 128,000 (Fig. 4). Moreover, rGST-cocktail serum showed cross-recognition of heterologous rGST proteins (rGST-Ra, rGST-Rm, and rGST-Hl) (Fig. 5). Interestingly, ELISA (Fig. 5) indicated that rGST-cocktail-induced serum showed between 60% and 48% cross-recognition of rGST-Hl, rGST-Ra, rGST-Rd, rGST-Rm, and rGST-Av, compared to serum induced separately against rGST-Av and rGST-Rd, respectively.

Fig. 6 shows the increase in avidity index (AI) of the rGST-cocktail serum during the immunization process. Increase in AI was observed testing the rGST-cocktail serum against rGST-Rd, rGST-Av, rGST-Ra, rGST-Rm, and rGST-Hl antigens. Put together, the data indicate that avidity increased during the immunization protocol, reaching the highest AI (>0.9) against all studied rGSTs after the 4th inoculation.

3.5. Effect of GST-cocktail vaccination on R. sanguineus infestation in rabbits

In order to analyze the rGST-cocktail potential to cross-protect against a *R. sanguineus* infestation, rGST-Av and rGST-Rd were tested as a multi-antigen vaccine. One week after the beginning of infestation, female ticks fed on vaccinated rabbits were smaller than those fed on the control group, with a scattered attachment pattern (Fig. 7). The average number of female ticks that finished engorgement in immunized and control groups was19.7 and 12.3, respectively (Table 1), corresponding to a statistically significant reduction of 37.29% in the vaccinated group. Female weight, egg laying, and hatched larvae parameters were not significantly affected by the immunization.

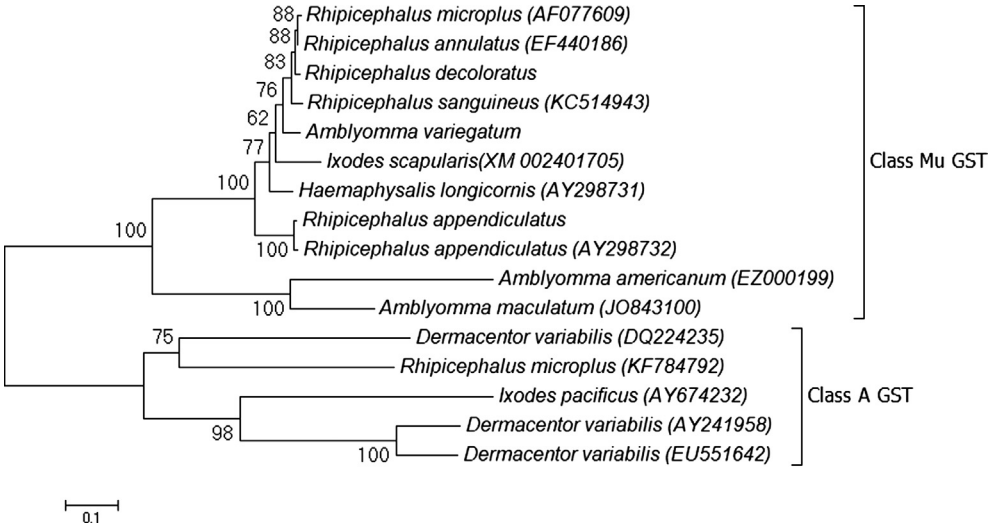

Fig. 2. Phylogenetic relationship of tick GSTs deduced amino acid sequences obtained in this study from *Rhipicephalus appendiculatus*, *Rhipicephalus decoloratus*, *Amblyomma variegatum,* and tick GSTs sequences retrieved from NCBI Genbank: *Rhipicephalus microplus* (AF077609), *Rhipicephalus annulatus* (EF440186), *Rhipicephalus sanguineus* (KC514943), *Ixodes scapularis* (XM002401705), *Haemaphysalis longicornis* (AY298731), *R. appendiculatus* (AY298731), *Amblyomma americanum* (EZ000199), *Amblyomma maculatum* (JO843100), *Dermacentor variabilis* (DQ224235), *R. microplus* (KF784792), *Ixodes pacificus* (AY674232), *D. variabilis* (AY241958), *D. variabilis* (EU551642). The phylogenetic relationship was inferred using the Neighbor-joining method. Bootstrap values are shown next to the branches (1000 bootstrap replications).

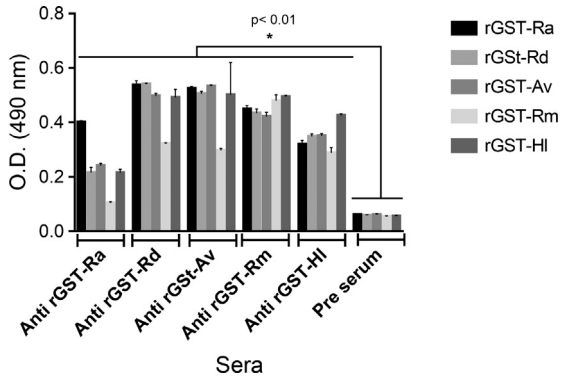

Fig. 3. Antigenicity of tick rGSTs and cross-reactivity of anti-rGST sera. Rabbits were immunized with one of the following rGST: *Rhipicephalus appendiculatus* (rGST-Ra), *Rhipicephalus decoloratus* (rGST-Rd), *Amblyomma variegatum* (rGST-Av), *Rhipicephalus microplus* (rGST-Rm) or *Haemaphysalis longicornis* (rGTS-Hl). Each serum produced against the rGSTs (anti rGST-Ra, anti rGST-Rd, anti rGST-Av, anti rGST-Rm or anti rGST-Hl) was tested against rGST-Ra, rGST-Rd, rGST-Av, rGST-Rm and rGST-Hl by ELISA. Negative control serum (pre-immunization serum) was probed against the same rGSTs. Statistical analysis was performed between the cross-recognition of each serum with reference to the pre-immunization serum. All anti-GST sera cross-recognized rGSTs ($p < 0.01$).

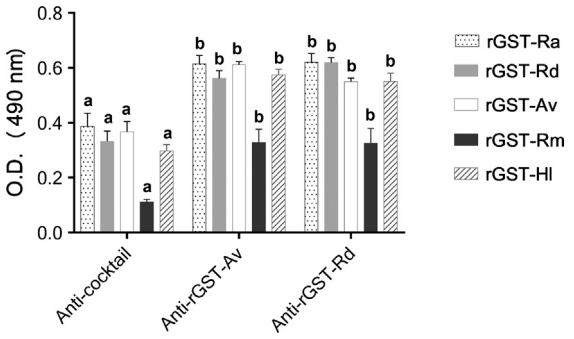

Fig. 5. Cross-reactivity of sera induced against rGSTs, analyzed by ELISA. Rabbits were immunized with rGSTs from *Rhipicephalus decoloratus* (rGST-Rd), *Amblyomma variegatum* (rGST-Av), and rGST-cocktail (comprised of rGSTs from *Amblyomma variegatum* (rGST-Av) and *Rhipicephalus decoloratus* (rGST-Rd). The sera produced (anti-cocktail, anti-rGST-Av and anti-rGST-Rd) were tested (1:1000 dilutions) against rGST-Rd, rGST-Av, and rGSTs from *Rhipicephalus appendiculatus* (rGST-Ra), *Rhipicephalus microplus* (rGST-Rm), and *Haemaphysalis longicornis* (rGST-Hl). Data represent mean and standard deviation of triplicate experiments. Multiple comparisons were performed by two-way ANOVA, and *a posteriori* Tukey's test was applied for pairwise comparisons. Different letters indicate significant difference ($p < 0.001$).

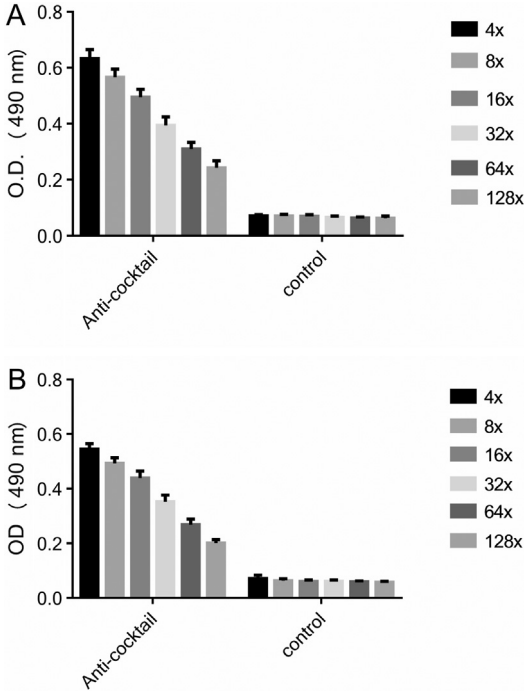

Fig. 4. Reactivity of anti rGST-cocktail serum analyzed by ELISA. Rabbits were immunized with rGST-cocktail comprised of rGSTs from *Rhipicephalus decoloratus* (rGST-Rd) and *Amblyomma variegatum* (rGST-Av). The serum produced against rGST-cocktail (anti-cocktail) was tested in dilutions ranging from 4000 to 128,000, and probed against (A) rGST-Av, and (B) rGST-Rd. Negative control serum (pre-immunization serum) also was probed against rGST-Av and rGST-Rd. Data represent mean and standard deviation of triplicate experiments.

4. Discussion

A number of candidate targets for single-antigen tick vaccines have been identified. However, only a few were characterized regarding their ability to induce a cross-reactive immune response against different tick species, in other words, a broad-spectrum tick vaccine. Bm86 and Subolesin, two important antigens characterized in *R. microplus*, can separately induce protection against other tick species infestations [7–11]. However, it is consensus that an effective vaccine against parasites [54,55], including ticks, requires more than one antigen [56,57]. Thus, it is essential to evaluate combination effects of more than one antigen in tick vaccines. Efforts to enhance protection offered by single antigens has led to cocktail-antigen tick vaccine formulations, but the anticipated results are yet to be verified. The Bm86 and Bm91 cocktail vaccine used in cattle vaccination only induced a moderate increase in protection over that with Bm86 alone [58].

A multi-antigenic vaccine based on *H. longicornis* GST (rGST-Hl), *Boophilus* yolk cathepsin (rBYC), and vitellin-degrading cysteine-endopeptidase (VTDCE) induced a moderate increase in protection when compared to immunization with rGST-Hl alone [20], suggesting an antigenic competition among the components. Interestingly, rGST-Hl was shown to cross-protect against *R. microplus* and *R. appendiculatus* [37,38], though this protection was limited and did not include protection against other closely related species, *e.g. R. sanguineus* [38]. Therefore, we sought to broaden the GST-based vaccine protection range by combining two or more tick GSTs, toward a broad-spectrum anti-tick vaccine. In this study, we constituted an immunogenic rGST-cocktail using *R. decoloratus* and *A. variegatum* GSTs, which was able to induce immune protection against tick infestation.

GST-Ra, GST-Rd, GST-Av, GST-Rm, GST-Hl showed high similarity with other tick sequences of class Mu GST deposited in Genbank. Similarity among class Mu GSTs from ticks supports the idea that one rGST antigen could partially cross-protect against different tick species, as has been demonstrated using rGST-Hl [37,38]. As proposed previously [18], a rGST-cocktail antigen should enhance the protection efficacy of rGST single-antigen tick vaccines, and broaden the protection range. Other studies along these lines have explored amino acid-based phylogenetic relationship to investigate the potential of Bm86 to cross-protect against a broad range of tick species other than *R. microplus* [59–61]. Accordingly, a rGST-cocktail tick vaccine could be used against infestation by multiple tick species, which is not uncommon in cattle in the field.

Immunogenicity analyses revealed that all tested rGSTs from different tick species were immunogenic in rabbits. Moreover, all tested anti-rGST sera showed cross-recognition of heterologous rGST proteins. Particularly, anti-rGST-Av and anti-rGST-Ra sera showed stronger cross-recognition of heterologous rGSTs,

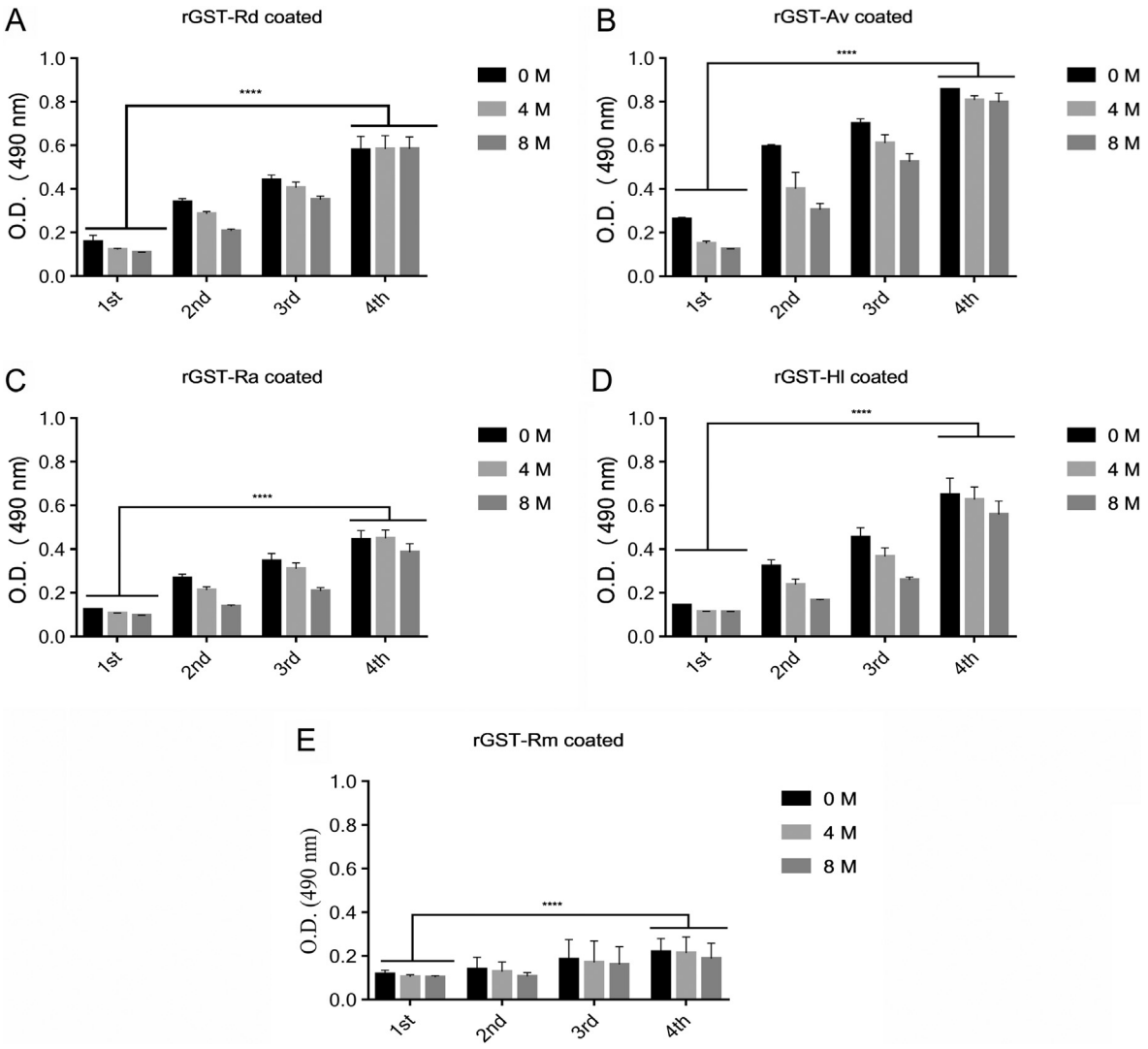

Fig. 6. Increasing avidity of rGST-cocktail serum during immunization, analyzed by ELISA. Rabbits were immunized with a rGST-cocktail comprised of rGSTs from *Amblyomma variegatum* (rGST-Av) and *Rhipicephalus decoloratus* (rGST-Rd). Sera were collected after the 1st, 2nd, and 3rd inoculations, as well as after *Rhipicephalus sanguineus* infestation (4th) and were probed (1:8000 dilution) against rGSTs from *Rhipicephalus decoloratus* (A), *Amblyomma variegatum* (B), *Rhipicephalus appendiculatus* (C), *Haemaphysalis longicornis* (D), and *Rhipicephalus microplus* (E). Data represent mean and standard deviation of triplicate experiments. All analyses were performed in triplicates. Multiple comparisons were performed by two-way ANOVA and *a posteriori* Tukey's test for pairwise comparisons. In all cases, avidity values after the forth immunization were statistically different from the values after the first immunization (with a $p < 0.0001$).

compared to anti-rGST-Rd, anti-rGST-Rm, and anti-rGST-Hl sera. This suggests that a single rGST, specifically rGST-Av or rGST-Rd, could cross-protect against a wider range of tick species, acting as potential broad-spectrum anti-tick vaccines. These data corroborates reports that rGST-Hl induces partial cross-protection against *R. microplus* and *R. appendiculatus* infestations [37,38]. The approach used to test rGST cross-recognition is similar to that previously used to test the potential of 64TRPs antigens to cross-protect against *Ixodes ricinus*, *R. sanguineus*, *R. microplus,* and *A. variegatum*, bringing forward a candidate antigen for a broad-spectrum tick vaccine [62]. The strong cross-recognition of anti-rGST-Av and anti-rGST-Rd sera further implies that both antigens are suitable to constitute a GST-cocktail vaccine. Since rGST-Hl serum also showed cross-recognition of heterologous rGSTs, and has been shown to induce tick cross-protection [37,38], the possibility that rGST-Hl could also be added in the constitution of an effective rGST-cocktail antigen should be kept in mind.

Rabbits immunized with a cocktail containing rGST-Av and rGST-Rd raised antibodies against both proteins, and the amount produced was similar to rabbits immunized with each protein separately. Densitometric analysis showed that over 50% of antibodies recognized each of the rGST-cocktail antigen constituents (rGST-Av and rGST-Rd). This observation suggests a limited competition between the antigens in the cocktail, not affecting the induction of immune response. Indeed, the rGST-cocktail was shown to be immunogenic, since rGST-cocktail serum recognized the constituting antigens (rGST-Av and rGST-Rd), as well as other rGSTs (rGST-Ra, rGST-Hl and rGST-Rm). In contrast, when rGST-HI, rBYC and rVTDCE cocktail was used in immunization experiments, serum analyses revealed lower production of antibodies against rBYC and rVTDCE in comparison with antibodies produced against rGST-HI [20]. Likewise, investigations into formulation of pathogen vaccines showed that antigenic competition reduces the protection efficacy of multi-antigen pathogen vaccines [63–65]. In view of the low antigenic competition among rGST-cocktail constituents, it is possible to hypothesize that rGST-cocktail vaccination could result in high protection against a wide range of tick species.

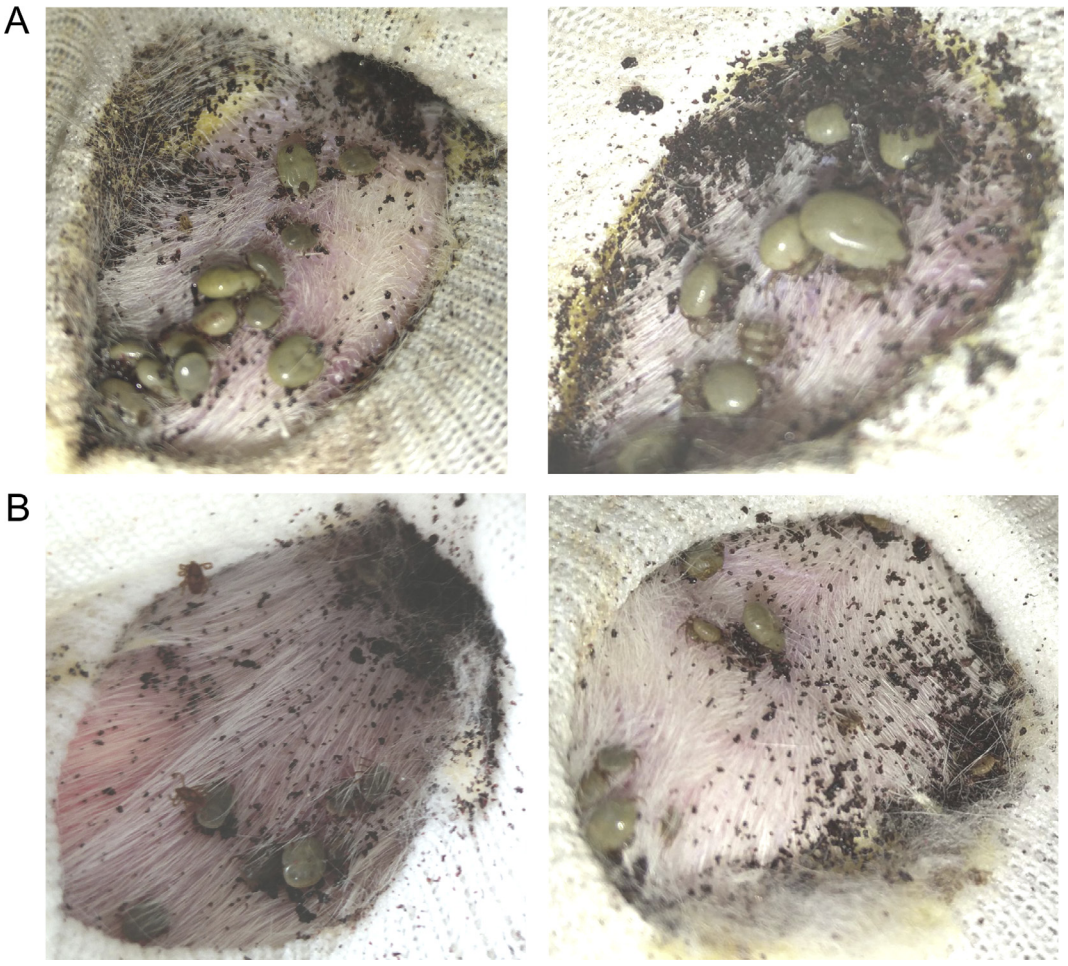

Fig. 7. Biological effect of rGST-cocktail on *Rhipicephalus sanguineus* infestation. Tick engorgement was analyzed in rabbits immunized with PBS (A) or with rGSTs cocktail (B) composed of rGSTs from *Amblyomma variegatum* (rGST-Av) and *Rhipicephalus decoloratus* (rGST-Rd).

Table 1
Biological parameters of *Rhipicephalus sanguineus* fed in GST-cocktail vaccinated and control rabbits.

Group	Rabbit	Tick number[a]	Tick weight[b]	Egg laying[c]	Egg fertility[d]
Control	1	23	83.1	0.555	0.076
	2	19	91.32	0.600	0.114
	3	17	82.12	0.571	0.08
	Mean	19.67	85.52	0.575	0.09
	S.D.	3.05	5.05	0.023	0.021
Vaccination	1	13	103.53	0.588	0.113
	2	10	94.05	0.565	0.099
	3	14	93.79	0.53	0.068
	Mean	12.33	97.12	0.561	0.093
	S.D.	2.08	5.55	0.029	0.023
	Difference[e]	**37.29%**[*]	**−13.57%**	**2.49%**	**−3.74%**

[a] = Number of engorged ticks recovered on rabbits.
[b] = Average weight (mg) of engorged ticks.
[c] = Total egg weight (mg) per total females weight.
[d] = Total larvae weight (mg) per total egg weight.
[e] = Difference (%) = 100 × (1 − mean value of vaccination group/control group).
[*] $p < 0.05$ = statistical significance: analysis performed using Student's *t*-test.

Despite the difficulties that arise from using a multi-antigenic vaccine, there is a consensus that an effective vaccine needs to be multi-antigenic. Therefore, it is important to characterize the effect in the immune response induced by combining more than one antigen in a vaccine. This strategy has multiple implications, since a vaccine based on more than one antigen could induce an immune response against a tick species, or even a cross-protective immune response, inducing protection against a wider range of tick species.

Other important factor in obtaining an adequate immune response is the avidity of antibodies induced by immunization. We show that rGST immunization induced an increase in serum avidity between the first and fourth inoculations. The high-affinity antibody response is an indicative of a good immuniza-

tion/vaccination protocol. Avidity is a common parameter used for differentiating viral vs. bacterial blood infection [52,66,67], for evaluating vaccination protocols, or in vaccine development [68,69]. Moreover, avidity has been used as criteria to characterize experimental vaccines against parasitic worms and unicellular parasites. For instance, it was reported that cattle with high-avidity antibodies after experimental vaccination showed low *Fasciola hepatica* infection burden, demonstrating a correlation between avidity and protection [70]. Also, affinity is an important parameter observed during anti-malaria immunization [71]. Despite being used in research on other parasites, avidity is not commonly used in tick vaccine development; however, it is possible to speculate that high-avidity anti-GST antibodies could strongly bind to tick GST enzymes, consequently interfering with GST biological activities.

Based on previous and present results, rGST-Av and rGST-Rd were selected as cocktail constituents to test immunization against *R. sanguineus* infestation in rabbits. The rGST-cocktail vaccine induced an immune response, reducing by 35.3% the number of adult female tick during infestation. However, it did not significantly affect adult female weight, reproductive parameters (egg viability), or hatched larvae. A GST vaccine exclusively affecting tick female numbers was already observed when rGST-Hl was tested against *R. microplus* infestation [37]. Moreover, rGST-Av and rGST-Rd protection against *R. sanguineus* infestation was similar to the one obtained when rabbits were immunized with rGST-Hl and challenged with *R. appendiculatus*, but not *R. sanguineus* [38]. Interestingly, GST-RNAi-treated *R. sanguineus* were susceptible to lower concentrations of acaricides compared to control non-treated tick [26]. Together, these results suggest that interfering with GST biological functions may induce physiological alterations in the tick, affecting survival when ticks are challenged by the host immune system, or by acaricides.

The present study illustrates a systematic approach that could be used for constituting cocktail-antigen tick vaccines. We have thereby developed a rGST-cocktail antigen immunogenic against a range of tick species. The vaccine was able to reduce the size of parasite population, likely by inducing multiple biological effects, and can potentially further enhance tick susceptibility to acaricides. This and previous work demonstrate that tick GSTs have a potential to be used as antigens in a broad-spectrum tick vaccine.

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Conflict of interest

The authors certify that they have no affiliations with, or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.02.039.

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