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VACINA DE GLUTATIONA S-TRANSFERASES COMO ESTRATÉGIA DE CONTROLE DE CARRAPATO

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LISTA DE ABREVIATURAS

cDNA: Ácido desoxirribonucleico complementar

DNA: Ácido desoxirribonucleico

EDTA: ácido etilenodiaminotetracético

ELISA: Enzyme-linked immunosorbent assay

IgG: Imunoglobulina G

mRNA: Ácido ribonucleico mensageiro

NBT: Cloreto de tetrazólio-nitroazul

ORF: Fase de leitura aberta

PBS: Tampão fosfato-salina

PBS-T: Tampão fosfato-salina contendo Tween

PCR: Reação em cadeia da polimerase

PVDF: Fluoreto de polivinilideno

qPCR: Reação em cadeia da polimerase quantitativa

RNA: Ácido ribonucleico

SDS-PAGE: Eletroforese em gel de poliacrilamida na presença de SDS

RESUMO

A glutationa S-transferase (GST) constitui uma superfamília de enzimas que desempenham um papel na desintoxicação de compostos endógenos e exógenos. Portanto, em carrapatos, presume-se que as GSTs promovam resistência aos acaricidas e demonstrem induzir proteção cruzada parcial contra carrapatos. Esta tese teve como objetivo constituir uma vacina baseada em GST multi-antígeno para proteção de diferentes espécies de carrapatos. Os antígenos constituintes do coquetel (antígeno) foram selecionados com base na reatividade do soro anti-rGST com antígenos rGST homólogos e heterólogos de *Rhipicephalus appendiculatus* (rGST-Ra), *Rhipicephalus decoloratus* (rGST-Rd), *Amblyomma variegatum* (rGST-Av), *Rhipicephalus microplus* (rGST-Rm) e *Haemaphysalis longicornis* (rGST-Hl). Subsequentemente, usando algoritmos baseados em sequência e em estruturas, foram previstos os soros anti-rGST epítopos de células B de reação cruzada nas sequências de GST. Além disso, a imunogenicidade dos péptidos correspondentes ao epítopo foi validada in vivo. Investigou-se o potencial das vacinas em induzir proteção contra a infestação de *Rhipicephalus sanguineus* e *R. appendiculatus* em coelhos. Os candidatos para a constituição dos coquetéis foram os antígenos rGST-Rd, rGST-Av e rGST-Hl. Particularmente, o coquetel 1 foi constituído por rGST-Av, rGST-Rd e rGST-Hl, enquanto o coquetel 2 por rGST-Av e rGST-Rd. Semelhante ao rGST-Hl, os coquetéis 1 e 2 tiveram impacto nos parâmetros biológicos (número e peso dos carrapatos, peso e fertilidade dos ovos) da infestação dos coelhos por *R. appendiculatus*. No entanto, uma diferença estatística no número de carrapatos (12,28%) e postura (37,17%) foi induzida apenas com o coquetel 1 e GST-Hl, respectivamente. O coquetel 2 impactou ainda mais os parâmetros biológicos: número de carrapatos (37,29%) e peso de ovos (2,49%), da infestação por *R. sanguineus*. Além disso, os epítopos de GST de células B previstos foram localizados na superfície dos modelos de GST e os peptídeos correspondentes induziram uma resposta imune nos coelhos. Além disso, os soros anti-rGST reagiram contra o conjugado BSA-peptídeo correspondente. Estes dados sugerem que um multi-antígeno pode ser constituído com base nos epítopos-peptídeos. Por fim, os coquetéis constituídos podem induzir proteção contra os carrapatos de importância econômica para a Uganda, África (*R. decoloratus*, *R. appendiculatus* e *A. variegatum*) e o Brasil, América do Sul (*R. microplus*).

ABSTRACT

Glutathione S-transferase (GST) constitute a superfamily of enzymes that play a role in detoxifying endogenous and exogenous compounds. Hence, in ticks, GSTs are presumed to foster acaricide resistance and are shown to induce partial cross-protection against ticks. This thesis, therefore, aimed to constitute a multi-antigen GST based vaccine toward multiple tick specie protection. The cocktail (multi-antigen) constituent antigens were selected based on the anti-rGST sera reactivity with homologous and heterologous rGST antigens of *Rhipicephalus appendiculatus* (rGST-Ra), *Rhipicephalus decoloratus* (rGST-Rd), *Amblyomma variegatum* (rGST-Av), *Rhipicephalus microplus* (rGST-Rm) and *Haemaphysalis longicornis* (rGST-Hl). Subsequently, using sequence and structural-based algorithms, the anti-rGST sera cross-reacting B-cell epitopes within the GST sequences were predicted. Additionally, the immunogenicity of the epitope corresponding peptides was validated *in vivo.* The potential of the cocktail vaccines to induce protection against *Rhipicephalus sanguineus* and *R. appendiculatus* rabbit infestation was investigated. The candidates for constituting the cocktails were rGST-Rd, rGST-Av and rGST-Hl antigens. Particularly, cocktail 1 and cocktail 2 were made up of rGST-Av, rGST-Rd and rGST-Hl and rGST-Av and rGST-Rd, respectively. Similar to rGST-Hl, cocktail 1 and 2 impacted on the biological parameters (tick number, tick weight, egg weight, fertility) of *R. appendiculatus* rabbit infestation. However, a statistical difference in tick number (12.28%) and egg laying (37.17%) was only induced with cocktail 1 and GST-Hl respectively. Cocktail 2 further impacted on the biological parameters: tick number (37.29%) and egg weight (2.49%), of *R. sanguineus* rabbit infestation. Moreover, the predicted B-cell GST epitopes were located on the surface of the GST models and the corresponding peptides induced an immune response in rabbits. Furthermore, the anti-rGST sera reacted against the corresponding BSA-peptide conjugate. These data suggest that a multi-antigen could be constituted based on the epitope-peptides. Ultimately, the constituted cocktails could induce protection against the cattle ticks of economic significance to Uganda, Africa (*R. decoloratus*, *R. appendiculatus* and *A. variegatum*) and Brazil, Souther America (*R. microplus*).

1. Introdução

Carrapatos são ectoparasitas hematófagos capazes de transmitir uma variedade de organismos patogênicos, como protozoários, vírus, fungos, helmintos e bactérias, para animais e para o homem (JONGENJAN & UILENBERG, 2005). Apesar dos carrapatos estarem presentes em todos os continentes, a sua distribuição varia dependendo da região geográfica.

A ação espoliativa desses parasitos pode resultar em anemia aos animais de produção, além de causarem doenças reduzindo a produção de carne, leite e couro, constituindo-se assim numa limitante para a produção pecuária. Por exemplo, no continente Americano, o carrapato *Rhipicephalus microplus*, vulgarmente conhecido como o "carrapato do boi", é o hospedeiro intermediário de *Anaplasma* spp. e *Babesia* spp, patógenos que causam a anaplasmose e a babesiose, respectivamente (PIERCE, 1956; FUTSE *et al*., 2003). Por outro lado, na África oriental encontram-se os carrapatos *Rhipicephalus appendiculatus* (transmissor dos protozoários *Theileria* spp*,* agente da ¨East coast fever¨) (COWDRY & HAM, 1932; OLDS *et al*., 2018), *Amblyoma variegatum* (transmissor da bactéria *Ehrlichia ruminantium,* agente da "cowdriosis" (ALLSOPPl, 2010); e *Rhipicephalus decolaratus* (transmissor dos protozoários *Babesia* spp. e da bactéria *Anaplasma* spp, agentes da babesiose e anaplasmoses, respectivamente) (OKON *et al*., 2011; AKINBOADE *et al*., 1981).

Entretanto, *R. microplus* tem sido reportado como uma espécie invasora de novas regiões como na África ocidental (DE CLERCQ *et al*., 2012; MADDER *et al*., 2012; ADAKAL *et al*., 2013), na África central (SILATSA *et al*., 2019), leste de África (LYNEN *et al*., 2008) e na África austral (NYANGIWE *et al*., 2017; TØNNESEN *et al*., 2004). Do mesmo modo que em outras regiões, o carrapato *R. microplus* causa perdas elevadas na indústria pecuária na África. Contudo, ainda não existem dados epidemiológicos suficientes sobre o real impacto das doenças transmitidas pelos carrapatos no continente africano.

Apesar da diferente distribuição das espécies de carrapatos, o uso de acaricidas ainda é a principal forma de controle em todas regiões geográficas onde se pode encontrar carrapatos de importância para o homem e para os animais. O uso indiscriminado dos acaricidas acelera a seleção de populações de carrapatos resistentes. Há casos reportados de resistência dos carrapatos aos compostos dos acaricidas frequentemente usados no sul e

centro do continente americano, na África e na Oceania (ABBAS *et al*., 2014). Além disso, existe a preocupação com a contaminação dos produtos de origem animal e poluição ambiental. Devido a esse fato, alternativas no controle de carrapatos têm sido sugeridas (MANJUNATHACHAR *et al*., 2014; GOSH *et al*., 2007).

Dos carrapatos de interesse econômico, nessa tese focamos em três espécies africanas (*R. appendiculatus, Amblyoma variegatum* e *R. decoloratus*), uma asiática (*Haemaphysalis longicornis*) e a mais importante das Américas (*R. microplus*), esta também presente na região sul e central de África.

1.1. Ciclo de vida dos carrapatos em estudo

Os carrapatos podem ser classificados baseados nos seus ciclos de vida. Como exemplo, *R. appendiculatus*, *H. longicornis* e *A. variegatum* são carrapatos de três hospedeiros, enquanto que *R. decoloratus* e *R. microplus* são carrapatos de um hospedeiro. As descrições dos ciclos de vida dos carrapatos de interesse do presente estudo são apresentadas abaixo.

1.2. Carrapatos de três hospedeiros

O termo carrapato de três hospedeiros deriva do fato de que durante os estágios de desenvolvimento de larva, ninfa e adulto, alguns carrapatos alimentam-se em três diferentes hospedeiros (Figura 1). Durante o desenvolvimento, estes carrapatos podem alimentar-se em uma variedade de hospedeiros como roedores, veados, cavalos, ovelhas, cabras, gatos e coelhos, entre outros animais.

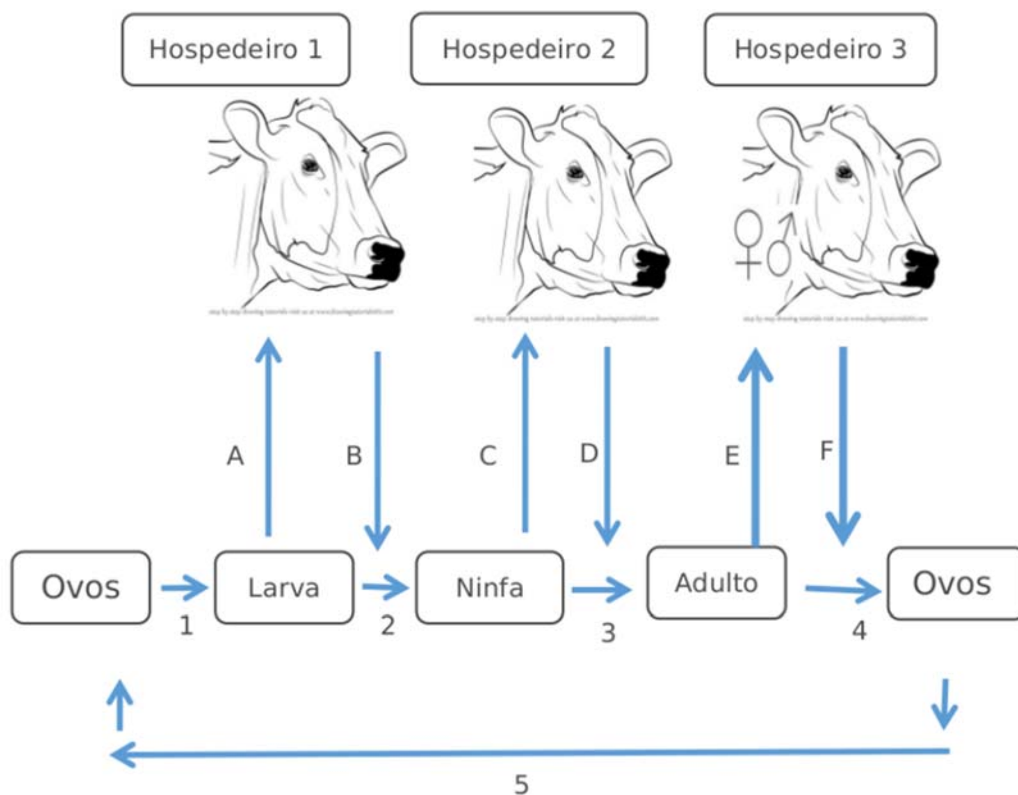

Figura. 1. Representação esquemática do ciclo de vida de carrapato de três hospedeiros. As larvas eclodem dos ovos (1). As larvas encontram (A) o hospedeiro 1, fixam-se e alimentam-se até que fiquem completamente ingurgitadas. Depois de ingurgitadas, a larva cai (B) ao solo e realiza a muda para ninfa (3). As ninfas encontram (C) o hospedeiro 2, fixam-se e alimentam-se até ficarem completamente ingurgitadas. As ninfas ingurgitadas caem (D) para o solo e mudam para adultos. O carrapato adulto macho ou fêmea encontra um hospedeiro (E) ocorre o acasalamento e as fêmeas alimentam-se e caem ao solo para a postura.

O ciclo descrito na Figura 1 é comum e genérico para carrapatos de três hospedeiros. Todavia, cada espécie tem características próprias, como descrito a seguir. Diferenças entre *R. appendiculatus* e *A. variegatum* foram obtidas do protocolo de manutenção do ciclo de vida dos carrapatos do instituto de pesquisa Internacional Livestock Research Institute Nairobi, Quênia (personal communication). Diferenças entre *H. longicornis* obtidas (ACG Health, 2015).

Tabela 1. Características dos carrapatos de três hospedeiros

Amblyomma variegatum	*Rhipicephalus appendiculatus*	*Haemaphysalis longicornis*
1. Tempo para eclosão das larvas (30°C): 2-6 semanas, mas as larvas demoram para eclodir em baixas temperaturas. **2.** Tempo para ingurgitamento de larvas: 5-10 (em coelhos) ou 8 dias (em bovinos). **3.** Tempo para muda de larva para ninfa: 2 semanas. **4.** Tempo para Ingurgitamento de ninfa 5-8 dias (em coelhos ou bovinos). **5.** Tempo para muda de ninfa para adultos: pelo menos 4 semanas. **6.** Tempo para machos adultos fixarem-se antes da fêmea começarem a alimentar-se: 6 dias. **7.** Tempo para ingurgitamento da fêmea: 10-14 dias. Tempo para postura de ovos: 3 semanas. **8.** Tempo para eclosão das larvas é dependente da umidade (em média 90). **9.** Antes de alimentação os adultos começam a exibir comportamento de procura do hospedeiro.	**1.** Tempo para eclosão das larvas: 7-10 dias. **2.** Tempo para ingurgitamento da larva: 5-8 dias. **3.** Tempo para muda de larva para ninfa: 8-10 dias. **4.** Tempo para ingurgitamento de ninfa: 4-5 dias. **5.** Tempo para muda de ninfa para adulto: 12-15 dias. **6.** Tempo para fêmea adulta ingurgitada: 7-13 dias (em coelhos) ou 6-11 dias (em bovinos). **7.** Tempo para postura de ovos a 28°C: 10-14 dias. **8.** Postura de ovos não é afetado pela umidade. **9.** Os carrapatos adultos exibem comportamento de procura de hospedeiro. **10.** A ausência do macho fixado não afeta o processo de alimentação da fêmea. **11.** Machos e fêmeas podem começar a alimentar-se ao mesmo tempo.	**1.** Tempo para eclosão das larvas: 60-90 dias (de 28-32°C). **2.** Tempo para ingurgitamento de larvas: 3-9 dias. **3.** Tempo para ingurgitamento de ninfas: 3-8 dias. **4.** Tempo para muda de ninfa para adultos: 40 dias. **5.** Tempo para ingurgitamento de adultos: 7-14 dias. **6.** Fêmeas adultas de alguns isolados são partenogenéticas, consequentemente a alimentação dessas não dependem da presença dos machos.

1.3. Carrapatos de 1 hospedeiro

O termo carrapato de 1 hospedeiro deve-se ao fato de que durante todas as fases de desenvolvimento de larva, ninfa e adulto, essas espécies alimentam-se somente em um hospedeiro. Semelhante aos carrapatos de três hospedeiros, durante o desenvolvimento os carrapatos de 1 hospedeiro podem alimentar-se em uma vasta gama de hospedeiros como veado, cavalos, ovinos, felinos e outros animais.

O ciclo descrito na Figura 2 é comum e genérico para carrapatos de um hospedeiro. Todavia, cada espécie tem características próprias. As características da espécie *R.*

decoloratus foram obtidas do protocolo de manutenção do ciclo de vida dos carrapatos do Internacional Livestock Research Institute Nairobi, Quênia (documento interno, não publicado). As características do *R. microplus* foram obtidas de SENBIL *et al*. (2018).

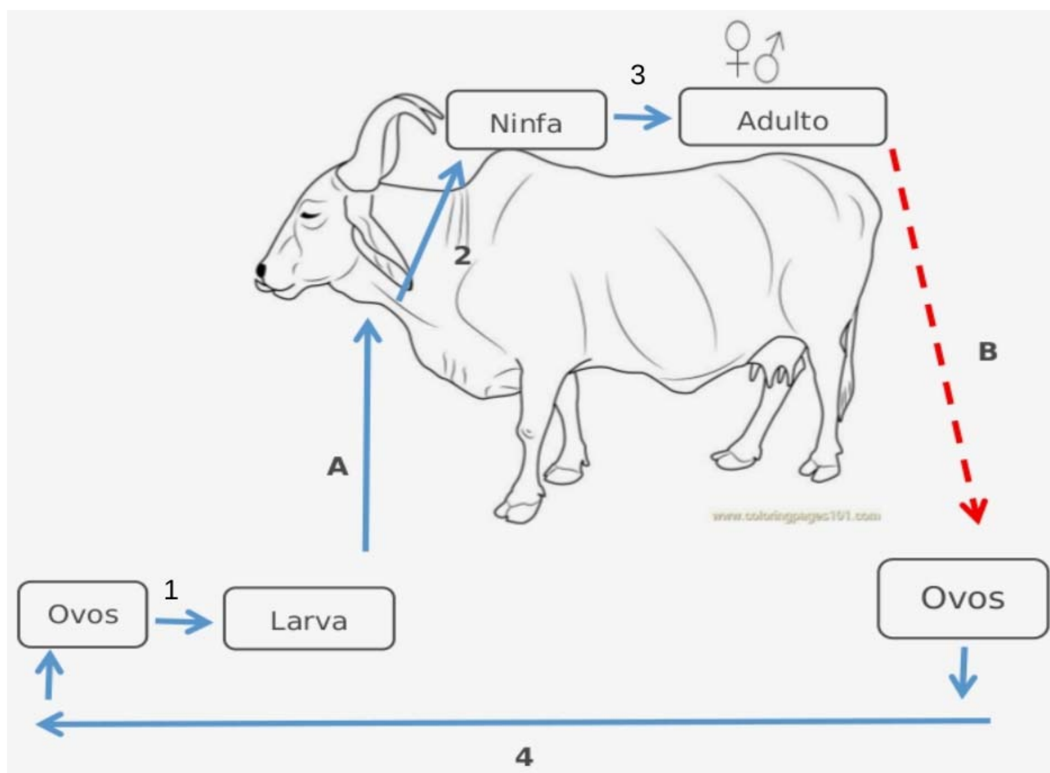

Figura 2. Representação esquemática do ciclo de vida de carrapato de 1 hospedeiro. As larvas eclodem dos ovos (1). A larva encontra (A) o hospedeiro, fixa-se, e alimenta-se até ficar totalmente ingurgitada. Depois de ingurgitada, as larvas mantem-se fixada no hospedeiro e depois faz a muda para ninfa (2). As ninfas alimentam até que essas ficam completamente ingurgitadas. As ninfas ingurgitadas mantem-se fixadas no hospedeiro e mudam para adultos (3). Enquanto ainda no hospedeiro, a fêmea e o macho cruzam e se alimentam, até a fêmea ficar completamente ingurgitada. A fêmea ingurgitada cai (D) no solo e esconde-se na vegetação para postura de ovos.

Tabela 2. Características dos carrapatos de 1 hospedeiro

Rhipicephalus decoloratus	*Rhipicephalus microplus*
1. Tempo para alimentação, muda de larva para ninfa: 3-4 dias. **2.** Tempo para alimentação, ninfa para adulto: 4-5 dias. **3.** Tempo para adulto ingurgitado: 7-10 dias	**1.** Tempo para alimentação, muda de larva para ninfa: 7-8 dias. **2.** Tempo para alimentação, muda de ninfa para adultos: 10-11 dias. **3.** Tempo para alimentação de adultos: 7-8 dias.

1.4. Métodos principais de controle de carrapatos

1.4.1. Controle químico

Atualmente o método químico pelo uso de acaricidas é o mais utilizado para combater infestação de animais pelos carrapatos. Mas, antes da introdução de acaricidas industriais, foram usadas outras substancias químicas para controlar carrapatos. Por exemplo, nos Estados Unidos, as substâncias usadas no passado para controlar carrapatos foram: banha com enxofre, banha com querosene, óleo de semente de algodão, querosene com enxofre, emulsão de querosene 10% e óleo de semente de algodão com petróleo (GEORGE *et al*., 2008). Na Austrália, bovinos eram imersos em tanques de banho com óleo minerais e ácido fênico (ANGUS, 1996).

As primeiras formulações químicas de acaricidas foram feitas com base de compostos arsênicos (GEORGE, 2000). Por serem mais efetivos, os acaricidas arsenicais foram adotados internacionalmente para controle de infestação dos animais por carrapato. Resistência aos compostos arsenicais pelo carrapato *R. microplus* foram reportados pela primeira vez na África do Sul (WHITEHEAD, 1958) e na Austrália (NEWTON, 1967).

Na década de 1940, os inseticidas organoclorados, tais como diclorodifeniltricloroetano (DDT) e os hexacloreto benzeno (BHL), foram introduzidos para o controle dos carrapatos (SHAW, 1970). Além disso, os organoclorados persistem no ambiente (DENIS & EDWARDS, 1964; JAYARAJ *et al*., 2016), tornando-se um risco de bioacumulação, além de contaminação de produtos de origem animal. Por estes motivos, os produtos organoclorados foram retirados do mercado mundial (GRAHAM & HOURRIGAN, 1977; AKTAR *et al*., 2009). Os organoclorados foram substituídos pelos organofosforado e piretroides sintéticos. Esses compostos são mais rapidamente metabolizados e requerem um pequeno período de carência (GRAF *et al*., 2004). Entretanto, após alguns anos de uso, foram identificadas populações carrapatos resistentes aos organoclorados piretroides sintéticos. Desde então, outras classes de acaricidas têm sido introduzidas ou produzidas, como carbamatos (Carbaryl, promacyl), formamidinas (amitraz), fenilpirazol (fipronil) e lactonas macrociclicas (invermetina).

Levando-se em consideração a resistência aos acaricidas, a rotação dos acaricidas (THULLNER *et al*., 2007) e a combinação de acaricidas (DUMONT *et al*., 2015; FOURIE *et al*., 2011) são propostas atrativas para combate aos carrapatos resistentes. Apesar disso,

ambas as estratégias podem ser de alto custo e apresentam limitações**.** Como exemplo, podemos citar a ausência de dados consistentes relativos ao intervalo de troca no uso de novos acaricidas, assim como a concentração na constituição da combinação de acaricidas, acarretando no risco de múltipla resistência aos acaricidas.

Indubitavelmente, o uso de acaricidas químicos continua sendo a abordagem com maior sucesso para controle dos carrapatos. Mas o uso indiscriminado dos mesmos químicos levanta a preocupação de potencial carcinogênico, poluição ambiental e contaminação de produtos de origem animal e a falta de meios de monitorização para a múltipla resistência aos acaricidas (GRAF *et al*., 2004). Tais limitações aumentam a necessidade de métodos alternativos para o controle dos carrapatos.

1.4.2. Controle imunológico dos carrapatos

Em busca de formas alternativas aos acaricidas químicos para o controle de carrapatos, a estratégia imunológica é considerada um método ambientalmente amigável e sustentável. O conceito de vacinação para o controle de carrapatos foi demonstrado pela primeira vez por TRAGER (1939). Até o momento, inúmeros antígenos dos carrapatos foram identificados para comporem tais vacinas (DE LA FUENTE & KOCAN, 2006; NUTTALL et al., 2006; VALLE & GUERRERO, 2018). Entre essas moléculas identificadas, pode-se classifica-las entre antígenos ocultos e expostos (NUTTALL et al., 2006). Antígenos ocultos são aqueles que não são expostos ao sistema imune do hospedeiro durante a alimentação do carrapato, enquanto que os antígenos expostos são moléculas dos carrapatos que entram em contato com o sistema imune dos hospedeiros, principalmente presentes na saliva.

Presumivelmente, o fundamento das vacinas contra carrapatos é que, durante a alimentação, o carrapato ingere sangue que contém anticorpos contra moléculas presente em diferentes tecidos do parasito. De fato, há evidências de que anticorpos podem atravessar a membrana do epitélio intestinal do carrapato e se difundir para diferentes tecidos (DA SILVA VAZ et al., 1996). Relatos demonstram, ainda, que os anticorpos podem se ligar às proteínas de diferentes tecidos do carrapato (TRIMNELL et al., 2002). Especula-se, portanto, que os anticorpos possam interferir na funcionalidade das proteínas teciduais as quais interagem. E consequentemente, dependendo da função da proteína do carrapato, a

vacina pode afetar diferente parâmetros biológicos. Isso inclui a redução no número de carrapatos ingurgitados, ingestão de sangue, número e viabilidade dos ovos e período de ingurgitamento.

Além de afetar os parâmetros biológicos, também foi investigada a possibilidade de as vacinas interferirem na transmissão de patógenos (LABUDA et al., 2006). Ademais, foi relatado que algumas vacinas contra carrapatos induzem proteção contra diferentes espécies de carrapatos (PIPANO et al., 2003; DE VOS et al., 2001; PARIZI et al., 2011; SABADIN et al., 2017). Apesar do fato de numerosas moléculas dos carrapatos terem sido relatadas para comporem um antígeno vacinal, a proteína BM86 continua sendo a mais bem-sucedida em condições de campo (DE LA FUENTE et al 2007). Também foi sugerido que a combinação de pelo menos 2 antígenos poderia aumentar a eficácia das vacinas contra carrapatos (WILLADSEN, 2008). De fato, o conceito foi adotado (COUMOU et al., 2015) e, no entanto, o resultado esperado ainda está para ser alcançado. Entretanto, é improvável que o controle total seja alcançado usando-se apenas um método, mas sim uma combinação entre diferentes metodologias. Por exemplo, o uso em conjunto de acaricidas químicos com vacinas (THULLNER, et al., 2007).

1.5. Glutationa S-transferases

Glutationa S-transferases (GST) são uma classe de enzimas virtualmente presentes em todos os organismos vivos. Essas enzimas foram relatadas pela primeira vez em humanos (BOOTH *et al*., 1962) e, desde então, têm sido estudadas em diferentes organismos. Por exemplo, em insetos, as GST são classificadas em citosólica e microssomal (ENAYATI *et al*., 2005; RANSON *et al*., 2001). Em *Ixodes scapularis*, 35 genes de GST foram relatados (NIRANJAN *et al*., 2011). Em outras espécies de carrapatos também foram identificados dois genes de GSTs (HERNANDEZ 2018; DREHER-LESNICK *et al*., 2006). No entanto, as classes de GST em carrapatos não estão definidas. Em insetos, as classes citosólicas e microssomais das GST desempenham um papel na conjugação de compostos eletrofílicos à glutationa reduzida (SALINAS & WONG 1999). Evidências mostram que através da reação de conjugação, as GST desempenham um papel fundamental na desintoxicação de xenobióticos e endobióticos (HABIG *et al*., 1972). Por essa razão, as GST foram implicadas na resistência a inseticidas (PRAPANTHADARA et al., 1993; HUANG et al., 1998;

VONTAS et al., 2001). Da mesma forma, em carrapatos, GST foram sugeridas para desempenhar um papel na desintoxicação de acaricidas (DA SILVA VAZ *et al*., 2004; HERNANDEZ *et al*., 2018; DUSHER *et al*., 2014). Portanto, presume-se que as GST participam da resistência a acaricidas, porém o mecanismo de ação ainda não está claro. Dado o papel na desintoxicação, as GSTs foram exploradas como um alvo para o desenvolvimento de vacinas contra patógenos (BALLOU *et al*., 1987; PREYAVICHYAPUGDEE *et al*., 2008; SEXTON *et al*., 1990) e carrapatos (PARIZI *et al*., 2011; SABADIN *et al*., 2017). Em particular, a GST recombinante de *H. longicornis* mostrou induzir proteção parcial cruzada contra *R. appendiculatus* e *R. microplus*. Nesta tese, foi analisado se uma combinação 2 ou mais GST poderia induzir proteção contra outras espécies de carrapatos.

2. Objetivo geral

Estudo do potencial de utilização de GST para vacinas multi antigênicas universais no controle dos carrapatos endêmicos da Uganda, Àfrica (*R. appendiculatus, A. variegatum* e *R. decoloratus*) e Brasil, América do Sul (*R. microplus*).

2.1. Objetivos específicos

- Desenvolvimento de uma vacina multi-antigênica baseada em diferentes GST.
- Predição dos epítopos de célula-B altamente conservados nas sequências de GST dos carrapatos.
- Investigação do potencial de proteção das vacinas multi-antigênicas da GST contra os carrapatos da África e do Brasil.

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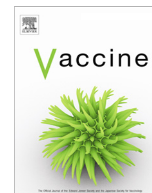

Constituting a glutathione S-transferase-cocktail vaccine against tick infestation

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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.

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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-

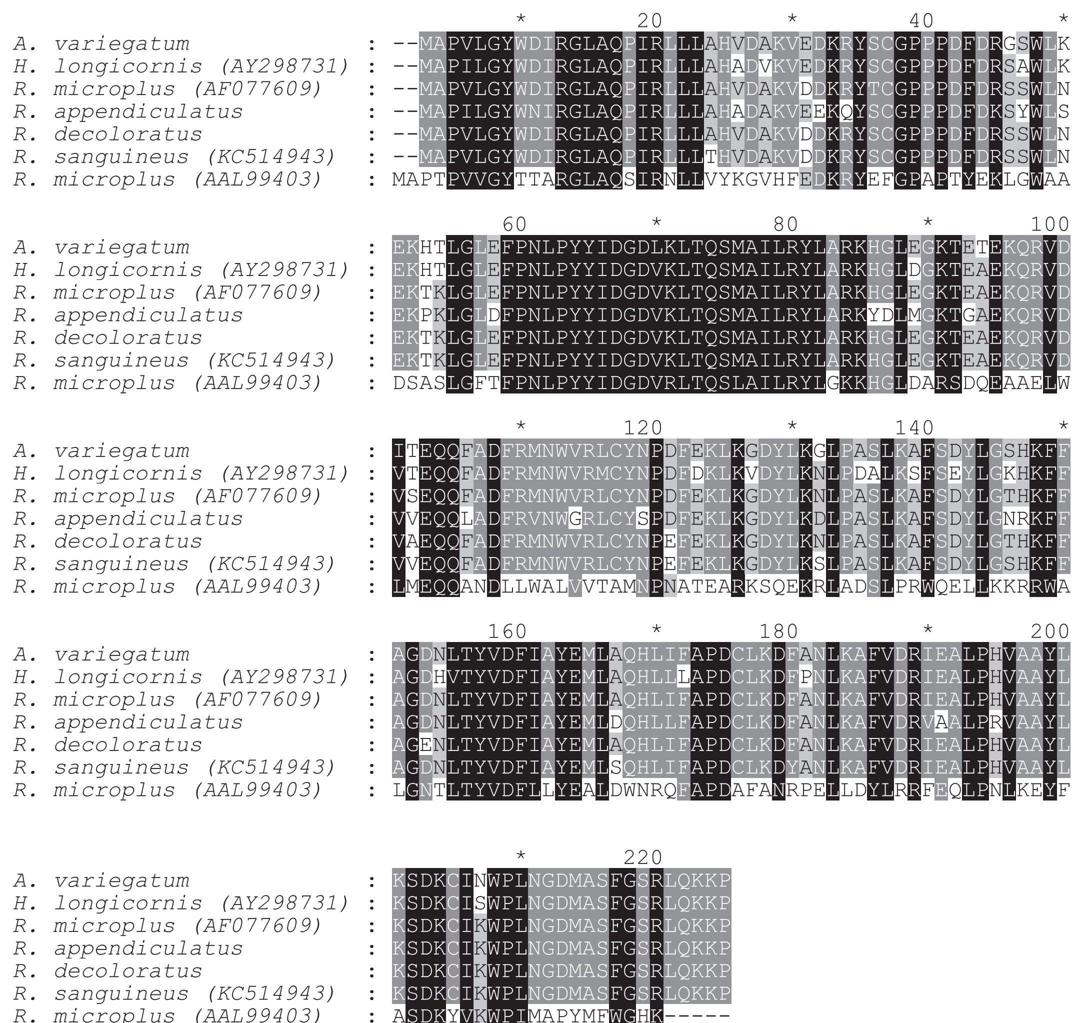

Fig. 1. Full-length GST amino acid sequence alignment. ClustalW alignment of sequences obtained in this study (*Rhipicephalus appendiculatus* MK133338, *Rhipicephalus decoloratus* MK133339, *Amblyomma variegatum* MK133337), and sequences retrieved from NCBI Genbank (*Rhipicephalus microplus* (AF077609 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, inter-species 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.

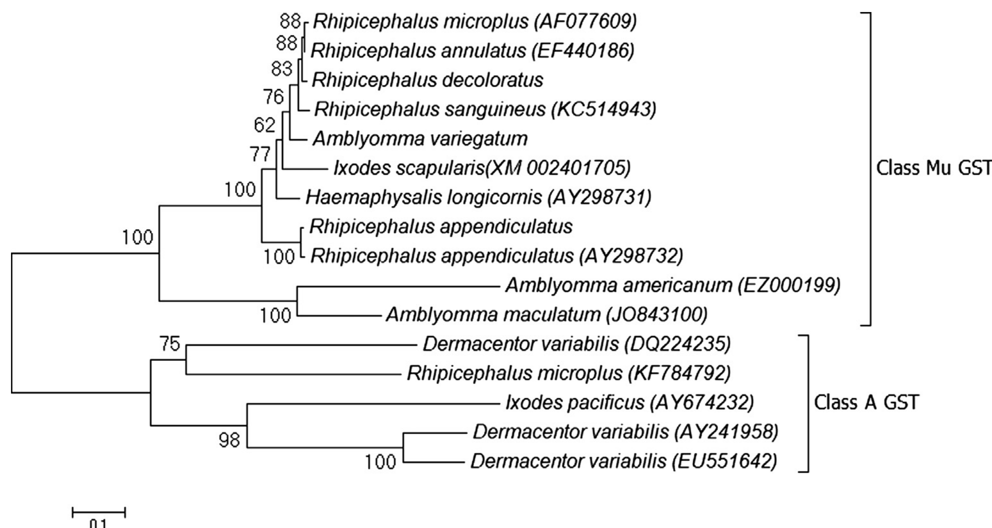

Fig. 2. Phylogenetic relationship of tick GSTs deduced amino acid sequences obtained in this study from *Rhipicephalus appendiculatus*, *Rhipicephalus decoloratus*, *Amblyomma variegatum*, and tick GSTs sequences retrieved from NCBI Genbank: *Rhipicephalus microplus* (AF077609), *Rhipicephalus annulatus* (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).

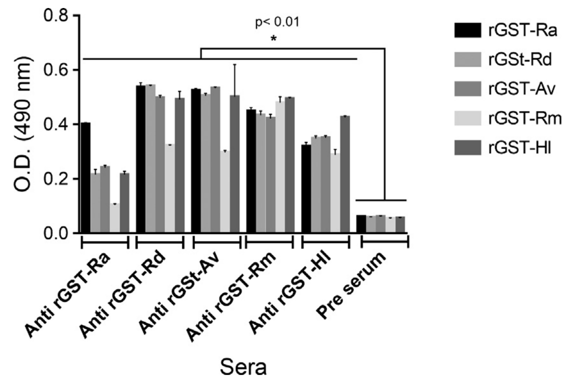

Fig. 3. Antigenicity of tick rGSTs and cross-reactivity of anti-rGST sera. Rabbits were immunized with one of the following rGST: *Rhipicephalus appendiculatus* (rGST-Ra), *Rhipicephalus decoloratus* (rGST-Rd), *Amblyomma variegatum* (rGST-Av), *Rhipicephalus microplus* (rGST-Rm) or *Haemaphysalis longicornis* (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 ($p < 0.01$).

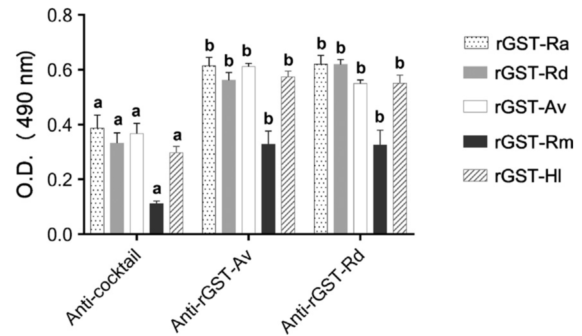

Fig. 5. Cross-reactivity of sera induced against rGSTs, analyzed by ELISA. Rabbits were immunized with rGSTs from *Rhipicephalus decoloratus* (rGST-Rd), *Amblyomma variegatum* (rGST-Av), and rGST-cocktail (comprised of rGSTs from *Amblyomma variegatum* (rGST-Av) and *Rhipicephalus decoloratus* (rGST-Rd). 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 ($p < 0.001$).

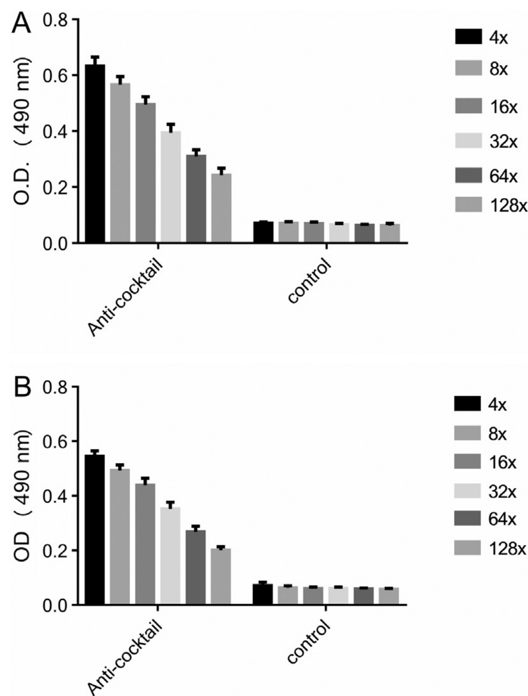

Fig. 4. Reactivity of anti rGST-cocktail serum analyzed by ELISA. Rabbits were immunized with rGST-cocktail comprised of rGSTs from *Rhipicephalus decoloratus* (rGST-Rd) and *Amblyomma variegatum* (rGST-Av). The serum produced against rGST-cocktail (anti-cocktail) was tested in dilutions ranging from 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,

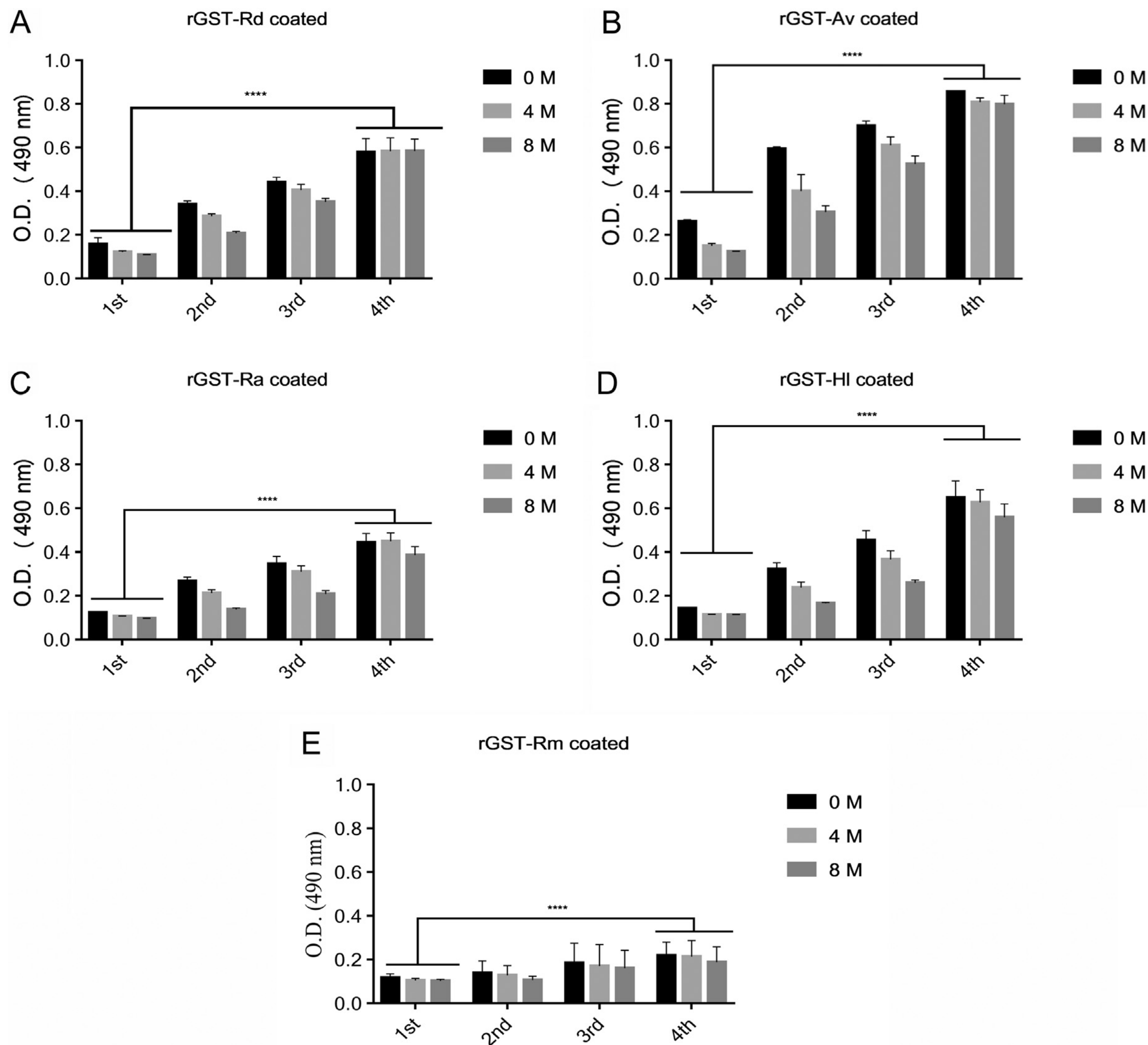

Fig. 6. Increasing avidity of rGST-cocktail serum during immunization, analyzed by ELISA. Rabbits were immunized with a rGST-cocktail comprised of rGSTs from *Amblyomma variegatum* (rGST-Av) and *Rhipicephalus decoloratus* (rGST-Rd). Sera were collected after the 1st, 2nd, and 3rd inoculations, as well 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.

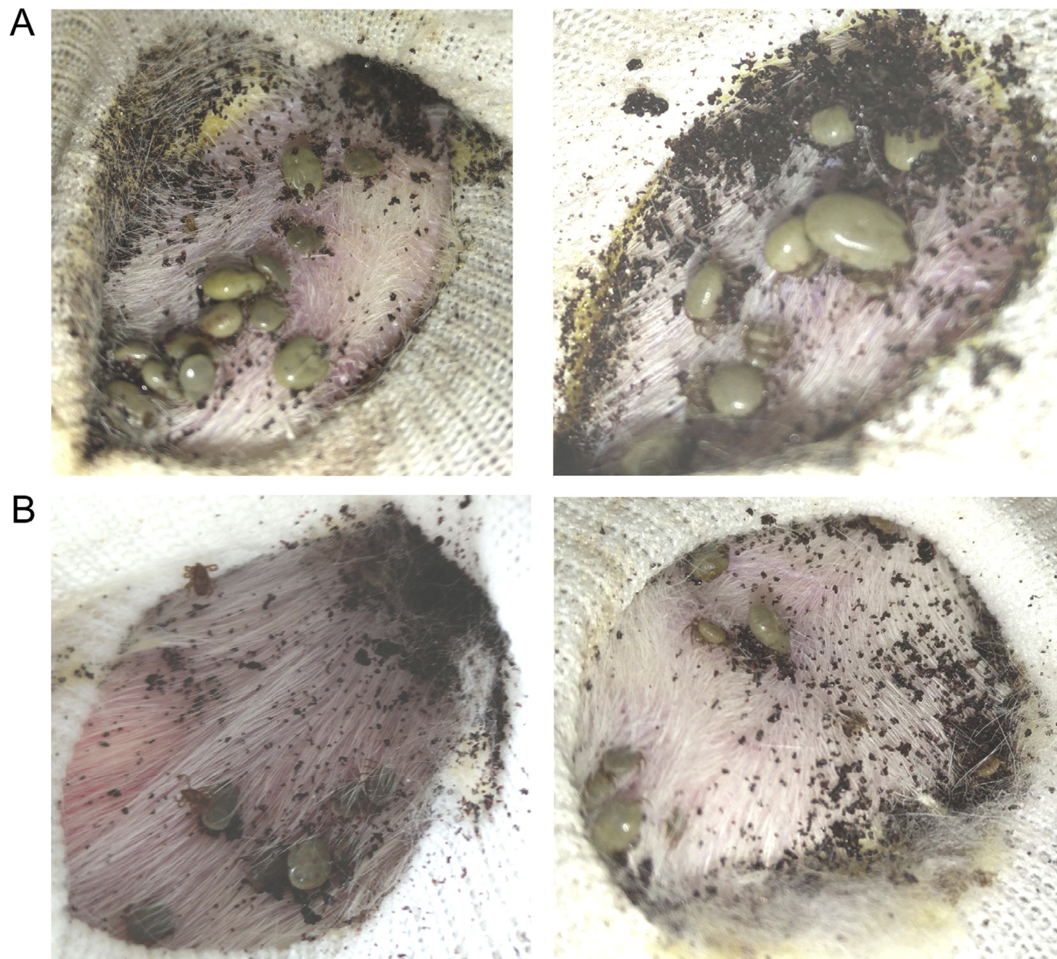

Fig. 7. Biological effect of rGST-cocktail on *Rhipicephalus sanguineus* infestation. Tick engorgement was analyzed in rabbits immunized with PBS (A) or with rGSTs cocktail (B) composed of rGSTs from *Amblyomma variegatum* (rGST-Av) and *Rhipicephalus decoloratus* (rGST-Rd).

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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3.0. CAPÍTULO 1: ¨Constituting a glutathione S-transferase-cocktail vaccine against tick infestation¨

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4. CAPÍTULO 2: "*In silico* prediction, mapping and validation of tick glutathione S-transferase B-cell epitopes" (Submitted to the Ticks and Tick-borne Diseases Journal)

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***In silico* prediction, mapping and validation of tick glutathione S- transferase B-cell epitopes**

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Abstract

In search of ways to address the increasing incidence of global acaricide resistance, tick control through vaccination is regarded as a sustainable alternative approach. Recently, a novel cocktail antigen tick-vaccine was proposed based on recombinant glutathione S-transferase (rGST) anti-sera cross-reaction against glutathione S-transferases of *Rhipicephalus appendiculatus* (GST-Ra), *Amblyomma variegatum* (GST-Av), *Haemaphysalis longicornis* (GST-Hl), *Rhipicephalus decoloratus* (GST-Rd) and *Rhipicephalus microplus* (GST-Rm). Therefore, the current study aimed to predict the shared B-cell epitopes within the GST sequences of *R. appendiculatus, A. variegatum, R. microplus, H. longicornis* and *R. decoloratus*. Prediction of B-cell epitopes and proteasomal cleavage sites was performed using immunoinformatics algorithms. The conserved epitopes predicted within the sequences were mapped on the homodimers of the respective tick GSTs, and the corresponding peptides were independently used for rabbit immunization experiments. Based on dot blot assays, the immunogenicity of the peptides and their potential to be recognized by the corresponding anti recombinant GST sera (rGST) were investigated. The study revealed that the predicted conserved B-cell epitopes within the five tick GST sequences were localized on the surface of the respective GST homodimers. The epitopes of GST-Ra, GST-Rd, GST-Av, and GST-Hl were also shown to contain a 7 amino acid-long peptide sequence with no proteasomal cleavage sites, whereas proteasomal digestion of GST-Rm was predicted to yield a 4-aa fragment. Given that few proteasomal cleavage sites were found within the conserved epitope sequences of the four GSTs, the sequences could also contain T-cell epitopes. Finally, the anti peptide and rGST sera reacted against the corresponding peptide, confirming their immunogenicity. These data support the hypothesis that the rGSTs used in the previous study contain conserved B-cell epitopes, which could explain why the anti rGST sera cross-reacted against non-homologous tick GSTs. Taken together, the data suggest that the B-cell epitopes predicted in this study could be useful for constituting epitope-based GST tick vaccines.

Keywords: glutathione S- transferase, immunoinformatics, epitopes.

1. Introduction

Ticks are blood feeding ectoparasites that severely impact humans and animals (de la Fuente 2008; Brites-Neto et al., 2015) and for which control methods have long relied on the use of acaricides. However, the incidence of global acaricide-tick resistance (Blair, 1989; Thullner et al., 2007), concerns over meat and milk contamination, and environmental pollution undermine the significance of acaricides in tick control for livestock animals. For those reasons, numerous alternative methods of tick control have been suggested and explored (Manjunathachar et al., 2014; Ghosh et al., 2007), among which vaccinating cattle against ticks is considered one of the most sustainable alternatives. Indeed, remarkable progress has been made toward identifying tick vaccine antigens (de la Fuente and Kocan, 2006; Merino et al., 2013; Nuttall et al., 2006; Valle and Guerrero, 2018). Strikingly, however, only a few antigens are reported to induce partial protection against multiple tick species (de la Fuente et al., 2013; Parizi et al., 2011; Sabadin et al., 2017; Trimnell et al., 2005; Kumar et al., 2017; Ndawula et al., 2019). For example, Bm86, a gut localized glycoprotein (Gough and Kemp, 1993), wasisolated from *Rhipicephalus microplus* (Rand et al., 1989) and its corresponding recombinant antigen was shown to induce a high protection, especially against *R. microplus* strains from Australia (Willadsen et al., 1995) and Cuba (Valle et al., 2004). The Bm86 recombinant vaccine was, however, reported to induce a lower protection against *R. microplus* strains from South America (Andreotti, 2006; García-García et al., 2000). The variation in protection could be attributed to differences in Bm86 amino acid sequences among the different populations (García-García et al., 1999; Freeman et al., 2010). Interestingly, a taxonomic revision of *R. microplus* has been performed where populations of *R. microplus* from Australia and other countries were renamed as *R. australis*. (Ali et al., 2016). Yet, Bm86 was shown to induce a high protection against *Rhipicephalus annulatus* (Fragoso et al., 1998), *Rhipicephalus decoloratus*, Hyalomma anatolicum anatolicum and Hyalomma dromedarii, but not against *Rhipicephalus appendiculatus* and *Amblyomma variegatum* (De Vos et al., 2001).

Considering that multiple tick species are present in most parts of the world, it is vital to develop vaccines that can induce cross-protection against different ticks. However, based on anti-tick vaccination reports, for instance using recombinant Bm86, it seems unlikely that single-antigen tick vaccines will induce a high cross-protection against multiple tick species. Therefore, constituting cocktail vaccines present a possibility to enhance the protection

range and the efficacy of anti-tick vaccines. However, how to select antigens for constituting efficacious cocktail tick vaccines is still a challenging question to researchers in the field. Some complicating factors are antigenic competition (Shaffer et al., 2016; Taussig et al., 1973), and the fact that combining two or more antigens increases the amount of non-protective epitopes exposed to the immune system, hence altering the system potency (Vyas et al., 2008; Gershoni et al., 2007) and humoral immune response (Childs et al.,2015). Epitope-based vaccines, by contrast, do not trigger undesirable immune responses, can induce a high specific immune response and, most importantly, they could induce longer immune protection (Childs et al., 2015; Vyas et al., 2008; Gershoni et al., 2007). The idea of epitope-based vaccines was first introduced in 1985 (Jacob et al., 1985). Indeed, the concept of constituting epitope-based vaccines has also been demonstrated toward tick control (Patarroyo et al., 2002; Aguirre et al., 2016). Therefore, it is plausible that replacing conventional or whole antigen-based vaccines by an epitope-based approach could enhance the potency of cocktail anti-tick vaccines. The fundamental question, however, remains how to identify and locate epitopes among the sequences of the conventional or whole antigen-based anti-tick vaccine antigens.

There are numerous methods for locating B-cell epitopes, including mass spectrometry, nuclear magnetic resonance, surface plasmon resonance, computer docking, pepscan, binding assays, mutagenesis (Ahmad et al., 2016; Gershoni et al., 2007). Epitope location based on protein structure is regarded as the gold standard (Gershoni et al., 2007), however, x-ray crystallographic data on the structure of tick antigens remains scanty. Nevertheless, with the advent of bioinformatics, the potential of using computer-based tools as an alternative approach to locate epitopes has been exploited (Soria-Guerra et al., 2014). In contrast to crystallography, epitope prediction using *in silico* prediction algorithms is cheaper, quicker and readily applicable. Indeed, numerous *in silico* epitope prediction tools have been reported (Potocnakova et al., 2016; Sun et al., 2019), of which the linear prediction tools are commonly used. For instance, in search for an immunogen against *R. microplus* 'ATAQ' protein, sequence based epitope prediction tools were used (Aguirre et al., 2016). It has been suggested, however, that most of the sequence-based predicted epitope peptides constitute the conformational-based predicted epitopes (Potocnakova et al., 2016). Therefore, the predictions based both on protein structure and linear peptide sequence are likely to give more accurate and reliable results (Assis et al., 2014).

In this work, a combination of three sequence and conformation-based epitope prediction tools was used. These were selected because they regarded to be among the most accurate (Potocnakova et al., 2016; Sun et al., 2019) tools. Additionally, CBTOPE (Ansari and Raghava, 2010) and Scratch (Cheng et al., 2005) were specifically selected to complement the structural and conformation epitope prediction tools.

Previous work by our group has demonstrated that gluthatione-S-transferase is a suitable candidate for a cocktail tick vaccine, inducing non-homologous cross-reaction against different tick species (Ndawula et al., 2019). The purpose of this study was to predict and map conserved epitopes within the glutathione S-transferase sequence of *Rhipicephalus appendiculatus* (GST-Ra), *Rhipicephalus decoloratus* (GST-Rd), *Haemaphysalis longicornis* (GST-Hl), *Amblyomma variegatum* (GST-Av) and *Rhipicephalus microplus* (GST-Rm). The predictions could help elucidate the cross-reaction phenomenon observed. Most importantly, the study illustrates an approach to selecting epitopes toward developing epitope-based tick vaccine antigens.

2. Materials and Methods

2.1. Ethics Statement

Rabbits used in the experiments were housed at Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS). This research was conducted according to the ethics and methodological guidance, in agreement with the Norms for Animal Experimentation Ethics Committee of UFRGS (process number 38748).

2.2. GST protein sequences

The GST deduced amino acid sequences used herein were deduced from *R. appendiculatus* (MK133338), *R. decoloratus* (MK133339), *A. variegatum* (MK133337) (Ndawula et al., 2019), *R. microplus* (AF366931.1) (Rosa de Lima et al., 2002), *H. longicornis* (AY298731.1) (da Silva et al., 2004), *R. microplus* (HQ337623.1) (Paul et al., 2010)and *R. microplus* (AF077609.1) (He et al., 1999) that were retrieved from GenBank (Clark et al., 2016).

2.3. Sequence-based mapping

Sequence-based mapping was performed to determine B-cell linear epitopes

(continuous epitopes) (Benjamin et al., 1984). For each GST sequence, the prediction was conducted using the following three algorithms.

BCPREDS: The sequences were independently submitted to the BCPREDS web server 1.0 (http://ailab.ist.psu.edu/bcpred/predict.html). Considering that FBCpred (EL-Manzalawy et al., 2008) has the highest accuracy compared to AAP (Chen et al., 2007) and BCPred (EL-Manzalawy et al., 2008) scale, predictions were performed using the FBCpred scale. The prediction tool was set at 75% specificity, at default epitope length of 14 amino acids and with an overlap filter. The predicted antigenic sequences were noted in descending order of antigenicity.

CBTOPE: This algorithm predicts both linear and conformation B-cell epitopes based on the sequence (Ansari and Raghava, 2010). P The sequences were submitted together to the CBTOPE web server (http://crdd.osdd.net/raghava/cbtope/submit.php). Predictions were performed at a threshold of 5 and 0-9 probability scale. The sequences with at least four amino acids and an antigenicity of four were noted.

BepiPred: The GST sequences were independently submitted to the BepiPred (Jespersen et al., 2017) server 2.0 (http://www.cbs.dtu.dk/services/BepiPred-1.0/). Predictions were performed under 0.5 epitope threshold and the antigenic epitopes sequence with at least 6 – denoted E – were noted.

2.4. Structure-based mapping

Similarly, structure-based predictions were performed using three algorithms. The predictions that were performed using Ellipro and DiscoTope, were based on homology models of the tick GSTs tick GST while yet the predictions in Scratch (Cheng et al., 2005) were based on the GST sequences. The GST sequences of R. microplus (AF366931.1), R. decoloratus (MK133339), R. appendiculatus (MK133338), A. variegatum (MK133337) and H. longicornis (AY298731.1) were separately queried against the RCSB Protein Data Bank (Berman et al., 2000) using the Basic Local Alignment Search Tool for proteins (BLASTp; Altschul et al., 1990). From each query, the structure with the highest resolution and whose sequence showed the highest similarity and coverage was selected. The selected structure was then used for building the structural models of the five tick GSTs. The models were built using the homology (comparative) modeling strategy under MODELLER 9.16 software (Marti-Renom et al., 2000). Thereafter, the theoretical three-dimensional orientation and

stereo-chemical properties of the built GST homology models were determined, and the three-dimensional profile of the models were evaluated (Lüthy et al., 1992) using WHATCHECK (Hooft et al., 1996), with the constructed models being independently submitted to https://servicesn.mbi.ucla.edu/WHATCHECK/. Finally, the structurally assessed GST models were used for subsequent analyses. Tick GST homology models (PDB files) were separately submitted to the Ellipro prediction server (Ponomarenko et al., 2008; http://tools.iedb.org/ellipro/result/predict/). Predictions were performed under default conditions maximum distance (6 Å) and maximum score (0.5). Tick GST homology models (PDB files) were independently submitted to the DiscoTope -2.0 server (Kringelum et al., 2012); http://www.cbs.dtu.dk/services/DiscoTope/). The predictions were performed under default score (-3.7).

Finally the GST sequences were separately submitted to Scratch protein predictor (Cheng et al., 2005; http://scratch.proteomics.ics.uci.edu/). The amino acid sequences with a propensity score of at least 10 were noted.

2.5. GST homodimer modeling and B-cell epitope mapping

To obtain the GST homodimer models, two GST monomer chain models of a particular tick species were juxtaposed to the reference (template) dimer that was used for the homology modeling. Thereafter, to assess the stereo-chemical quality and structural integrity of the GST homodimers. Similarly, the constructed models were submitted to WHATCHECK (https://servicesn.mbi.ucla.edu/WHATCHECK/). All the structure manipulations were performed using PyMol 1.3 (Schrodinger LLC). Finally, the predicted conserved B–cell epitopes from each GST were mapped and highlighted on the three-dimensional protein structure of both monomeric and dimeric GST models.

2.6. Proteasome cleavage mapping

The presence of proteasome cleavage sites within the identified conserved epitopes was investigated using two algorithms, which are shown to be the most accurate available (Bhasin and Raghava 2004). The GST sequences of the five tick species were separately submitted to P cleavage (Bhasin and Raghava 2004) web server, available through http://crdd.osdd.net/raghava/pcleavage/. All predictions were based on the *in vitro* constitutive proteasomeat -0.1 threshold. Similarly, GST amino acid sequences were separately submitted to NetChop 3.1 server (Nielsen et al., 2005;

http://www.cbs.dtu.dk/services/NetChop/). All predictions were performed using the C term 3.0 methods at a threshold of 0.5.

2.7. *In vivo* evaluation of the predicted consensus epitopes

2.7.1. Peptide synthesis and rabbit immunization

Based on the predicted consensus sequences of *R. appendiculatus* (SCGPPPDFDKSY), *R. microplus* (GPAPTYE), *R. decoloratus* (SCGPPDFDRSS), *A. variegatum* (SCGPPPDFDRGS) and *H. longicornis* (GPPPDFDRSA), the corresponding peptides were obtained by solid-phase synthesis, using the Fmoc (N- (9-fluorenyl)-methoxycarbonyl) methodology, as previously described (Hirata et al., 1994; Korkmaz et al., 2008).

Using glutaraldehyde as a crosslinker, the peptides were coupled to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) carriers as described previously (Carter, 1996) with modifications. Carrier proteins (5 mg/ml) and peptides (5 mg/ml) were each resuspended in phosphate buffered saline, pH 7 (PBS). For chemical cross-linking, BSA/peptide and KHL/peptide mixtures were treated with 0.2% freshly prepared solution of glutaraldehyde for 1h at room temperature. The reactions were terminated by addition of 1 M glycine and then dialyzed overnight in PBS.

The peptides/KHL conjugates were independently mixed (1:1) with the adjuvant (Montanide 888 – Seppic and Marcol 52 – Exxon Mobil Corporation). Five New Zealand rabbits were independently inoculated with 500 µg of the cross-linked peptides/adjuvant mixture. Rabbits were immunized three times at an interval of 14 days. Before each inoculation, 200 µl of blood were drawn from rabbit ears, centrifuged at 16,000 x g, and the serum were stored at -20 °C until the subsequent analyses. Finally, the rabbits were euthanized as per the guidelines of the UFRGS ethical committee.

2.7.2. Peptide immunogenicity and anti rGST serum reactivity analyses

To determine whether the peptides were immunogenic, first, the anti peptide sera were tested against the corresponding BSA/peptide conjugate using dot blot assay. Briefly, 1 µg of each conjugated was added onto the nitrocellulose membrane (0.45 µm Bio-Rad) and dried at room temperature for 20 min. The membrane was incubated in blotto (5%) for 1 h at room temperature while gently agitating, then incubated overnight in blotto with the

corresponding peptide anti-sera, at 4 °C under shake incubation. The pre immunization sera (pre-sera) were diluted at 1:100, whereas the first (1st), second (2nd), and third (3rd) anti-sera (collected after the first, second and third KLH/peptide conjugate rabbit immunization, respectively) were diluted at 1:1,000. The membrane was then treated as follows: washed three times in blotto (nonfat dry milk) (5%); incubated in blotto with anti-rabbit IgG peroxidase 1:5,000 (A-6154 Sigma); washed in PBS; incubated in DAB (3,3'-diaminobenzidine tetrahydrochloride) substrate dissolved in 1% cobalt chloride and 30% hydrogen peroxide for 20 min in the dark, at room temperature with gentle agitation. Finally, the membrane was washed with distilled water and dried at room temperature.

The same dot blot procedure was used to analyze the sera from rabbits that were independently immunized with recombinant GST of *R. appendiculatus, R. decoloratus, R. microplus, A. variegatum,* and *H. longicornis* (Ndawula et al., 2019), at a 1:10,000 dilution, against the corresponding BSA/peptide conjugate.

3. Results and discussion

Until now, vaccinating cattle against ticks is regarded as one of the most promising tick-control alternative approaches to replace the use of acaricides. Indeed, researchers have identified numerous antigens (de la Fuente and Kocan, 2006; Merino et al., 2013; Nuttall et al., 2006; Valle and Guerrero 2018; Ndawula et al 2019), but none has so far matched the success exhibited by Bm86 in field conditions (de la Fuente et al., 2007, 1999, 1998, 2016). Combining antigens could potentially enhance the protection efficacy of tick vaccines (de la Fuente and Contreras, 2015; Willadsen, 2008; de la Fuente et al., 2016), but despite the efforts in constituting a cocktail anti-tick vaccine (Willadsen et al., 1996; Parizi et al., 2012; Imamura et al., 2006, 2008; Ndawula et al., 2019), substantial enhanced protection is still to be attained, for reasons that remain largely unclear.

Formulation of epitope-based cocktail-antigens is one of the approaches to improve cocktail anti-tick vaccines. However, it first requires the location of epitopes, within each potential vaccination antigen. Epitopes are classified into B-cell or T-cell cells. B-cell epitopes are solvent-exposed sequences or regions of an antigen (Sanchez-Trincado et al., 2017). On the other hand the T-cell epitopes are located within antigen-derived peptides that form complexes with major histocompatibility proteins (MHCs) that are presented for recognition by the T-cells (Sanchez-Trincado et al., 2017; Madden, 1995).

The current study aimed to predict B-cell epitopes conserved among GST sequences of *R. appendiculatus, R. decoloratus, A. variegatum, H. longicornis* and *R. microplus*, to help elucidating the previously observed (Ndawula et al., 2019) cross-reactivity of rGST anti-sera against non-homologous tick rGSTs . The B-cell epitopes were determined using *in silico* linear and conformational-based epitope predicting algorithms. The conceptual design followed in the epitope prediction investigations is outlined in Fig. 1. Furthermore, the immunogenicity of the corresponding peptides was investigated.

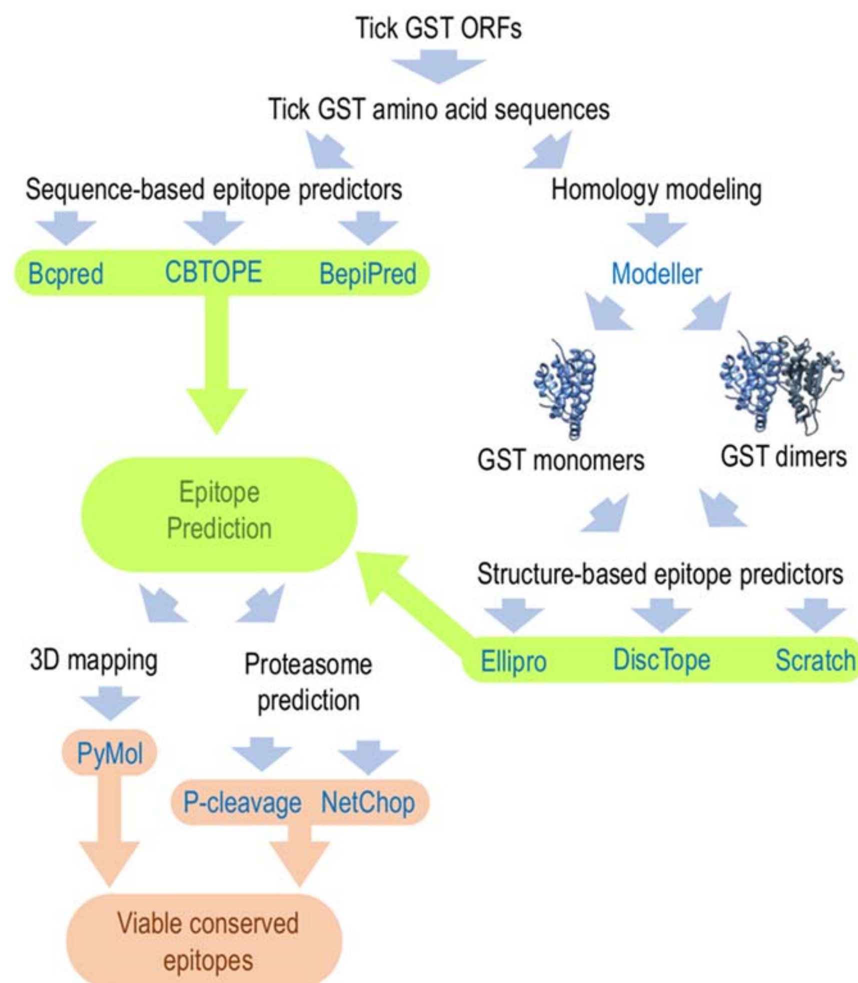

Figure 1: the conceptual design followed in the epitope prediction investigations

The findings obtained using the sequence-based predicting algorithms (BCPREDS, CBTOPE, BepiPred) for GST-Rd, GST-Av, GST-Hl and GST-Ra indicate the presence of consensus epitopes (Table 1). Strikingly, among the four tick GSTs, one epitope was consistently predicted by the three prediction algorithms. Two prediction algorithms

(BCPREDS and BepiPred) showed consensus epitopes within the GST sequence of *R. microplus* (GST-Rm). However, the epitope sequence consistently shown within GST-Rm was different from the consensus sequence reported for GST-Ra, GST-Av, GST-Rd and GST-Hl.

Conversely, the conformational-based predictions showed one consensus epitope sequence within GST-Ra, GST-Av, GST-Rd, GST-Rm and GST-Hl (Table 2). However, the consensus epitope within the sequence of *R. microplus* (GST-Rm) was only evidenced by DiscoTope and Scratch. The consistently predicted epitope was highly conserved among GST-Ra, GST-Av, GST-Rd and GST-Hl, but not in GST-Rm.

Table 1. Table 1. Sequence-based epitope prediction against the glutathione S-transferase (GST).

	BCPEDS	**BepiPrep**	**CBTOPE**
GST-Rd	^{86} LEGKTEAEKQRVDV ^{99} ^{33} **YSCGPPPDFDRSSW** ^{46} ^{107} NPEFEKLKGDYLKN ^{130} ^{56} EFPNLPYYIDGDVK ^{69} ^{132} PASLKAFSDYLGTH ^{145} ^{102}QQFADFRMNWVRLC ^{115}	^{28}VDDKR**YSCGPPPDFDRSSW**LNEKTKLGLEF ^{57} ^{85} GLEGKTEAEK ^{94} ^{116} YNPEFEKLKGD ^{126} ^{171} APDCLKDFA ^{179} ^{200} KSDKCIKWPVNGDMASFACRLQ ^{221}	^{28} VDDKR**YSC** ^{35} ^{37} **PPDFDRSSW**LNEKTK ^{52} ^{88} KTEAEKQRVDVAE ^{101} ^{115} CYNPEFEKLKGDYL ^{128} ^{174} CLKDFANL ^{181} ^{205} KWPVNGDM ^{219}
GST-Av	^{32} **RYSCGPPPDFDRGS** *^{45}* ^{132} PASLKAFSDYLGSH ^{145} ^{83} KHGLEGKTETEKQR ^{96} ^{108} RMNWVRLCYNPDFE ^{121} ^{51} PNLPYYIDGDLKLT ^{171}	^{28} VEDK**RYSCGPPPDFDRGS**WLKEKHTLGLE ^{56} ^{85} GLEGKTETEK ^{94} ^{116} YNPDFEKLKG ^{125} ^{171} YNPDFEKLKG ^{179} ^{119} KSDKCINWPLNGDMASFGSRLQ ^{220}	^{27} KVED**KRYSCGPPPDFDRGS**WLKEKHTL ^{53} ^{117} NPDFEKLKGDYL ^{128} ^{130} GLPASLKA ^{137} ^{174} CLKDFANL ^{181} ^{198} LSKDKCINW ^{206}
GST-Ra	^{27} K**VEEKQYSCGPPPD**^{40} ^{82} RKYDLMGKTGAEKQ ^{95} ^{127} YLKDLPASLKAFSD ^{140} ^{56} DFPNLPYYIDGDVK ^{69} ^{142} LGNRKFFAGDNLTY ^{155}	^{28} **VEEKQYSCGPPPD**FDKSYWLSEKPKLGLD ^{56} ^{86} LMGKTGAEK ^{94} ^{116} YSPDFEKLKGD ^{126} ^{169} LFAPDCLKDFA ^{179} ^{199} KSDKCIKWPLNGDMASFGSRLQ ^{220}	^{20} LLAHADAK**VEEKQYSCGPPPD**FDKSYWLSEKPKL ^{53} ^{56} DFPNLPYYIDG ^{66} ^{93}EKQRVDVVEQQLADFRVNWGRLCYSPDFEKLKGDYLKDLPASLKAFS^{139} ^{199} KSDKCIKWPL ^{208}
GST-Rm	^{119} NPNATEARKSQEKR^{132} ^{1} **MAPT**PVVGYTTARG ^{14} ^{38} **GPAPTYEKLGWAAD**	^{28} VHFEDKRYEF**GPAPTYEKLGWAAD**SASLG ^{56} ^{87} GLDARSDQEA ^{96} ^{119} NPNATEARKSQEKR ^{132} ^{170} RQF**APDAFANRPELLD**^{185}	^{86} HGLDARSD ^{93}

	^{51} ^{191} EQLPNLKEYFASDK ^{204} ^{137} L**PRWQELLKKRRW**A ^{150} ^{55} LGFTFPNLPYYIDG ^{68} ^{156} TYVDFLLYEALDWN ^{169} ^{908} ELWLMEQQANDLLW ^{111} ^{207} KWPIMAPYMFWGHK^{220}	^{188} RRFEQLPNLKEYFASDKYVKWPIM ^{211}	
GST-Hl	^{85} GLDGKTEAEKQRVD^{98} ^{36} **GPPPDFDRSAWLKE** ^{49} ^{132} PDALKSFSEYLGKH ^{145} ^{56} EFPNLPYYIDGDVK^{69} ^{113} RMCYNPDFDKLKVD^{126} ^{150} GDHVTYVDFIAYEM^{163}	^{28} VEDKRYSC**GPPPDFDRSAWLKE**KHTLGLEF^{57} ^{85} GLDGKTEAEK ^{94} ^{116} YNPDFDKLKVD ^{126} ^{171} APDCLKDFP ^{179} ^{199} KSDKCISWPLNGDMASFGSRLQ ^{220}	^{27} DVKVEDKRYSC**GPPPDFDRSAWLKE**KHT ^{52} ^{92} AEKQRV ^{97} ^{117} NPDFDKLKVDYLKNL ^{131} ^{199} KSDKCISW ^{206}

The sequences were from *Rhipicephalus decoloratus* (GST-Rd) (MK133339), *Amblyomma variegatum* (GST-Av) (MK133337), *Rhipicephalus appendiculatus* (MK133338), *Rhipicephalus microplus* (GST-Rm) and *Haemaphysalis longicornis* (GST-Hl) (AY298731.1). The numbers depict the position of the amino acid in the GST sequence. The predictions were performed using BCPREDS web server 1.0, CBTOPE web server and BepiPred server 2.0. The amino acid sequences shown in bold were also predicted in Table 2.

Table 2. Structural-based epitope predictions against the glutathione S- transferase models and the sequences.

	Ellipro	Scratch	DiscoTope
GST-Rd	^{34} **SCGPPPDFDRSS**WLNEKTKLGLEFPN ^{59} ^{117} NPEFEKLKGDYLKNLPA^{133} ^{82} RKHGLEGKTEAEKQRVD ^{98} ^{217} SRLQKKP ^{223} ^{176} KDFANLKA^{183} ^{136} KAFSDYLGTHKF ^{147} ^{24} VDAKVD ^{29} ^{149} AGENL^{153}	^{24} VDAKVDDKRY**SCGPPPDFDRSS**WLNEKTKLGLEFPN^{59} ^{117} NPEFEKLKGD ^{126} ^{143} GTHKFFA^{149} ^{175} LKDFANLKA^{183} ^{186} DRIEALPHVAAYLKSDKCIKWPV ^{208}	^{34} **SCGPPPDFDRSS** ^{45} ^{47} LNEKTK^{52}
GST-Av	^{34} **SCGPPPDFDRGS**WLKEKHTLGLEFPN^{59} ^{117} NPDFEKLKGDYLKGLPA^{133} ^{82} RKHGLEGKTETEKQR^{96} ^{136} KAFSDYLGSHKF^{147} ^{171} APDCLKDFANLKA^{183} ^{24} VDAKVE ^{29} ^{149} AGDNL ^{153} ^{217} SRLQKKP^{223}	^{24} VDAKVEDKRY**SCGPPPDFDRGS**WLKEKHTLGLEF ^{57} ^{62} YYIDGDLKLT ^{71} ^{116}YNPDFEKLKG^{125} ^{144} GSHKFFA^{149} ^{176} KDFANLK ^{182} ^{187}RIEALPHVAA^{196} ^{200} SDKCINWP^{207} ^{211} DMASFGSRL^{219}	^{34}**SCGPPPDFDRGS**^{45}
GST-Ra	^{34} **SCGPPPDFDKSY**WLSEKPKLGLDFPN^{59} ^{117} SPDFEKLKGDYLKDLPA^{133} ^{82} RKYDLMGKTGAEKQRVD^{98} ^{176} KDFANLKA^{183} ^{136} KAFSDYLGNRKF ^{147} ^{24} ADAKVE^{29} ^{149} AGDNL^{153} ^{217}SRLQKKP^{223}	^{24}ADAKVEEKQY**SCGPPPDFDKSY**WLSEKPKLGLDFPNLPYYIDGDVKL^{70} ^{117} SPDFEKLKG^{125} ^{143} GNRKFFA^{149} ^{168} LLFAPDCLKDFANLK^{182} ^{187} RVAALPRVAAY^{197} ^{200} SDKCIKWPL^{208} ^{211} DMASFGSRLQK^{221}	^{34}**SCGPPPDFDKSY** ^{45} ^{47} LSEKPK^{52}

GST-Rm	^{1} **MAPT** ^{4} ^{36} EF**GPAPTYE**KLGWA**ADSASLGFTF**PN^{61} ^{118} **MNPNATEARKSQ**EKRLAD^{135} ^{173} **APDAFANRPELLD**^{185} ^{84} KKHGLDARSDQEAAELW^{100} ^{138} **PRWQELLKKRRW** ^{149} ^{151} LGNTL^{155} ^{26} **KGVHFE**^{31}	^{1} **MAPT**PVVGYTTAR ^{13} ^{26} **KGVHFE**DKRYEF**GPAPTYE**KLG^{47} ^{50} **ADSASLGFTF**^{59} ^{118} **MNPNATEARKSQ**^{129} ^{171} QFA**PDAFANRPELLD**YLRRFEQLPNLKEYF^{200} ^{202} DKYVKWPIMAPYMFWGHK^{223}	^{38} **GPAPTYE**^{44}
GST-Hl	^{34} SC**GPPPDFDRSA**WLKEKHTLGLEFPN^{59} ^{117} NPDFDKLKVDYLKNLPD^{133} ^{82} RKHGLDGKTEAEKQRVD^{98} ^{176} KDFPNLKA^{183} ^{24} ADVKVE ^{29} ^{136} KSFSEYLGKHKF^{147} ^{149} AGDHV^{153} ^{217} SRLQKKP^{223}	^{14} ADVKVEDKRYSC**GPPPDFDRSA**WLKEKHTLGLEFPNLPYY^{63} ^{116} YNPDFDKLKV^{125} ^{175} LKDFPNL^{181} ^{187} RIEALPHVAAY^{191} ^{200} SDKCISWPL^{208} ^{211} DMASFGSRLQKKP ^{223}	^{36}**GPPPDFDRSA**^{45}

The sequences were from *Rhipicephalus decoloratus* (GST-Rd) (MK133339), *Amblyomma variegatum* (GST-Av) (MK133337), *Rhipicephalus appendiculatus* (MK133338), *Rhipicephalus microplus* (GST-Rm) (AF366931.1) and *Haemaphysalis longicornis* (GST-Hl) (AY298731.1). The numbers depict the position of the amino acid in the GST sequence. The predictions were performed (against the GST models) using DisoTope -2.0 web server and Ellipro prediction server and (against GST amino acid linear sequences) using scratch web server. The amino acid sequences shown in bold were also predicted Table 1.

A comparison encompassing all the prediction findings (Tables 1 and 2) reveals that the epitope sequence consistently found in each GST sequence was predicted using both the sequence and conformational-based prediction algorithms. Notably, within GST-Ra, GST-Av, GST-Hl, GST-Rd and GST-Rm the epitope sequence found using DiscoTope was consistently obtained using other prediction algorithms. Put together, the prediction data support the view that linear epitopes are usually made up of a few stretches of discontinuous epitopes (Potocnakova et al., 2016). Data further indicate that the predictions based both on the protein conformational structure and sequence are likely to give more accurate and reliable results than independently using one prediction approach (Assis et al., 2014).

Furthermore, the comparison between findings obtained using the sequence and conformational-based prediction algorithms reveals that - within GST-Ra, GST-Av, GST-Hl, GST-Rd and GST-Rm - DiscoTope found the smallest number of epitopes. The differences in output among the B-cell epitope prediction algorithms could be attributed to that fact that algorithms were developed based on different prediction models. Examples of models that were used to develop prediction algorithms used herein are Support Vector Machine (SVM) (Ansari and Raghava, 2010; El-Manzalawy et al., 2008; Cheng et al., 2005), Hidden Markov Models (HMM) (Larsen et al., 2006), Artificial Neutral Networks (ANN) (Saha and Raghava, 2006), Random Forest (Jespersen et al., 2017). A detailed account has been given on the differences between the sequence and conformational-based epitope predicting algorithms (Ansari and Raghava 2013; El-Manzalawy and Honavar, 2010; Gao and Kurgan, 2014).

Although the conserved epitope found within GST-Rm was not similar to that within GST-Ra, GST-Av, GST-Hl, and GST-Ra, it has been previously reported that rGST-Hl induced cross-protection against *R. microplus* (Parizi et al., 2011) and *R. appendiculatus* (Sabadin et al., 2017). Based on these reports, it is likely that highly conserved epitopes exist in common among GST-Rm, GST-Ra and GST-Hl sequences. To investigate this apparent discrepancy, the conserved epitopes found within GST-Ra, GST-Av, GST-Hl, GST-Rd and GST-Rm were aligned against different, previously characterized GST-Rm sequences: HQ337623.1 (Paul et al., 2010) and AF077609.1 (He et al., 1999). The alignment showed that the conserved sequence found within GST-Ra, GST-Av, GST-Hl, and GST-Rd, but not the one found in GST-Rm (AF366931), was also present in GST-Rm HQ337623 and AF077609 sequences. Indeed, GST paralogs have been reported in different tick species (Hernandez 2018; Dreher-Lesnick et al.,

2006; Niranjan et al., 2011), as well as in other arthropods, for instance mosquitoes (Lumjuan et al., 2007). It is possible that *R. appendiculatus*, *A. variegatum*, and *R. decoloratus* also to have GST paralogs, although they are still to be reported. On the other hand, it is also likely that *R. appendiculatus*, *A. variegatum*, *H. longicornis* and *R. decoloratus* could express GSTs that contain epitopes similar to those found in GST-Rm (AF366931.1).

In addition to predicting the conserved epitopes among GST-Ra, GST-Rd, GST-Av, GST-Hl, and GST-Rm sequences, the location (mapping) of the epitopes on the GST homology model structures was investigated. The prerequisites for epitope mapping are identification of epitope sequences and the presence of a characterized antibody. Although there are numerous methods to predict B-cell epitopes (Ahmad et al., 2016; Gershoni et al., 2007), x-ray crystallography is regarded as the golden standard (Gershoni et al., 2007). However, x-ray crystallography epitope mapping is expensive, laborious, and time consuming. Therefore, to circumvent those challenges, *in silico* conformational-epitope prediction algorithms, for instance DiscoTope (Kringelum et al., 2012), have been developed based on the x-ray crystallographic data. To attain better predictions with the conformational-based epitope prediction algorithms, it would be appropriate to have x-ray crystallographic structure data on the antigen of interest. In the event that there is no x-ray crystallographic data, it is essential to model the structure of the antigen of interest. However, as with most anti-tick vaccine antigens, the crystal structures of tick GSTs are still to be resolved. Therefore, to fulfill the requirements for *in silico* conformational epitope mapping, the homology structures of GST-Ra, GST-Av, GST-Hl, GST-Rd, and GST-Rm were searched and found in the RCSB Protein Data Bank.

The search for tick GST homology models revealed that GST-Ra, GST-Av, GST-Hl, GST-Rd, and GST-Rm showed a sequence coverage of 96% and 55-58% similarity to the *Homo sapiens* GST M2-3 (PDB ID 3GTU), which has a crystal structure at 2.5 Å resolution (Patskovsky et al., 1999). Put together, these findings suggest that the GST M2-3 structure was suitable for building tick GST models. First, the tick GST monomer models were built based on the single chain structure of GST M2-3. Considering that mu-GSTs are reported to be dimers (Ji et al., 1992; Hussey et al., 1991, 1993), tick GST homodimers models were built. Based on the WHATCHECK (Hooft et al., 1996) analyses, the constructed models were found to have a good stereo-chemical quality and structural integrity (G factor negative). The predicted conserved epitopes were located on the surface of the GST homodimer models (Fig. 2). These findings suggest that

predicted epitopes could be recognized by the corresponding anti rGST sera and that the corresponding peptides are immunogenic, a hypothesis that was herein further investigated *in vivo*, as presented below.

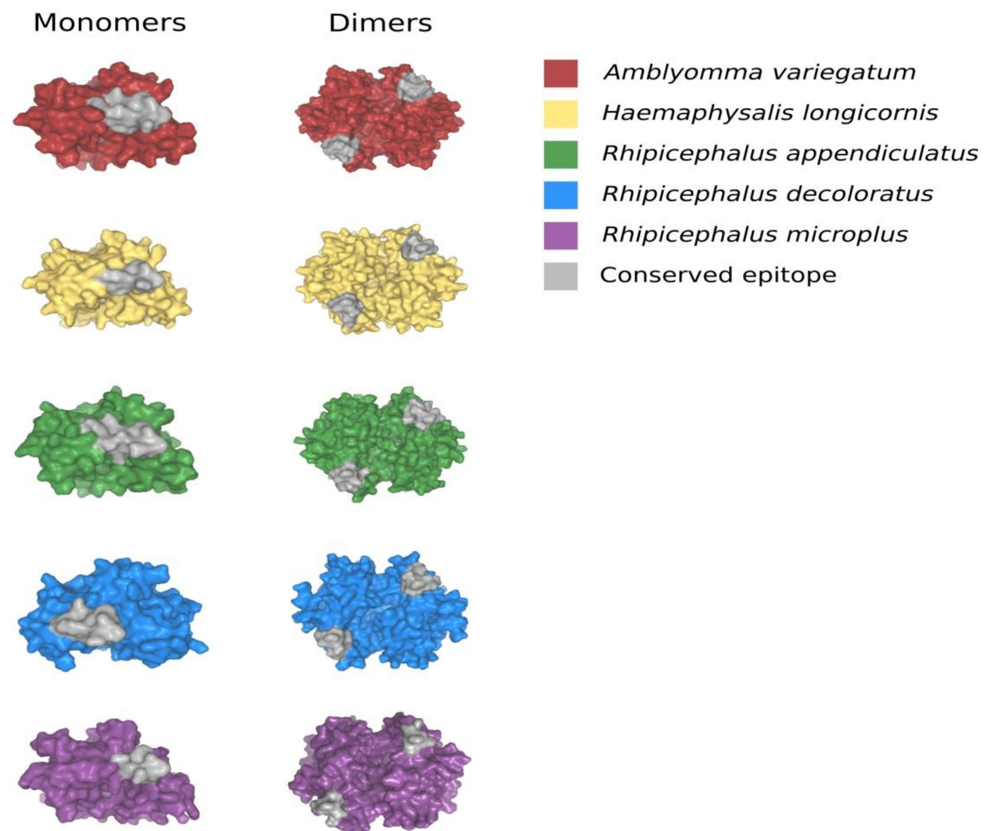

Figure 2. Area of the structure that is covered by the predicted conserved B-cell epitope. The GST 3-dimensional models were obtained from the amino acid sequences translated from the following nucleotides sequences: *Rhipicephalus decoloratus* (GST-Rd) (MK133339), *Amblyomma variegatum* (GST-Av) (MK133337), *Rhipicephalus appendiculatus* (GST-Ra) (MK133338), *Rhipicephalus microplus* (GST-Rm) (AF366931.1) and *Haemaphysalis longicornis* (GST-Hl) (AY298731.1). GST-monomer models were built using Modeller (9.16) based on the GST M2-3, PDB ID 3GTU (Patskovsky et al., 1999), upon which the monomers were juxtaposed to form GST homodimers; stereo-chemistry was verified using WHATCHECK. Epitope mapping was performed using PyMol Molecular graphics system version 1.3 Schrodinger, LLC. The mapped epitope (gray) is the region predicted using DiscoTope -2.0 web server (see Table 2). Additionally, in each GST, the mapped sequence was consistently reported using other structural- and the sequence-based epitope prediction algorithms (Tables 1 and 2).

Regarding tick vaccine antigens, investigations have been made toward predicting conserved antigenic B-cell epitopes using *in silico* epitope prediction algorithms (Sabadin et al., 2017; Parizi et al., 2011; Blecha et al., 2018; Aguirre et al., 2016). In these studies, epitope prediction was performed based not on the conformational structures, but on the amino acid sequences, implying that linear (continuous) epitopes were reported. It is important to note, however, that linear epitopes are just a smaller part

of the discontinuous epitope (Potocnakova et al., 2016) and therefore may not accurately mimic the entire epitope in the corresponding protein. Indeed, the peptide-based tick vaccine antigens derived from Bm86 sequence (Patarroyo et al., 2002), and ATAQ peptide (Aguirre et al., 2016) were shown to independently induce a humoral response in experimental animals and, most importantly, had an impact on experimental tick infestation. For instance, ATAQ peptide-based vaccine (Aguirre et al., 2016) had a higher impact against *R. sanguineus* infestation in rabbits and dogs compared to the whole recombinant ATAQ vaccine (Évora et al., 2017). However, in the case of Bm86 epitope-based vaccines, of the three peptides that were tested, only one induced a high protection against tick infestation in the vaccinated populations (Patarroyo et al., 2002).

The current study further aimed to establish, using *in silico* prediction algorithms, whether the conserved sequences could also a contain T-cell epitope. In principle, to induce a good immune response upon inoculation in an animal, a protein must have B-cell and T-cell epitopes (Hoffmeister et al., 2003). However, before forming epitope-MHC complexes, the immunogen undergoes proteasomal hydrolysis (Rock and Goldberg, 1999; Rammensee et al., 1995; Falk and Rötzschke, 1993). Therefore, in search of synthetic vaccines, a few algorithms have been developed to predict the proteasomal hydrolysis sites within antigen sequences, of which NetChop (Nielsen et al., 2005) and P cleavage (Bhasin and Raghava, 2004) are regarded among the most accurate (Saxová et al., 2003).

The analyses indicated that the five tick GST sequences contained numerous proteasomal cleavage sites (Fig. 3). Strikingly, a few cleavage sites were found within the predicted conserved epitope sequences. For example, within the 12-amino acid conserved epitope identified among GST-Ra, GST-Av, GST-Hl, and GST-Rd, a 7-aa sequence was found that did not contain any proteasomal cleavage site. On the other hand, only a 4-amino acid sequence was found devoid of cleavage sites within the 7-aa epitope identified in GST-Rm. The size of the cleaved peptide predicted from the conserved epitope of GST-Ra, GST-Av, GST-Hl, and GST-Rd (7 amino acids) was still slightly smaller than the anticipated size of APC-degradation peptides (8-10 amino acids) (Rammensee et al., 1995; Rock and Goldberg, 1999; Falk and Rötzschke, 1993). The data suggest that the conserved epitopes among the four GSTs could have a role in stimulating T-cells. These findings also suggest that the reported conserved B-cell epitopes within the four GSTs are immunogenic.

GST-Av
MAPVIGYWDIRGLAQPIRLLLAHVDAKVEDKRY**<u>SCGPPD</u>**FDRGSWLKEKHTLGLEFP
NLPYYIDGDLKITQSMAILRYLARKHGIEGKTETEKQRVDLTEQQFADFRMNWVRICY
NPDFEKLKGDYLKGLPASLKAFSDYLGSHKFFAGDNLTYVDFLAYEMLAQHLIFAPD
CLKDFANLKAFVDRIEALPHVAAYLKSDKCINWPLNGDMASFGSRLQKKP

GST-Rd
MAPVIGYWDIRGLAQPIRLLLAHVDAKVDDKRY**<u>SCGPPPD</u>**FDRSSWLNEKTKLGLEF
PNLPYYIDGDVKLTQSMAILRYLARKHGIEGKTEAEKQRVDVAEQQFADFRMNWVRL
CYNPEFEKLKGDYLKNLPASLKAFSDYLGTHKFFAGENLTYVDFIAYEMLAQHLIFAP
DCLKDFANLKAFVDRIEALPHVAAYLKSDKCIKWPVNGDMASFACRLQKKP

GST-Ra
MAPILGGYWNIRGLAQPIRLLLAHADAKVEEKQY**<u>SCGPPPD</u>**FDKSYWLSEKPKLGLD
FPNLTQSMAILARKYDIMGKTGAEKQRVDVVEQQIADFRVNWGRLCYSPDFEKLKGD
YLKDIPASLKAFSDYLGNRKFFAGDNLTYVDFIAYEMLDQHLFAPDCLKDFANLKAFV
DRVAALPRVAAYLKSDKCIKWPLNGDMASFGSRLQKKPLKAFVDRVAALPRVAAYL
KSDKCIKWPLNGDMASFGSRLQKKP

GST-Rm
MAPTPVVGYTTARGLAQSIRNLLVYKGVHFEDKRYEFG**<u>PAPT</u>**YEKLGWAADSASIGF
TFPNLPYYIDGDVRLTQSLAILRYLGKKHGIDARSDQEAAELWLMEQQANDLLWALV
VTAMNPNATEARKSQEKRLADSLPRWQELLKKRRWALGNTLTYVDFLLYEAIDWNR
QFAPDAFANRPELLDYLRRFEQLPNLKEYFASDKYVKWPIMAPYMAFWGHK

GST-Hl
MPAILGYWDIRGLAQPIRLLLAHADVKYEDKRY**<u>SCGPPPD</u>**FDRSAWLKEKHTLGLEF
PNLPYYIDGDVKLTQSMAILRYLARKHGLDGKTEAEKQRVDVTEQQFADFRMNWVR
MCYNPDFDKLKVDYLKNLPDALKSFSEYLGKHKFFAGDHVTYVDFIAYEMLAQHLLL
APDCLKDFPNLKAFVDRIEALPHVAAYLKSDKCISWPLNGDMASFGSRLQKKP

Figure 3: Prediction of proteasome cleavage sites within tick-GST amino acid sequences. The GST amino acid sequences were translated from GST nucleotide sequences of *Rhipicephalus decoloratus* (GST-Rd) (MK133339), *Amblyomma variegatum* (GST-Av) (MK133337), *Rhipicephalus appendiculatus* (MK133338), *Rhipicephalus microplus* (GST-Rm) and *Haemaphysalis longicornis* (GST-Hl) (AY298731.1). The cleavage site prediction was performed using NetChop 3.1 server and/or Pcleavage web server. The gray shaded amino acids are regions that contain cleavage sites. Amino acid sequences indicated in bold underlined font are regions within the conserved epitope sequences (reported in Tables 1 and 2 using both sequence- and structural-based algorithms) that do not contain proteasomal cleavage sites.

Finally, based on the predicted conserved epitopes, the corresponding peptides were synthesized conjugated with KLH protein carrier (Carter, 1996) and independently used for rabbit immunization. The peptide anti-sera were shown to react against the corresponding peptides/BSA conjugates. In particular, the reaction observed with GST-Ra, GST-Rd, GST-Av and GST-Hl was stronger than with GST-Rm (Fig. 4). Conversely, tests were performed to determine whether the rGST anti-sera, raised in previous work (Ndawula et al., 2019), could react against the corresponding peptide. Similarly, in comparison to the anti-sera of *R. microplu*s (rGST-Rm), *R. appendiculatus* (rGST-Ra), *A. variegatum* (rGST-Av), *H. longicornis* (rGST-Hl) and *R. decoloratus*

(rGST-Rd) showed a stronger reaction against the peptide/BSA conjugates (Fig. 4). Therefore, unlike GST-Rm, the peptide from GST-Ra, GST-Rd, GST-Av, and GST-Hl was found to be more immunogenic.

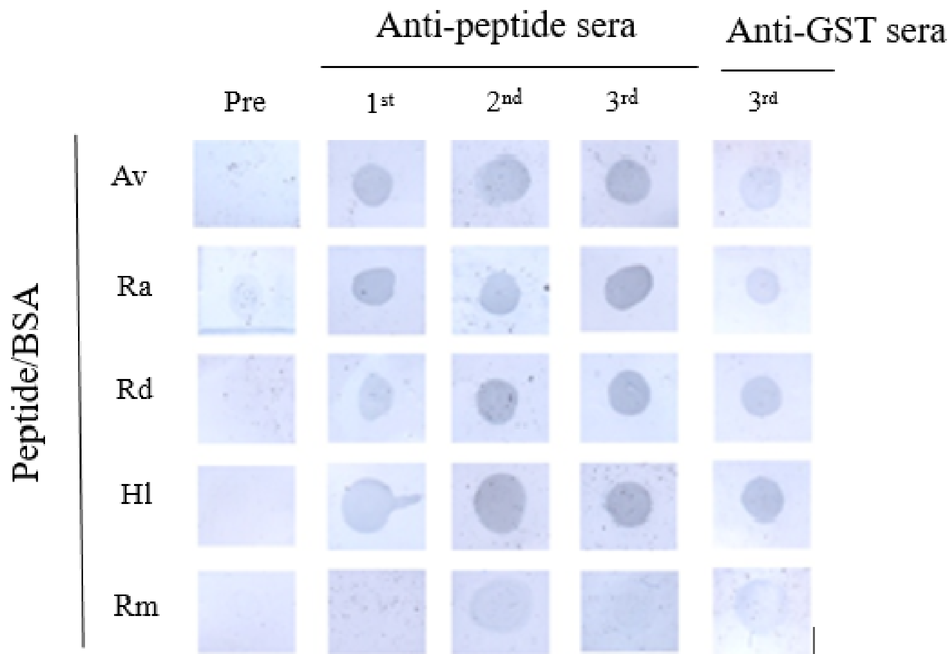

Figure 4: Dot-blot analysis of tick GST peptide immunogenicity. The sera (1:1,000) that were raised after the first (1st), second (2nd) and third (3rd) GST peptide/keyhole limpet hemocyanin (KLH) rabbit-immunization reacts against the corresponding GST peptide/BSA conjugate. The full-sequence recombinant anti-rGST sera (Ndawula et al., 2019) also recognize the GST peptide/BSA conjugates. The pre anti-sera (1:100) does not recognize the GST peptide/ BSA conjugates. The anti-peptide sera were collected at 2-week intervals and the GST epitopes were predicted from the GST sequence of *Amblyomma variegatum* (MK133337), *Rhipicephalus decoloratus* (MK133339), *Rhipicephalus appendiculatus* (MK133338), *Rhipicephalus microplus* (AF366931.1) and *Haemaphysalis longicornis* (AY298731.1).

4. Conclusion

Ultimately, the data presented here could support the hypothesis that GST-Ra, GST-Rd, GST-Hl, GST-Rm and GST-Av contain conserved epitopes, explaining the cross-reaction among tick GSTs (Ndawula et al., 2019). Additionally, the study brings forward an approach to selecting B-cell epitopes. Most importantly, the epitopes predicted herein were found to be immunogenic. Based on these findings, we hypothesize that the identified epitopes could be used to constitute epitope-based antigen tick vaccines against *R. appendiculatus*, *A. variegatum* and *H. longicornis*.

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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.

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5.0. CAPÍTULO 3: ¨Vaccine efficacy of different recombinant glutathione S-transferase formulations against Rhipicephalus appendiculatus ¨ (In preparation)

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Vaccine efficacy of different recombinant glutathione S-transferase formulations against *Rhipicephalus appendiculatus*

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Abstract

Nowadays, ticks and tick-borne diseases are a significant global economic burden. Despite the increasing phenomenon of tick resistance, acaricide use remains the principal method of tick control. In search of ways to address tick acaricide resistance, several alternative tick-control measures have been investigated, being vaccination one of most promising. To date, several tick vaccine antigens have been shown to induce partial protection against particular tick species. For instance, glutathione S-transferase of *Haemaphysalis longicornis* (GST-Hl) has been shown to induce partial protection against *Rhipicephalus microplus* and *Rhipicephalus appendiculatus,* but not against *Rhipicephalus sanguineus.* Therefore, the goal of the current study is to investigate whether cocktail GST vaccines could increase protection against different tick species. The vaccination experiments were performed in rabbits, by use of two GST antigenic cocktails and a mono antigen GST-Hl vaccine. Cocktail 1 contained the recombinant GSTs of *Amblyomma variegatum* (rGST-Av), *Rhipicephalus decoloratus* (rGST-Rd) and rGST-Hl, while cocktail 2 contained rGST-Av and rGST-Rd. The anti-cocktail sera reacted against the cocktail constituting antigens and against the rGST protein of *R. appendiculatus*. Overall, a difference in biological parameters was noted among the ticks that were fed on cocktail 1, cocktail 2 and the GST-Hl treated rabbits in comparison to the those fed on the control rabbits. However, a statistical difference was only shown in the tick number (12.28%) and egg laying (37.17%) of ticks that were fed on rabbit treated with cocktail and GST-Hl respectively. These findings raise hope, first, that the cocktail vaccines could impact on the biological parameters of *R. microplus*, *A. variegatum*, *R. decoloratus* and *H. longicornis*. Second, that the cocktails could be a potential candidate for anti-tick cattle vaccination.

Keywords: Cocktail vaccines, glutathione S-transferase, tick control.

1. Introduction

Ticks are blood-feeding ectoparasites capable of transmitting a range of pathogens such as bacteria, protozoa, fungi and viruses, that severely impact humans and animals (de la Fuente et al., 2008; Brites-Neto et al., 2015). For instance, *Rhipicephalus appendiculatus* (brown tick)is a three-host tick specie that transmits *Theileria* spp. (Koch et al., 1983; Young & Purnell, 1973) pathogens that cause East Coast Fever (one of the most devastating cattle disease that affects East, Central and Southern Africa) (Mukhebi et al., 1992), Corridor disease and January disease (Uilenberg, 1999; Jura & Losos, 1980). Additionally, during feeding, *R. appendiculatus* secretes and inoculates saliva containing neurotoxins, chemicals that can cause brown ear tick paralysis in cattle (Mans et al., 2004).

Currently, the use of acaricides is the main approach to tick control. Worldwide, however, there are reports of acaricide tick resistance (Higa et al., 2015; Abbas et al., 2014). Moreover, the excessive use of acaricides further raises concern over contamination of meat, milk and the environment (Graf et al., 2004). Therefore, numerous alternative tick control approaches have been suggested (Abbas et al., 2014), of which anti-tick vaccination is regarded as the most sustainable approach. Indeed, the lists of tick potential vaccine antigens have been compiled (de la Fuente & Kocan, 2006; Merino et al., 2013; Nuttall et al., 2006; Valle & Guerrero, 2018). However, of the identified antigens, Bm86 remains the most outstanding under field conditions (de la Fuente et al., 2007; 1998). It is proposed, therefore, that combining at least two antigens could enhance the protection efficacy of tick vaccines (Willadsen et al., 2008). To date, multi-antigenic tick vacciness have been constituted (Hope et al., 2010; Parizi et al., 2012) and yet substantial enhanced protection is still to be reported under field conditions. Strikingly, reports in the afore-cited studies indicate reduction in antibodies induced against one of the cocktail constituting antigens,which could partially explain why most cocktail tick vaccine antigens induce a slight or no substantial enhanced protection. Such immunological response developed for multiple antigens raises doubts on how the cocktail tick vaccine constituting-antigens can be selected.

Recently, an approach to constitute cocktail antigens was demonstrated (Ndawula et al., 2019). In addition to revealing that rGST-Rd (recombinant GST from Rhipicephalus decoloratus) and rGST-Av (recombinant GST from Amblyomma variegatum) were the best candidates to constitute a cocktail rGST-based vaccine, the study suggested that rGST-Hl (recombinant GST from Haemaphysalis longicornis) could be used to constitute cocktail tick vaccines. Evidence that the anti-cocktail (rGST-

Rd and rGST-Av) sera strongly cross-react against the rGSTs of other tick species such as R. appendiculatus, Rhipicephalus microplus, H. longicornis (Ndawula et al., 2019), suggests that the cocktail has potential for inducing cross-protection against different tick species. Moreover, earlier reports indicate that rGST-Hl induces cross partial protection against R. microplus and R. appendiculatus (Parizi et al., 2011; Sabadin et al., 2017). The current study, therefore, addresses the question whether combining two or three rGST vaccine antigens could enhance protection against R. appendiculatus in rabbits. In this way, the potential of vaccinating against a wide range of tick species using cocktail rGST antigens was illustrated.

2. Materials and Methods

2.1. Animals

The vaccination experimental design was approved by the Institutional Animal care and use Committee of the International Livestock Research Institute (ILRI, Nairobi, Kenya).The *R. appendiculatus* ticks used in the study were pathogen free and were obtained from the tick colony of the ILRI Tick Vector Laboratory. In this study, three-month old and pathogen free New Zealand rabbits weighing 2.5 kg were used. The rabbits were obtained from the rabbit colony of the ILRI Small Animal Unit Farm. All the experiments were conducted at the ILRI Tick Vector Laboratory.

2.2. Vaccine preparation

2.2.1. GST protein expression

The recombinant proteins used herein were separately expressed using pET-43.1a (Novagen) constructs with the glutathione S-transferase open reading frame (ORF) inserts of *R. appendiculatus* (*GST-Ra*) (MK133338), *R. decoloratus* (*GST-Rd*)(MK133339), *A. variegatum* (*GST-Av*) (MK133337) (Ndawula et al., 2019) and *H. longicornis* (*GST-Hl*) (AY298731.1) (da Silva Vaz et al., 2004). The constructs (pET-43.1a/*GST-Av*, pET-43.1a/*GST-Rd*, pET-43.1a/*GST-Av* and pET-43.1a/*GST-Hl*) were independently transformed into *Escherichia coli* AD494 (DE3) (Novagen Inc USA) competent cells using the thermic shock method (Maniatis et al., 1985). The protein expression was performed as previously described (Ndawula et al., 2019). In brief, the protein expression was induced for 6 h using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) after which the culture broth was harvested, and centrifuged at 6,000 x g at 4ºC for 10 min. The pellet was retained, washed 3 times in phosphate buffered saline (PBS) pH 7.2 and kept at -20 ºC until purification.

2.2.2. GST protein purification

The pellets were separately suspended in 15 ml of PBS pH 7.2 and lysed at 30 pulses for 30 sec, 36 % amplitude, using an ultrasonicator (Pulse Sonics Vibra-cell VCX 500–700). The supernatant was applied onto a GSTrap™ 4B column(GE Healthcare Bio-Sciences USA) and purified by affinity chromatography as previously described (Ndawula et al., 2019). Through western blot analysis, the purified recombinant proteins rGST-Hl, rGST-Rd, rGST-Av, and rGST-Ra were confirmed with the corresponding previously raised anti-rGST sera (Ndawula et al., 2019).

2.2.3. Vaccination composition

The antigens used herein were selected based on the previous report (Ndawula et al., 2019). The vaccines were constituted of 200 µg of protein in total as follows: cocktail 1 containing rGST-Hl, rGST-Rd and rGST-Av (67 µg* 3); cocktail 2 containing rGST-Rd and rGST-Av (100 µg* 2); mono-antigenic vaccine containing rGST-Hl (200 µg). The placebo was made up of PBS pH 7.2. To make the immunization doses, the above formulations were independently mixed with the adjuvant (1:1) (Montanide ISA 61 VG seppic, Paris, France) using the Eppendof ThemoMixer C (Themo Fisher Scientific) until the formation of homogenized emulsions.

2.3. Rabbit vaccination

Eight rabbits were randomly distributed among four experimental groups. One week prior to vaccination, 1 ml of blood was drawn from the rabbit ears. The samples were centrifuged at 16,000 x g at 4ºC and serum kept at -20ºC until further use. To vaccinate, 1 ml of the each antigen prime dose was inoculated subcutaneously on the rabbit back. In all sessions, the inoculations were blindly performed by the same person. The rabbits were boasted two times at an interval of 2 weeks with the same antigen dose. Two weeks after each inoculation, blood samples were collected from the rabbits and processed as described above.

The tick-feeding cotton bags were attached to the rabbit ears using glue (Pattex, Henkel Chemical, Nairobi, Kenya). Two weeks after the last inoculation, each rabbit was infested with 60 unfed *R. appendiculatus* adult ticks (30 females and 30 males). The ticks that had not attached 24 h after infestation were removed and counted. The ticks were let to feed for 10 days during which the tick females that spontaneously detached were collected and weighed. The female ticks were placed in separate sterile microfuge tubes and kept in an incubator at 30ºC under 80% humidity (Branagan, 1973). Considering

that *R. appendiculatus* fully feeds and detach within 5-7 days (Bailey, 1960; Joyner & Purnell, 1968), the ticks that did not detach after 10 days were considered not to have successfully fed. Two weeks after harvesting all the ticks, blood samples were collected from the rabbits, centrifuged at 16,000 x g at 4ºC and serum kept at -20ºC.

The ticks were further kept in the incubator for 10 to 14 days for egg laying. Females that died during egg laying were quantified. The eggs were weighed and kept in the incubator for 2 weeks at 30ºC under 80% humidity to larva hatching (Branagan, 1973). The larvae were further frozen at -20ºC. Finally, under the stereoscope, the larvae were carefully sorted from egg shells and dead eggs and the weight of the sorted larvae was determined.

2.4. Immunological analyses

In this study, the immunological analyses were performed using indirect ELISA as previously described (Ndawula et al., 2019; Parizi et al., 2011), but with slight modifications.

2.4.1. Determining the vaccine-sera titre

To determine the titre, all the antisera were tested against the rGST-Hl antigen. This antigen was selected because it was earlier reported to induce humoral immune response in rabbits and cattle as well as protection against *R. appendiculatus* (Sabadin et al., 2017) and *R. microplus* (Parizi et al., 2011), respectively. The assay was performed as follows.

First, the flat bottom immulon^{TM} 2HB 96 microtitre plates (Dynatec USA) were coated with 50 µl of 0.1 µg/µl of rGST-Hl in 50 mM of carbonate bicarbonate buffer pH 9.6 and incubated for 12 h at 4 ºC. Next, the coating antigen solution was poured off and the excess solution removed. The plates were then washed three times with 200 µl of PBS pH 7.0 -TWEEN 0.05%, filled with 200 µl of PBS-TWEEN 0.05% and incubated at 37ºC for 1 h. After, the plates were washed with PBS and then 100 µl of pre, 1st, 2nd, 3rd or 4th sera of a particular group was added. The pre-immunization sera (pre sera) was processed from blood collected before rabbit vaccine inoculation. The first (1st), second (2nd) and third (3rd) sera were processed from blood collected after the priming dose (the first dose), the 1st booster (second dose) and 2nd booster dose (third dose) vaccination, respectively. The fourth (4th) sera were processed from blood collected 2 weeks after the end of the infestations. The sera used in the assay were diluted 1:8,000, 1:16,000, 1:32,000, 1:64,000 and 1:128,000 in PBS-TWEEN 20. The plates were incubated for 2 h at 37 ºC, washed 3 times with PBS-TWEEN 0.05%, and then 100 µl of Goat anti-

Rabbit IgG (H+L) secondary antibody at a 1:5,000 dilution (# 31460 Invitrogen USA) was added and incubated at 37ºC for 1 h. After, the plates were washed 3 times using PBS-TWEEN 0.05%, and 100 µl of SIGMA FAST^{TM} OPD substrate (Sigma–Aldrich USA) added and incubated in the dark at room temperature for 15 min. The substrate was prepared as per the manufacturer's instructions. After incubation, 50 µl of 3 M HCl was added to stop the reaction. Finally, the reaction optical density reading was determined at 490 nm using an ELISA plate reader (Synergy HT BioTech USA). The vaccine sera were confirmed reactive (positive) when the optical density reading (490 nm) of the test serum was greater the average obtained for the negative control serum plus two standard deviations.

2.4.2. Rabbit vaccination sera analyses

The analyses were performed following the indirect ELISA protocol described in section 2.6.1 but with slightly modifications, to determine the response of the rabbits to the respective vaccines. The sera processed from blood samples that were collected from group 1 (rabbit 1 and 2) were separately tested against the rGST-Hl, rGST-Rd and rGST-Av antigens. The sera obtained from group 2 (rabbit 3 and 4) were separately tested against rGST-Rd and rGST-Av. Finally, the sera obtained from group 3 (rabbit 5 and 6) were separately tested to rGST-Hl. All assays, the pre, 1st, 2nd and 3rd sera were used.

The sera were diluted as follows: pre sera (1:8,000), 1st sera (1:16,000), 2ndand 3rd (1: 64,000). The sera dilutions used herein were selected based on titre values that were obtained in the above experiment (2.6.1). All assays were performed in triplicates. Statistical analyses were performed in GraphPad prism 8.0 (La Jolla California USA) using two-way ANOVA ($p < 0.005$) to determine the significance of the humoral response for vaccine antigens. The statistical significance was determined by comparing the reactivity of the sera that were collected at the same time from rabbits of the same group.

2.4.3. Assessing the sera cross-reactivity against the vaccine rGSTs constituting antigens

The immunized animal sera cross-reactivity analyses were performed as described in 2.6.1 but with slight modifications. In particular, the assays were performed using only the pre immune sera (1:8,000) and 3rd antisera (1:64,000). The sera obtained from rabbits of group 1 (1 and 2), group 2 (3 and 4) and group 3 (5 and 6) was separately tested against rGST-Av, rGST-Rd, rGST-Ra and rGST-Hl. The rGST-Ra was prepared as in previous study (Ndawula et al., 2019). All experiments were performed in

triplicates. The statistical significance of the sera cross-reactivity was assessed in GraphPad prism 8.0 software (La Jolla California USA) using two-way ANOVA ($p < 0.0001$). The statistical significance was determined by comparing the cross-reactivity of the 3rd sera that were collected from rabbits of the same group.

2.5. Determining the vaccine effect on rabbit infestation

The effect of the vaccines was determined in reference to the impact induced on the tick biological parameters: tick feeding, oviposition and egg fertility. The statistical significance analyzes of the vaccine on the tick parameters was performed in Excel software using the Student's *t*-test with unequal variance ($p < 0.05$). The difference (percentage) in the vaccine effect over the biological parameters was calculated as follows: Difference (%) = 100 × (1 − mean value of vaccination group/control group).

3. Results

3.1. rGST expression and purification

The GST proteins expressed and purified herein depicted a single band of 25 kDa (data not shown). Additionally, the anti-rGST sera that were previously raised (Ndawula et al., 2019) reacted against the homologous rGSTs expressed and purified in the current study: rGST-Hl, rGST-Rd and rGST-Av (data not shown) which further confirmed that proteins expressed and purified herein were rGSTs.

3.2. Immunological response to rGST vaccination

3.2.1. The vaccination-sera titre analyses

The sera against cocktail 1, cocktail 2 and rGST-Hl were obtained from group 1, 2 and 3 respectively, cross-reacted against the rGST-Hl antigen at 1:128,000 dilution (data not shown). On the other hand, the sera that were obtained from group 4 (adjuvant/PBS immunized) rabbits showed no reaction against the rGST-Hl antigen.

3.2.2. Rabbit humoral immune response

The sera induced in the rGST immunized rabbits was shown to react against the vaccine constituting antigens. In particular, the sera against cocktail 1 (rGST-Av, rGST-Rd and anti-rGST-Hl) sera, induced in rabbit 1 and 2, independently reacted against rGST-Av, rGST-Rd and rGST-Hl (Figure 1). Similarly, the sera against cocktail 2 (rGST-Rd and rGST-Av), induced in rabbit 3 and 4, independently reacted against rGST-

Rd and rGST-Av (Figure 2). The anti-rGST-Hl sera, induced in rabbit 5 and 6, reacted against rGST-Hl (Figure 3). In all the analyses $p<0.005$ (Two-way ANOVA).

Notably, in comparison to anti-GST-Hl sera (Figure 3), the anti-cocktail 1 sera (Figure 1 C) showed a weak recognition of rGST-Hl antigen. Similarly, in comparison to the anti-cocktail 2 sera (Figure 2), the anti-cocktail 1 sera showed a weak recognition of rGST-Av and rGST-Rd antigens (Figure 1 A and B).

A difference in the sera reactivity for vaccine constituting GST antigens was evident among the rabbits that were subjected to the same immunization. Cocktail 1 antisera induced in rabbit 1 showed a stronger cross-reaction against the corresponding cocktail constituting antigens than the sera induced in rabbit 2 (Figure 1). A similar pattern was revealed with anti-cocktail 2 sera induced in rabbit 3 and 4 respectively (Figure 2), but the difference in cross-reaction was not so evident as shown with anti-cocktail 1 sera (induced in rabbit 1 and 2) (Figure 1 A and B). By contrast to the anti-cocktail sera, the anti-rGST-Hl sera showed a slight difference in reactivity against the rGST-Hl antigen. Precisely, the anti-rGST-Hl sera induced in rabbit 5 showed a slight stronger reaction against the rGST-Hl antigen than with sera induced in rabbit 6 (Figure 3). Finally, in all assays, the sera that was obtained prior to the rGST rabbit immunization did not cross-react against the vaccine constituting antigens.

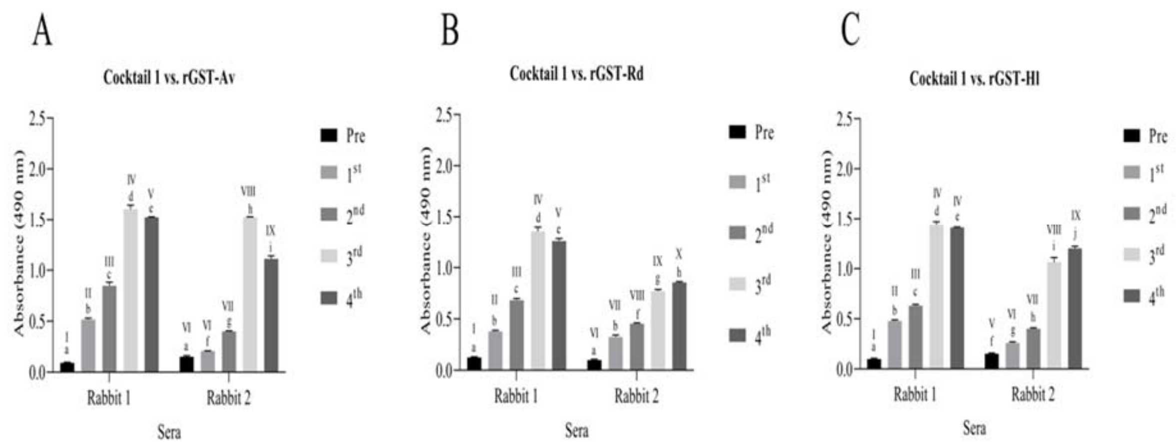

Figure 1. Analyzes of cocktail 1 immunogenicity. In group 1, rabbits 1 and 2 were immunized with the cocktail 1 vaccine that contained the glutathione S-transferase of *A. variegatum* (rGST-Av), *R. decoloratus* (rGST-Rd) and *H. longicornis* (rGST-Hl). The sera raised before immunization (pre), after the first (1st), second (2nd) and third (3rd) inoculation and after the end of infestation (4th) were tested against the cocktail 1 constituting antigens. The sera were diluted at 1:8,000 (pre-sera), 1:16,000 (1st sera) and 1: 64,000 (2nd, 3rd and 4th sera). $p<0.005$ (Two-way ANOVA), a-j: comparison between equivalent sera from different rabbit, I-X: comparison between sera from the same rabbit.

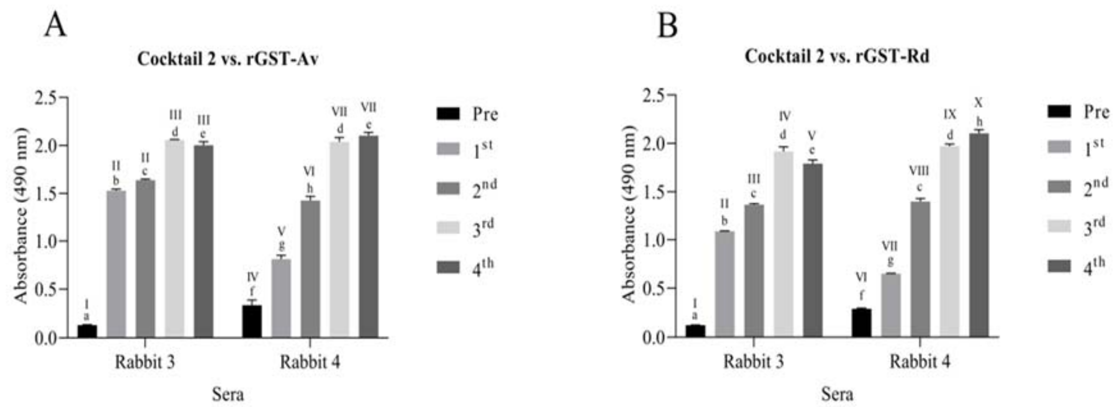

Figure 2. Analyzes of cocktail 2 immunogenicity. In group 2, rabbits 3 and 4 were immunized with the cocktail 2 vaccine that contained the glutathione S-transferase of *A. variegatum* (rGST-Av), and *R. decoloratus* (rGST-Rd). The sera raised before immunization (pre), after the first (1st), second (2nd) and third (3rd) inoculation and after the end of infestation (4th) were tested against the cocktail 2 constituting antigens. The sera were diluted at 1:8,000 (pre-sera), 1:16,000 (1st sera) and 1: 64,000 (2nd, 3rd and 4th). $p<0.005$ (Two-way ANOVA), a-h: comparison between equivalent sera from different rabbit, I-X: comparison between sera from the same rabbit

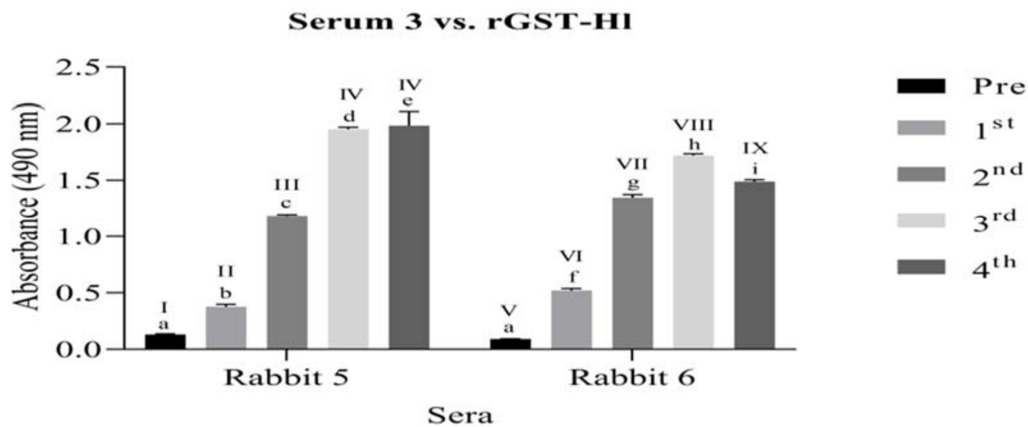

Figure 3. Analyze of the single glutathione S-transferase vaccine immunogenicity. In group 3, rabbits 5 and 6 were immunized with the GST of *H. longicornis* (rGST-Hl). The sera raised before immunization (pre), after the first (1st), second (2nd) and third (3rd) inoculation and after the end of infestation (4th) were tested against the rGST-Hl antigen. The sera were diluted at 1:8,000 (pre-sera), 1:16,000 (1st sera) and 1: 64,000 (2nd, 3rd and 4th). $p<0.005$ (Two-way ANOVA), a-i: comparison between equivalent sera from different rabbit, I-IX: comparison between sera from the same rabbit.

3.2.3. Antiserum cross-reaction against the non-homologous tick rGSTs

The anti-GST sera were shown to react against the vaccine constituting antigens and the rGST of *R. appendiculatus* (rGST-Ra) that was previously prepared (Ndawula et al., 2019). Shown in figure 4A, the anti-cocktail 1 sera cross-reacted against the four rGST antigens. A stronger cross-reaction was shown against rGST-Av, followed by rGST-Hl, rGST-Rd, and rGST-Ra. There was a slight difference between the cross-reaction against rGST-Hl and rGST-Rd and against rGST-Rd and rGST-Ra antigens. In all the analyzes the statistical significance of $p<0.0001$ was shown (Two-way ANOVA).

Strikingly, the anti-cocktail 1 sera that was induced in rabbit 1 exhibited a stronger cross-reaction against the four rGST antigens compared to the sera induced in rabbit 2.

Similarly, anti-cocktail 2 sera cross-reacted against the four rGST antigens. A stronger sera cross-reaction was noted against rGST-Av, followed by rGST-Rd, rGST-Hl and rGST-Ra (Figure 4B). A slight difference in sera cross-reaction was shown against rGST-Rd and rGST-Hl and against rGST-Ra and rGST-Hl. The anti-cocktail 2 sera induced in rabbit 3, showed a stronger cross-reaction against the four rGST antigens than the sera that was induced in rabbit 4. Overall, the anti-cocktail 2 sera exhibited a stronger cross-reaction against the four rGST antigens than with the anti-cocktail 1 sera.

The anti-rGST-Hl sera, on the other hand, also showed cross-reaction against the four rGST antigens (Figure 4C). As expected, anti-rGST-Hl sera strongly reacted against the homologous rGST-Hl, followed by the cross-reaction against non-homologous rGST-Av, rGST-Rd and rGST-Ra antigens respectively. There was also a small difference between the anti-rGST-Hl sera cross-reaction against of rGST-Hl and rGST-Av, but a bigger difference in cross-reaction was shown against rGST-Rd and rGST-Ra. Furthermore, rabbit 5 sera showed a stronger cross-reaction against the four rGST antigens compared to the anti-rGST-Hl sera induced in rabbit 6.

Put together, the data revealed that all the anti-rGST sera similarly cross-reacted against rGST-Ra, although anti-rGST-cocktail 2 sera showed a slightly higher cross-reactivity. Moreover, all pre-immunization sera showed no reaction against the four rGST antigens.

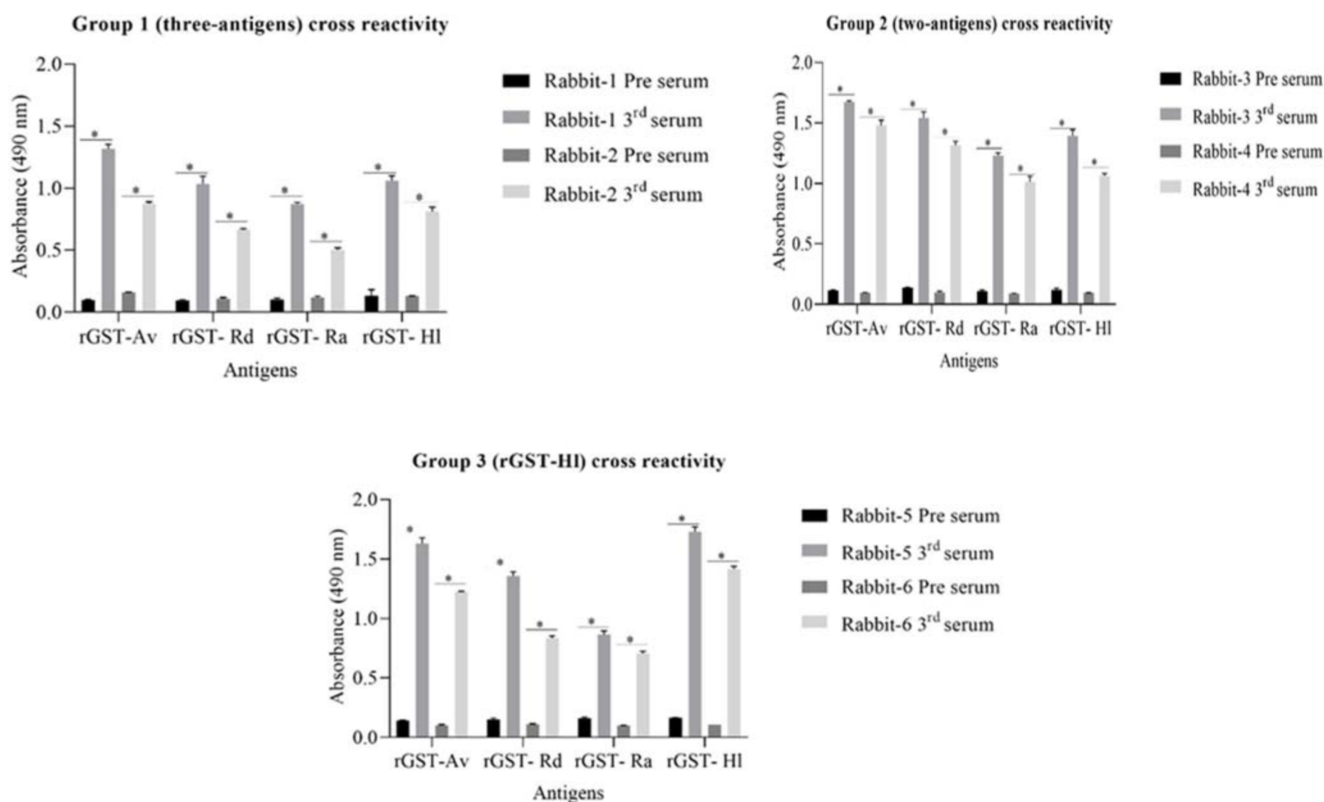

Figure 4. Assessing the antisera cross-reactivity with the tick glutathione S-transferase antigens. The group 1 sera (rabbits 1 and 2) immunized with cocktail 1 vaccine (glutathione S-transferase of *A. variegatum* (rGST-Av), *R. decoloratus* (rGST-Rd) and *H. longicornis* (rGST-Hl)). The group 2 sera (rabbits 3 and 4) immunized with cocktail 2 vaccine (rGST-Av and rGST-Rd). The group 3 sera (rabbits 5 and 6) immunized with rGST-Hl. It was tested sera before the immunization (pre), diluted at 1:8,000, and after the third (3rd) inoculation, diluted 1:64,000, against the vaccine constituting antigens (rGST-Av, rGST-Rd and rGST-Hl) and the GST of *R. appendiculatus* (rGST-Ra). The statistical significance was determined by the comparison between the cross-reactivity of the 3rd sera that were collected from rabbits of the same group showed a statistical significance *$p<0.0001$ (Two-way ANOVA).

3.3. Effect of the vaccination in rabbits

The vaccination trial against R. appendiculatus showed a statistically significant decrease in tick number (12.28%) and egg laying (37.17%) in cocktail 1 and GST-Hl groups, respectively (Table 1). Other parameters were not significantly affected by the immunization. In addition, the immunization with cocktail 2 did not significantly induce a reduction in biological parameters.

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

Group	Rabbit	Tick number[a]	Tick weight[b]	Egg laying[c]	Egg fertility[d]
Cocktail 1	1	26	338.02	0.126	0.374
	2	24	408.11	0.413	0.351
	Mean	25	373.06	0.269	0.362
	S.D.	1,41	49.56	0.2	0.016
	Difference[e]	**12.28*%**	**23.73%**	**49.91%**	**15.20%**
Cocktail 2	3	28	274.87	0.201	0.426
	4	28	515.75	0.5	0.444
	Mean	28	395.31	0.35	0.435
	S.D.	0.0	170.33	0.211	0.013
	Difference[e]	**1.75%**	**19.18%**	**34.85%**	**-1.75%**
rGST-Hl	5	22	500.32	0.369	0.418
	6	15	273.56	0.307	0.289
	Mean	18.5	386.94	0.338	0.353
	S.D.	4.95	160.34	0.043	0.091
	Difference[e]	**35.09%**	**20.89%**	**37.17%***	**17.31%**
Control	7	30	499.09	0.568	0.466
	8	27	479.19	0.508	0.389
	Mean	28.5	489.14	0.538	0.427
	S.D.	2.12	14.07	0.042	0.054

a= Number of engorged ticks recovered on rabbits.

b= Average weight (mg) of engorged ticks.

c= Total egg weight per total females weight.

d= Total larvae weight 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.

4. Discussion

Glutathione S-transferases are a class of enzymes that play a role in cellular detoxification of xenobiotics and endobiotics (Hayes et al., 2005; Sharma et al., 2004). Despite the reports that GSTs are involved in resistance, it remains unclear about how GSTs contributes for tick resistance to acaricides. Nonetheless, GSTs have been exploited as targets for the development of tick (Parizi et al., 2011; Sabadin et al., 2017) and endo-parasite (Brelsford et al., 2017; Veerapathran et al., 2009; Riveau et al., 1998; Paykari et al., 2002) vaccines. In the current study, therefore, the potential of vaccine composed by GST antigens to induce protection against *R. appendiculatus* rabbit-infestation has been investigated. When used as a single vaccine antigen, rGST-Hl induced protection against *R. microplus* (Parizi et al., 2011) and *R. appendiculatus* (Sabadin et al., 2017). The vaccine, however, did not induce a statistically significant impact against *R. sanguineus* (Sabadin et al., 2017). In another study, when rGST-Hl was combined with Vitellin-Degrading Cysteine Endopeptidase (VTDCE) and Boophilus Yolk pro-Cathepsin (BYC) and tested against *R. microplus* cattle infestation under field conditions, the protection was similar to single rGST-Hl vaccine (Parizi et al., 2012).

The findings presented herein regarding GST-Hl are similar to the earlier report that used the same antigen that showed protection against *R. appendiculatus* infestation (Sabadin et al., 2017). In a related recent study, a cocktail of rGST-Rd and rGST-Av induced a reduction on the tick number, but not on the other biological parameters of the *R. sanguineus* adult female (Ndawula et al., 2019). By contrast, the data presented herein reveal that cocktail 2 showed no statistically significant effect on all the *R. appendiculatus* biological parameters. There are multiple GST isoforms in tick, so maybe currently cocktail did not affect the *R. sanguineus* physiology equally than other GST vaccines previously tested (Dusher et al., 2014).

Generally, although the cocktails did not a statistical significance effect, a substantial significance was noted among the different vaccinated rabbits. To illustrate (table 1), out of the 27 ticks that separately attached onto rabbit 1 and 2 (cocktail 1 vaccinated group), the live female ticks recovered were 11 and 22 respectively. By contrast, out of the 29 and 30 ticks that separately attached onto rabbit 3 and 4 (cocktail 2 vaccinated group), 18 and 27 live female ticks were recovered respectively. In addition, out of the 22 and 15 ticks that separately attached onto rabbit 5 and 6 (single antigen

treated group) 15 and 10 live female ticks were recovered respectively. However, although a lower recovery was also shown in ticks that were obtained from the single antigen treated rabbits, there was no substantial significance in the average weight of tick egg and larvae average. Rather a substantial significance was shown in the average weight of the eggs and larvae that were obtained from the ticks of the cocktail vaccinated rabbit (1 and 3). Despite the substantial significance exhibited, it is evident that there were differences in tick attachment among the rabbits. Therefore, the questions are: why were there differences in tick attachment among the rabbits? And why were there differences in the live tick recovery among the rabbits that were inoculated with the same vaccine?

Intriguingly, although all the vaccines were immunogenic, a difference in immune response was shown among the rabbits that were inoculated with the same vaccine (Figure 1-3). Furthermore, a high humoral immune response was shown in the rabbits with a low tick recovery. Similarly, the efficacy of single tick-antigen vaccines has been associated to a high humoral immune response (Lambertez et al., 2012). Nevertheless, put together, the differences could suggest that there were differences in the genetic makeup of the rabbits used in this study. Although this phenomenon is yet to be reported in line with tick vaccines, the differences in immune response has been attributed to the differences in the genetic makeup of the experimental animals. Indeed, the effect of genetic control on immune response has been extensively discussed (McDevitt, 2000). Evidence indicates that the differences in humoral immune response are highly likely among the inbred experimental animals (Tuttle et al., 2018). However, we cannot rule the possibility that the animals used in this study were inbred.

Previously GST-Hl vaccination trial in rabbits against *R. appendiculatus*, damaged tick ovary and salivary glands, resulting in reduction of biological parameters (Sabadin et al., 2017). The overall protection achieved with rGST-Hl against adult *R. appendiculatus* infestation was 62.7% (based on number of engorged ticks) or 67.1% (based on the weight of engorged ticks). As shown herein and in earlier papers with rGST-Hl (Parizi et al., 2011; Parizi et al., 2012; Sabadin et al., 2017), immunization with rGST-Hl can affect tick physiology.

Interesting, in another study, *R. appendiculatus* infestation of rabbits immunized with a *R. microplus* cystatin (rBrBmcys2c) showed to induce damage in the gut, ovary and salivary glands (Parizi et al., submitted). However, it was observed a small reduction of 11.5% in tick number. Similarly, immunization of cattle against *Babesia bovis*,

combining a multi-epitope modified vaccinia Ankara virus and a recombinant protein, induced strong Th1 cell responses, but fails to trigger neutralizing antibodies required for protection (Ortiz et al., 2019).

Through immunological assays, it was shown that all the rGST vaccines (in the current and previous experiments (Ndawula et al., 2019) were immunogenic and the cocktail GST antisera reacted against each GST present in the cocktail vaccine. However, there was a distinct antisera reactivity against vaccine constituting antigens. The highest difference in antisera reactivity was illustrated with the anti-cocktail 1 (rGST-Av, rGST-Rd and rGST-Hl) sera, followed by anti-cocktail 2 (rGST-Av and rGST-Rd) sera. The difference in anti-cocktail sera reactivity could be due to the competition among the cocktail constituting rGST antigens. Despite the fact that there was a difference in cocktail antisera reactivity, all antigens in rGST based vaccines are immunogenic. Interestingly, it was observed that GST used in this cocktail have similar B-cell epitope (submitted). The antigenic competition is commonly reported among combined vaccine antigens (Insel, 1995; Halperin et al., 1999; Sesadic et al., 1991 Parizi et al., 2012). Parizi et al., (2012) showed that bovine sera developed after 78 days of immunization showed an increase reaction against rGST-Hl, rVTDCE and rBYC. However, at the end of the experiment (day 127), there was a decrease in reaction against rVTDCE and rBYC, but not rGST-Hl.

Previously, the potential use cocktail tick vaccine has been investigated (Parizi et al 2012; Lambertz et al., 2012; Coumou et al., 2015; Olds et al., 2016). Additionally, in comparison to the current study, the cocktails that were investigated in the aforementioned studies were constituted by non-similar proteins. On the contrary, herein, there was a smaller reduction in the serum induced for cocktail constituting antigens in comparison to the aforementioned studies. However, similar to other past studies, the cocktail vaccine did not improve the vaccine effect in the parasite biological parameters.

Conclusion

Despite fact that the cocktail GST vaccines impacted on the biological parameters of *R. appendiculatus* the findings are not conclusive. Therefore, further vaccination experiments against *R. appendiculatus* using more experimental animals are required. Further more, investigation are required to determine whether the cocktail vaccines can impact on other tick species: *R. decoloratus*, *A. variegatum*, *R. microplus*, and *H. longicornis*.

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7. Discussão

Glutationa S-transferases são enzimas que estão envolvidas na detoxificação de xenobióticos e endobióticos (LI, 2009). Essas enzimas estão presentes em virtualmente todos os organismos vivos e são normalmente reguladas durante a detoxificação de drogas. Por essa razão, as enzimas GST estão amplamente implicadas na resistência a medicamentos (LO & ALI 2007; LUI *et al*., 2001), herbicidas (CUMMINS *et al*., 1999) e inseticidas (ENAYATI *et al*., 2005; CHE-MENDOZA *et al*., 2009). Dado o seu papel na detoxificação, as GST têm sido estudadas na busca de biomarcadores para resistência (BERNIG *et al*., 2016; BROGDON & BABER, 1990; MOROU *et al*., 2010), aumento da suscetibilidade a inseticidas (PASAY *et al*., 2009) e no controle imunológico de endoparasitas (BRELSFOD *et al*., 2017; PAYKARI *et al*., 2002; RIVEAU *et al*., 1998).

Apesar de pesquisas demonstrarem que as GST podem estar envolvidas na detoxificação de acaricidas (HERNANDEZ *et al*., 2018; DA SILVA VAZ *et al*., 2004 a), dados sobre o papel destas enzimas nos carrapatos permanecem escassos. Interessantemente, o silenciamento de GST por RNAi induziu suscetibilidade à acaricidas em *R. sanguineus* demonstrando que essas enzimas são úteis no processo de detoxificação nesse carrapato (DUSCHER *et al*., 2014).

Já foi demonstrado, em coelhos e bovinos, o potencial de rGST-Hl para induzir proteção imunológica contra carrapatos (SABADIN *et al*., 2017; PARIZI *et al*., 2011). Baseado nisso, o objetivo geral dessa tese foi estudar o potencial de utilização de uma vacina universal composta por multiplas GST no controle dos carrapatos endêmicos da Uganda, Africa (*R. appendiculatus, A. variegatum* e *R. decoloratus*) *e* Brasil, América do Sul (*R. microplus*).

Inicialmente, os candidatos ao coquetel vacinal foram selecionados com base na reação cruzada de soros anti-rGST contra GST de diferentes espécies de carrapatos. O coquetel foi construído utilizando as rGST de *A. variegatum e R. decoloratus*, uma vez que foram consideradas imunogênicas, pois apresentaram uma maior reatividade cruzada de seus respectivos anti-soros contra as outras GST, além de apresentarem um alto índice de avidez. Em trabalhos anteriores, a imunização com rGST-Hl foi relatada como não tendo impacto sobre *R. sanguineus* em coelhos (SABADIN *et al*., 2017). Em contraste, a vacina relatada neste trabalho induziu efeito sobre a alimentação de *R. sanguineus* em animais vacinados.

A análise *in silico* das sequências de GST de *R. appendiculatus* (GST-Ra)*, R.*

decoloratus (GST-Rd), *A. variegatum* (GST-Av) e *R. microplus* (GST- Rm) e *H. longicornis* (GST-Hl) revelou um epítopo de células B altamente conservado em GST-Ra (MK133338), GST-Av (MK133337), GST-Rd (MK133339) (NDAWULA *et al.*, 2019) e GST-Hl (AY298731.1) (DA SILVA VAZ *et al.*, 2004). No entanto, em GST-Rm (AF366931.1) (ROSA DE LIMA *et al.*, 2002) o mesmo não foi encontrado. Além disso, foi demonstrada *in vitro* a reação dos soros contra os peptídeos sintetizados baseados em regiões imunogênicas de GST-Ra, GST-Av, GST-Rd e GST-Hl. Dessa forma, os dados confirmam que as sequências de rGSTs contêm epítopos conservados de célula B.

Finalmente, foi analisado se uma vacina baseada em diferentes rGSTs poderia aumentar a proteção contra diferentes espécies de carrapatos. Os coquetéis 1 (rGST-Rd, rGST-Av e rGST-Hl) e 2 (rGST-Rd e rGST-Av) e o antígeno único rGST-Hl (PARIZI *et al.*, 2011) foram testados em coelhos contra a infestação por *R. appendiculatus*. Além disso, a imunogenicidade das vacinas de coquetel também foi avaliada. As vacinas compostas pelo coquetel 1 e o antígeno único tiveram impacto no número de teleóginas ingurgitadas e a postura de ovos, respectivamente. Apesar disso, devido ao pequeno número de animais utilizados e a grande variação entre os grupos vacinais, não é possível definir que uma vacina coquetel de GST protege mais que uma vacina com apenas uma GST. O impacto da vacina sobre os parâmetros biológicos foi maior entre os carrapatos alimentados em coelhos com maior nível de anticorpos, o que é coerente como medida da eficácia da vacina e proteção clínica contra patógenos (NAUTA *et al.*, 2009). Isto já tinha sido observado em outra vacina contra carrapato (VARGARS *et al.*, 2010).

Quando comparadas com uma vacina coquetel previamente testada composta pela combinação de rGST-Hl, vitellin-degrading cysteine endopeptidase (VTDCE) e *boophilus* yolk pro-cathepsin (BYC), utilizada para proteger bovinos contra a infestação por *R. microplus* (PARIZI *et al.*, 2012) a proteção obtida pelos coquetéis não foram aparentemente maiores. Uma hipótese para esse fato é a imunogenicidade diferencial entre os antígenos durante o processo de imunização.

8. Conclusões

Os resultados mostram a possibilidade da GST de induzir uma resposta imune protetora contra carrapatos, demonstrando o potencial de utilização de uma vacina de GST multi antigênica no controle dos carrapatos endêmicos da Uganda, Africa (*R. appendiculatus, A. variegatum* e *R. decoloratus*) e Brasil, América do Sul (*R. microplus*).

Em decorrência dos estudos realizados sugere-se que a dos epítopos de célula-B podem ser utilizados em diferentes vacinas antigênicas como forma de prevenção. E também indica a possibilidade de utilização desses epítopos tanto em GSTs quanto em vacinas alterantivas.

9. Perspectivas

Os resultados apresentados sugerem que é possível induzir proteção cruzada contra diferentes espécies de carrapatos, entre os quais carrapatos de importância econômica para Uganda, África (*R. appendiculatus*, *R. decoloratus* e *A. variegatum*) e Brasil, América do Sul (*R. microplus*).

No entanto, será necessário aprofundar os estudos afim de determinar a concentração apropriada dos antígenos rGST constituintes do coquetel vacinal. Também é essencial, realizar experimentos para determinar se as vacinas podem impactar nos parâmetros biológicos das espécies de carrapatos que não foram utilizadas nesta pesquisa de doutorado: *R. microplus*, *A. variegatum* e *R. decoloratus* ou mesmo *H. longicornis.*

Finalmente, é fundamental investigar tanto a eficácia quanto a proteção das vacinas contra os carrapatos em bovinos. Além disso, é possível examinar através da vacinação os possíveis peptídeos GST (epítopo) que possam induzir proteção cruzada, especialmente contra as espécies de carrapatos acima mencionadas. Por fim, tendo como base os resultados das experiências de vacinação composta por epítopos, as perspectivas de constituir antígenos quiméricos baseados em epítopos GST devem ser realizadas.

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ARTIGOS COMPLETOS PUBLICADOS

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RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

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