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Research paper

Design of the ATAQ peptide and its evaluation as an immunogen to develop a *Rhipicephalus* vaccine

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ABSTRACT

Tick infestation may cause several problems including affecting domestic animal health and reducing the production of meat and milk, among others. Resistance to several classes of acaricides have been reported, forcing researchers to search for alternative measures, such as vaccines against ticks, to ensure tick control while having no or at least low negative impacts on the environment and public health. However, the current commercially available vaccines in different strains of *Rhipicephalus microplus* are reported to be of low efficacy. Fortunately, reverse vaccinology approaches have shown positive results in the new generation of vaccines. On this basis, a synthetic peptide from the ATAQ protein, which is present in the gut and Malpighi tubes of *R. microplus*, was synthesized. The ATAQ proteins were isolated, characterized and sequenced from several species of the genus *Rhipicephalus*. The alignment showed 93.3% identity among DNA sequences of ATAQs from these species. Because of this, immunization trials with this peptide were conducted on mice, rabbits and cattle to evaluate the humoral immune response and the efficacy against *Rhipicephalus sanguineus* in addition to *R. microplus*. Based on recent results, we conclude that reverse vaccinology is a promising approach because it is more accurate and faster than conventional methods in the detection of potential antigens to use in anti-tick vaccines. It is not only applicable against *R. microplus* but also against tick species that play important roles in spreading other diseases. ATAQ proteins should be considered as the antigen in new trials to develop a multi-antigenic vaccine. Although these peptides behave as hapten and are not able to be recognized by the immune system on its own, using carriers and adjuvants helps its presentation and induces strong immune responses. Furthermore, an efficiency of 35% reduction in overall life cycle parameters was reported for *R. microplus* (98% for ELISA responder animals) and 47% for *R. sanguineus*. Although not yet enough to prevent the environment to infestation of ticks, this still constitutes a promising strategy that could be applied to integrated measures on tick control and in new research that develops anti-tick vaccines.

1. Introduction

Rhipicephalus microplus control has been conducted mainly by acaricide application, resulting in the selection of resistant ticks and contributing to environmental pollution (De La Fuente and Kocan, 2006). Because of this, studies have started to focus on vaccine development. The current main antigen used for anti-tick vaccines is the glycoprotein Bm86 (Willadsen and Kemp, 1988; Willadsen et al., 1988), which is predominantly located in the membrane of gut cells of ticks (Gough and Kemp, 1993).

Recombinant Bm86 is the basis for the commercial vaccines TickGARD and Gavac™ (Rodriguez et al., 1995; De La Fuente et al., 2007). Several vaccine formulations have been developed with this protein (Rand et al., 1989; Rodriguez et al., 1995; De La Fuente et al., 1999; Patarroyo et al., 2002). However, variable percentages of efficacy against strains of *R. microplus* were found in different geo-

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graphical locations, which may be due to natural allelic variations in the gene Bm86 (De La Fuente and Kocan, 2006).

A difference of 3.4% in Bm86 sequence among different strains could be enough to cause inefficient immune response against the strains (Garcia-Garcia et al., 1999). The percentage of efficacy of TickGARD and Gavac™ in a stall test with a regional isolate of *R. microplus* was 46.4% and 49.2%, respectively (Andreotti, 2006). These results are lower than what was observed in other regions of the world (Rand et al., 1989; Richardson et al., 1993; De La Fuente et al., 1999; Patarroyo et al., 2002). There is a difference in Bm86: the hydrophobic sites of CG (Campo Grande strain) have the potential to interfere with antibody binding, which can explain the low efficacy of Gavac™ to prevent tick infestation in cattle from the Campo Grande region (Andreotti, 2006).

Due to recent reports about the low efficacy of commercial vaccines in different strains of *R. microplus*, studies for new potential antigens have increased over recent years. Several vaccines have used whole proteins as antigens, such as trypsin inhibitors, subolesin, akirin, kallikrein, elastase inhibitors and peptides designed by reverse vaccinology approaches in post genomic studies. Interest has also been shown in strategies of compound vaccines (Tanaka et al., 1999; Sasaki and Tanaka, 2008; Prudencio et al., 2010; Andreotti et al., 2012; Carreón et al., 2012).

A recent study described the ATAQ protein, a putative Bm86 homolog with high similarity in primary and secondary structures. Despite the RNAi experiments showing a very weak phenotype for both Bm86 and ATAQ, Bm86 caused strong protection when used as an antigen (Nijhof et al., 2006). We hypothesized that the ATAQ protein is also a potential antigen for vaccine development because it is present in the gut and Malpighian tubes of all tick instars from *R. microplus*, *Rhipicephalus annulatus*, *Rhipicephalus decoloratus* and *Rhipicephalus evertsi evertsi* (Nijhof et al., 2010).

Reverse vaccinology is a promising strategy to discover new immunogen candidates for vaccines and is gaining increasing attention (Rappouli, 2000). The novel feature of this methodology is a bottom-up approach to identify potential antigens before animal testing, which is enabled by bioinformatics tools that can process huge amount of data from parasite genomics. Reverse vaccinology analysis usually searches for proteins and domains present in the host-parasite molecular interface (extracellular portion) and is capable of being recognized by the host immune system (presence of linear B and T cell epitopes). Great advances using the reverse vaccinology approach have been made in several disease agents that, up until now, lack effective means of control and whose vaccine development has not been successful using traditional methods. These diseases include meningitis B, malaria, tuberculosis, syphilis and hepatitis C (Rappouli, 2000; Adu-Bobie et al., 2003).

In the case of tick vaccines, one study targeting the Bm86 glycoprotein found a specific domain (Bm7462) and had 81.05% efficacy against *R. microplus* (Patarroyo et al., 2002). Maritz-Olivier et al. (2012) performed the transcriptome analysis of *R. microplus* and used the VaxiJen algorithm to analyze properties of the translated proteins to find 25 peptides with high antigenic properties. Among them all, three peptides were recognized by polyclonal serum against *R. microplus* gut protein extract.

Despite the fact that the ATAQ protein has not been detected in *Rhipicephalus sanguineus* (e.g., NCBI BioSystems Database, Geer et al., 2010), our *in silico* analysis identified sequences similar to ATAQ in other *Rhipicephalus* species, suggesting that this protein is likely to exist in these species.

This study aims to find an antigenic peptide from the ATAQ protein of *R. microplus* described in GenBank using a reverse vaccinology approach and characterize the immune response against such peptide. Using distinct adjuvants and testing in different host species, we have surveyed the vaccine effectiveness of this peptide against *R. sanguineus* in rabbits and *R. microplus* in cattle.

2. Materials and methods

2.1. Animals

Eighteen female BALB/c mice, aged between six to eight weeks, were used to test the immune response against the peptide. They were kept in cages (three mice per cage) with individual ventilation, controlled room temperature (22 °C), an exhaustion system and photoperiod (12 h of light and 12 h of dark). They were fed with sterilized commercial ration and sterile water *ad libitum*.

Nine female white cross-breed rabbits, aged between 12 to 20 weeks and weighing around two kilograms, were used. The animals were kept in individual cages and fed with commercial ration and sterile water *ad libitum*.

We selected eight male cattle Holstein-Friesan breed from a squad maintained at Embrapa Beef Cattle, aged between twelve to eighteen months old. These animals were kept in stalls and were fed with corn silage supplemented with mineral salt and water *ad libitum*.

All animals were maintained at Embrapa's biotery during the trials. All procedures with animals were approved by the ethical committee of animal use from Universidade Federal de Mato Grosso do Sul under protocol number 595/2014 and were carried out in accordance with the International Guiding Principles for Biomedical Research Involving Animals issued by the Council for the International Organizations of Medical Sciences.

2.2. Bioinformatics analyses and peptide synthesis

To detect potentially immunogenic peptides from the ATAQ protein of *R. microplus* (access number GenBank: ADR01301.1) we used various bioinformatics programs that added biological information about the potential immunogenicity of a peptide targeting the humoral immune response. The task of identifying immunogenic peptides can be defined as detecting protein peptides that have features that potentially increase the immunogenicity of this peptide. We used tools for the prediction of linear B-cell epitopes to detect properties that increase the probability of immunogenicity of a peptide (Bepipred program, IEDB Analysis Resource, cutoff 1.0 for 9 consecutive amino acids), the prediction of extracellular regions (signalp, tmhmm and GPI programs, cutoff of 0.5 for all) and the prediction of intrinsically unstructured proteins (IUPs, program IUPred, cutoff of 0.3 for 9 consecutive amino acids). These were intended to predict regions that could be constantly exposed in the whole protein at the tertiary structure level. This kind of feature is desirable because it indicates that the peptide is in a region of the protein that could be exposed to the host immune system. For the prediction of properties that diminish the immunogenicity, we used the BLAST program for the detection of possible regions of the ATAQ protein that have high similarity with *Bos taurus* proteins (e-value less than 0.5) in order to avoid possible cross-reaction with host proteins and intracellular portions as predicted by the tmhmm software. These regions would be less exposed to the humoral immune response (Fig. 1).

Other analyses of the ATAQ protein were also performed using algorithms that already existed. The software Geneious Pro 4.8.5 (Biomatters, Auckland, New Zeland) includes tools that predict antigenic properties from the primary protein structure, considering features such as hydrophobic and linear regions, and predict the secondary structure (alpha helix, beta strand and coils). The probability of an amino acid sequence region to be exposed on the surface of the protein was calculated by the Emini Surface Acces-

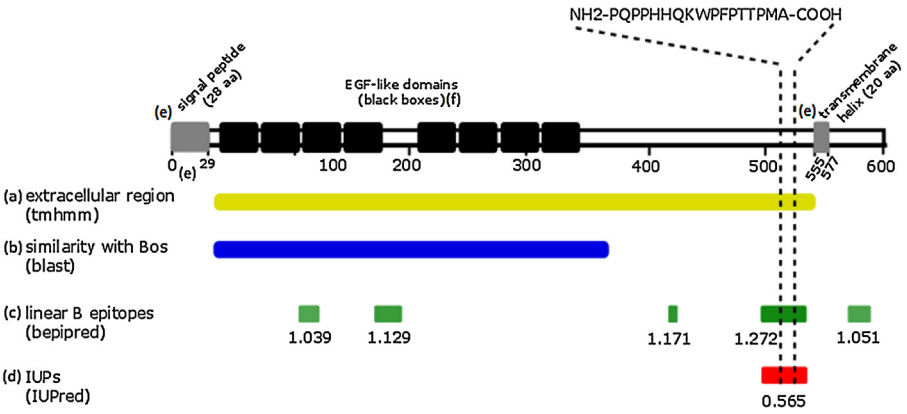

Fig. 1. Localization properties predicted for ATAQ proteins from *Rhipicephalus microplus*. The boxes/rhombuses indicate the localization of some sites of interest for immunogenicity. Yellow boxes are extracellular sites predicted by tmhmm (a); blue boxes are sites with similarities with *Bos Taurus* proteins (b); green boxes have color intensities proportional to scores of possible B cell epitopes predicted by Bepipred (c); red boxes are possible intrinsically unstructured proteins predicted by iupred (d); grey boxes are signal peptides and the transmembrane helix anchor site predicted by signalp (e); and black boxes are EGF-like domains (f).

sibility Scale (IEDB Analysis Resource) in order to ensure that the peptide region is exposed to the antibody when it is in native conditions. Despite the lack of information in the literature on predicting T and B cell epitopes based on Bovine Leukocyte Antigen (BoLA), especially in the case of presentation through MHC class II (our strategy), and based on previous data that have successfully predicted cattle T and B cell antigens through virtual matrices based on Human Leukocyte Antigens-DR (HLA-DR), we used MHC-II Binding Predictions (IEDB Analysis Resource) and the ProPred – MHC Class-II Binding Prediction Server to predict regions on the ATAQ protein that are recognizable by MHC class II host molecules (Vordermeier et al., 2003; Nene et al., 2012; Rodriguez-Valle et al., 2013). The alignment with ATAQ orthologs was performed with ClustalW2 to check whether the peptide sequence has enough identity to induce an immune response against other *Rhipicephalus* ATAQs. This allowed us to survey if the same peptide had some efficacy against *R. sanguineus*.

The predicted peptide was synthesized by Mimotopes (Melbourne, Australia) in two forms: pure peptide and conjugated to Keyhole Limpet Hemocyanin (KLH). To conjugate to KLH, a cysteine was added to the peptide amino-terminus, and a *m*-maleimidobenzoyl-*N*-hydoxysuccinimide linker was added to conjugate the peptide to KLH through the amino-terminus. A total amount of 10 milligrams (mg) of freeze dried pure peptide was synthesized, with a purification grade of 70%. The KLH-conjugated peptide was synthesized and freeze-dried in a total amount of 59.4 mg, 44 mg of which corresponded to the carrier KLH and 15.4 mg to the peptide with the purification grade of 90%. The pure and KLH-conjugated peptides were solubilized in 1 mL and 59.4 mL of sterile distilled water to reach a final concentration of 10 mg/mL and 1 mg/mL, respectively, and stored at −18 °C until use in immunological assays.

2.3. Animal inoculations

The mice were randomized in three groups of six females, each ranging from six to eight weeks old: peptide conjugated to KLH carrier and adjuvant (K); pure peptide and adjuvant (P); and phosphate buffer saline (PBS) and adjuvant as control group (C). The animals received, subcutaneously, three doses of 10 μg (the K group received 38.6 μg of the KLH-conjugated peptide to ensure 10 μg of peptide) of the peptide in 100 μL final dose volume (peptide, adjuvant and water) at an interval of 15 days. Complete Freund's adjuvant was used for the first dose and Incomplete Freund's adjuvant for the following two doses. All animals received a 50/50 proportion of adjuvant/antigen.

For rabbit inoculation, the pure and KLH-conjugated peptide were diluted in PBS and emulsified with Montanide (Seppic, Paris) adjuvant for all doses, with an adjuvant/peptide proportion of 60/40. Each animal received a total dose of 500 μL, containing 100 μg of peptide (the K group received 386 μg of the KLH-conjugated peptide to ensure 100 μg of peptide). The rabbits were randomized into three groups with three animals each: one inoculated with the peptide conjugated to the KLH carrier and adjuvant (K); one with pure peptide and adjuvant (P); and PBS and adjuvant as the control group (C). The vaccination schedule was performed the same as described in mice, but using intramuscular injection instead.

The cattle were divided into two groups of four animals each: one with the peptide conjugated with KLH and adjuvant (K); and one with PBS and adjuvant as control (C). These animals were inoculated with 200 μg of peptide in a final volume of 2 mL (the K group received 772 μg of the KLH-conjugated peptide to ensure 200 μg of peptide), containing a proportion of 60/40 adjuvant/antigen. The vaccination schedule was performed the same as described in rabbits. The adjuvant used for both rabbits and cattle was Montanide ISA 61 VG (Seppic®, Paris).

The day of the first dose was considered as day 0.

2.4. Immunoassays

Animal serum samples were obtained periodically, in same days of the inoculations, up to 20 days after the third vaccinal dose, which was the same days of the tick infestations (for rabbits and cattle). The mouse blood was collected via the orbital plexus, using a Pasteur glass pipette. For rabbits and bovines, the jugular vein was chosen. After collection, the blood samples were kept at 4 °C for 12 h for clot formation and then centrifuged at 3000 *g* for five minutes to separate cells from serum.

ELISA plates were coated with 1 μg of the sole peptide per well, diluted in 20 mM sodium carbonate buffer (pH 9.6) and incubated overnight at 4 °C. Briefly, plates were blocked with 5% skimmed milk, primary antibodies (animal sera) were diluted 1:300 in PBS with 0.05% tween 20 (PBS-T). Secondary antibodies (Anti-Mouse IgG, Anti-Rabbit IgG and Anti-Bovine IgG, whole molecule, Sigma-Aldrich, Saint Louis, MO) were diluted 1:2000 in PBS-T. The reaction was revealed with *ortho*-phenylenediamine (OPD) and stopped with sulfuric acid at 2.5 Normal. Duplicate samples of pooled sera from all animals of each group were tested. Subsequent procedures

of incubation were performed as described previously (Prudencio et al., 2010).

2.5. Tick's maintenance and life cycle survey

R. sanguineus and *R. microplus* ticks used for this study were obtained from Embrapa Beef Cattle's colonies. Ticks were maintained at 28 °C and 80% relative humidity for either colony maintenance or life cycle survey after experimental challenges.

Rabbits and cattle from Embrapa's biotery and squad, respectively, were used in these trials for tick colony maintenance when the parasitic stage was required.

2.6. Calculation of vaccine effectiveness against R. microplus and R. sanguineus

Rabbits were challenged with *R. sanguineus* 20 days after the third inoculation. Sixteen female and 10 male adults were put into artificial chambers a day before the challenge (Bechara et al., 1995). Chambers were checked daily until the last engorged tick detached. The life cycle parameters observed were total number of detached engorged females, average weight of engorged females, total weight of mass of eggs and hatchability in percentage.

The cattle were challenged 20 days after the third inoculation with 20,000 *R. microplus* larvae per animal, as described previously (Andreotti et al., 2002). For this tick species, the life cycle parameters observed were the same as described above for *R. sanguineus*.

2.7. Statistical analysis

Data on female *R. microplus* reproductive parameters were analyzed using a *t*-test for unequal variances. Data on female *R. sanguineus* reproductive parameters were analyzed using Kruskal–Wallis. Differences were considered significant when $p < 0.05$.

The calculation of the percentage of efficacy of the peptide in reducing the tick's life cycle parameters was carried out for both *R. microplus* and *R. sanguineus*. The formula to calculate efficacy for both *R. sanguineus* and *R. microplus* was used as described previously: $E\% = 100\ [1 - (CRT \times CRO \times CRF)]$ (Canales et al., 1997; Andreotti, 2007). We showed each parameter of this formula separately (see Tables 1 and 2 in the next section).

3. Results

3.1. Characterization of the ATAQ peptide sequence

Bioinformatic analyses showed that a peptide sequence, with 17 amino acids, between residues 531 and 547 of the ATAQ protein (Fig. 1, peptide sequence: NH2-PQPPHHQKWPFPTTPMA-COOH), had several antigenic properties. As described by Nijhof et al. (2010), the ATAQ protein has a signal peptide in its N-terminal portion (Fig. 1), followed by a large extracellular portion (Fig. 1, yellow box), which is anchored to the membrane in its C-terminal portion by a transmembrane helix (GPI-anchor).

We detected five potential B cell epitopes (Fig. 1). Two of them were located in the EpidermalGrowth Factor-like (EGF-like) domain region that possessed similarity to the predicted *Bos taurus* proteins (Fig. 1) and were removed from further analyses. One potential B cell epitope (rightmost green box on Fig. 1) was removed due to its co-location with the C-terminal intracellular region. From the two predicted epitopes remaining, we selected the one that had the highest prediction value for linear B epitopes, which was collocated with an intrinsically unstructured protein region (IUP region, red bar, Fig. 1), in the extracellular region (golden bar, Fig. 1) and with no detected similarity to *Bos taurus* proteins (blue bars, Fig. 1).

The software Geneious Pro 4.8.5 (Biomatters) predicted that the mean identity between the ATAQs coding sequences from *R. microplus* and their orthologs was 93.3%. The same software also predicted that the 17 amino acid peptide was potentially antigenic (data not shown). The high genetic identity was also reflected in the high identity among these ortholog proteins, as shown in the alignment on ClustalW2 (Fig. 2).

Additionally, the algorithms Emini Surface Accessibility Scale and Bepipred Linear Epitope Prediction showed that the region in which this peptide was included was located on the surface of the ATAQ protein and is also a linear epitope (Figs. 3 and 4).

MHC-II Binding Predictions and MHC Class-II Binding Prediction Server showed that this peptide had a high score rank in the potential of this peptide to bind to human MHC II alleles HLA-DBR1*01:01 and HLA-DBR1*03:01 (data not shown) (Rodriguez-Valle et al., 2013).

3.2. Evaluation of antibody production by ELISA

Initially, the pure and KLH-conjugated peptide were tested in two species: mice and rabbits. We did not observe IgG detecting the adsorbed pure peptide in ELISA plates in the pure peptide injected group (P) in either species. However, in the KLH-conjugated peptide injected group (K), some animal sera showed specific antibodies binding peptides in ELISA, but some did not. We observed the same situation in both species (first and second graphics in Fig. 5).

Hence, we performed a controlled test using cattle in stables to test the efficiency of this candidate antigen (KLH-conjugated peptide) in inducing the immune response to reduce the *R. microplus* life cycle parameters in laboratory conditions (28 °C and 90% of humidity). We found the same result that was previously observed in mice and rabbits, where some animals developed a consistent immune response against the peptide, and others did not.

Overall, even with the carrier, not all animals from the KLH-conjugated peptide injected groups in the three tested species developed a consistent response. This explains the large standard deviation in the pathogen's optical density (O.D.) values on the last days (Fig. 5).

3.3. Evaluation of the tick's life cycle and calculation of vaccine effectiveness

When we tested the *R. microplus* ATAQ peptide as a candidate antigen in rabbits against *R. sanguineus*, we reported only a reduction in detached engorged females recovered from ELISA responder animals from the group vaccinated with the KLH-conjugated peptide (group K) (Table 1). We observed an overall reduction of 47% in life cycle parameters of ticks fed on animals from this group. We also observed that the ticks that had more significant reductions in their life cycle parameters were the same that were fed on animals that showed higher IgG responses in ELISA (66% efficacy, data not shown).

Of all the parameters of the *R. microplus* life cycle that were evaluated, we observed the same effect as previously reported against *R. sanguineus*: the diminishing of detached engorged females, only in two animals, which were the same animals that showed higher IgG production in ELISA (Table 2). The efficiency of the immune response in reducing *R. microplus* life cycle parameters for these animals that responded as shown by ELISA was 98%. This was an efficiency that could control environmental tick infestation. This (from four animals, two with decreased tick recovery) resulted in a large standard deviation of the parameters within the whole vaccinated group (K), a possible reason that no difference had been reported between the vaccinated and control groups, leading to an overall reduction of 35%. However, when we compared the female reproductive parameters of these two ELISA responder animals (RA

Table 1
Vaccine efficacy of the pure ATAQ peptide (P) and conjugated to KLH (K) against *Rhipicephalus sanguineus* fed on rabbits and its effect on female reproductive parameters.

Animals	Total detached engorged females			Tick mean weight (mg)			Egg mean weight (mg)			Larvae hatchability (%)		
Groups	K	P	C	K	P	C	K	P	C	K	P	C
1	6.25	7.0	7.5	168.5	118.4	151.3	91.2	68.6	116	98	98	98
2	2.5	5.0	7.0	166.3	180.1	137.5	128	107.2	87.8	95	96	99
3	1.25	10.0	8.0	171.5	163.4	157.2	168	63.6	118	99	92	97
Mean ± SD	**3.3 ± 2.6**	**7.3 ± 2.5**	**7.5 ± 0.5**	**168.8 ± 2.6**	**154 ± 31.9**	**148.6 ± 10.1**	**129.1 ± 38.4**	**79.8 ± 23.9**	**114 ± 5.3**	**97.3 ± 2.1**	**95.3 ± 3.1**	**98 ± 1**
Kruskal-Wallis (*p* value)	0.11			0.2			0.11			0.11		
% Reduction (Group K)	55.6			−13.5			−20.3			−20.3		
% Reduction (Group P)	2.2			−3.6			25.6			25.6		

Efficacy (K) = 100 × [1 − (3.3/7.5) x (129.1/107.3) x (97.3/98)] = 47%.
Efficacy (P) = 100 × [1 − (7.3/7.5) x (79.8/107.3) x (95.3/98)] = 29%.

Table 2
Vaccine efficacy of the ATAQ peptide conjugated to KLH (K) against *Rhipicephalus microplus* fed on cattle in a stall test and its effect on female reproductive parameters.

Animal	Total detached engorged females		Tick mean weight (mg)		Egg mean weight		Larvae hatchability (%)	
	K	C	K	C	K	C	K	C
1	438	137	141	140	98	98	98	98
2[a]	8	382	156	141	92	98	92	98
3[a]	4	278	133	140	93	95	93	95
4	213	263	163	136	97	96	97	96
Mean ± SD	166 ± 206	265 ± 100	148 ± 14	139 ± 2	95 ± 2.9	97 ± 1.5	95 ± 2.9	97 ± 1.5
Mean RA[a] + SD	**6 ± 2.8**		**145 ± 16**		**92.5 ± 0.7**		**92.5 ± 0.7**	
t-Test (*p* value)	0.216		0.183		0.134		0.172	
t-Test RA (*p* value)	**0.007**		0.400		0.353		**0.005**	
% Reduction	37.5		−5.2		−6.7		1.8	

Efficacy = 100 × [1 − (166/265) × (148/139) × (95/97)] = 35%.
Efficacy RA = 100 × [1 − (6/265) × (145/139) × (92.5/97)] = 98%.
[a] RA = ELISA Responders Animals.

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gi|312838467|gb|ADR01301.1|   PNLCPQDCICIPDAEKPYKCDCRENTTAKTPQPPHHQKWPFPTTPMAPQR 550
gi|312838469|gb|ADR01302.1|   PNLCPQDCICIPDAEKPYKCDCRENTTAKTPQPPHHQKWPFPTTPMAPQR 550
gi|312838471|gb|ADR01303.1|   PNLCPQDCICIPDAEKPYKCDCRENTTAKTPQPPHHHKWPFPTTPTVTQR 550
gi|312838473|gb|ADR01304.1|   PNLCPQDCICIPDAEKPYKCDCRENTTAKTPQPPHHHKWPYPTTPTVSQR 550
gi|312838477|gb|ADR01306.1|   PNPYPLDCICEPDVVTSYKCNCRENTTAKTPQPPHHHKWSLPTTPPVSQR 550
                              **  * **** **. ..***:***************:**. **** .:.**
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Fig. 2. ClustalW2 alignment. From up to down, represented are the peptide sequences (square) from ticks from *Rhipicephalus* genus: *R. microplus*; *R. annulatus*; *R. decoloratus*; *R. evertsi evertsi* e *R. appendiculatus*, respectively. Within the 17 amino acids, twelve are identical (71% identity).

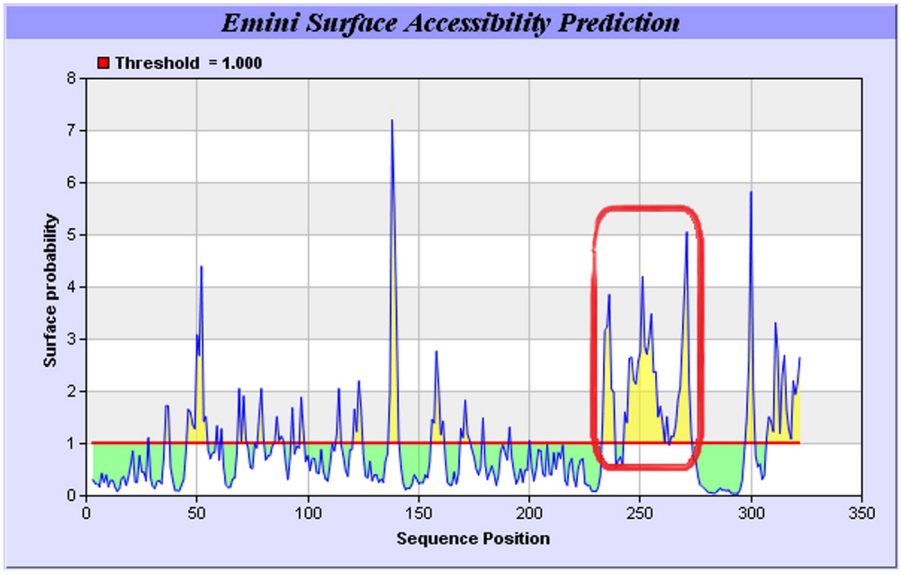
in Table 2) with the control group by one-tailed *t*-test, we observed a *p* value less than 0.5 (highlighted in bold in Table 2) in total detached engorged females and larvae hatchability. This suggested that anti-ATAQ peptide IgG was not only able to cause direct losses during the tick blood meal but could also reduce environmental tick burden by reducing the number of viable larvae derived from the few engorged females that were able to lay eggs. There was no significant reduction in the mean weight of ticks and eggs in either the vaccinated group or their responder animals. Thus, this reduction is due only to two parameters in the formula.

4. Discussion

Currently, *in silico* analysis provides tools that make the process of identifying potential antigens/immunogens faster, once the programs have been trained with a pre-analysis of an immune assay, avoiding unnecessary assays with antigens that are not immunogenic by predicting sites that have the most antigenic properties cited above. However, there is the need of subsequent assays using recombinant proteins or peptides not only to validate this approach but also to improve techniques for the prediction of new immunogen candidates to anti-tick vaccine by these algorithms (Maritz-Olivier et al., 2012).

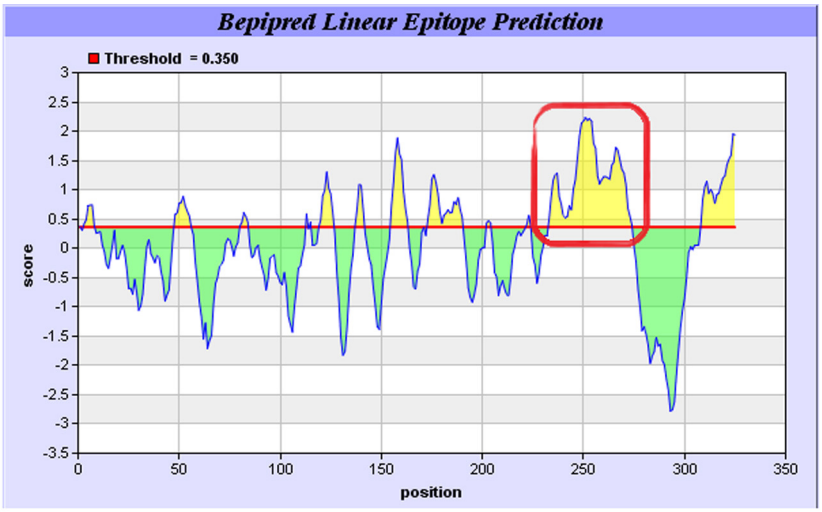
Carriers are large molecules that have the feature of being easily detectable by antigen presenting cells (APC); thus the whole molecule containing some target peptides could be phagocytosed by these APCs, enabling the peptide processing and presentation that was almost impossible previously (Briand et al., 1985). The ELISA result suggests that the peptide sequence is able to be presented through the MHC molecule by APCs to T lymphocytes and B-cell receptors once it was detected by host-specific IgGs. This confirms previous reports where peptides were so small they went undetected by the antigen presenting cells (APCs), being incapable to induce immune response by itself (Friede et al., 1993) despite *in silico* analyses that showed that the peptide had antigenic properties. For this reason, studies using peptides as immunogens began employing conjugation to molecular carriers, including not only the carrier used in this study (KLH) but also Bm95, ovalbumin, purified proteic derived (PPD) and proteasomes (De Silva et al., 1999; Prinz et al., 2004; Rodríguez-Valle et al., 2014).

Other systems of presenting peptides have shown interesting results, such as the presentation by liposomes, virosomes, adenoviruses and phage-displayed systems (Friede et al., 1993; Toes et al., 1996; Van Houten et al., 2006; Kopp et al., 2010). Some of these systems were already tested against ticks and showed some efficacy. Prudencio et al. (2010) explored the phage-displayed system; they used phage clones as carriers to present distinct protein peptides from *R. microplus*. Nevertheless, their result only found egg laying reduction and morphological modification, such as internal bleeding.



No.	Start Position	End Position	Peptide	Peptide Length
1	46	54	PLKWDSTKQ	9
2	156	161	SSSDPR	6
3	233	238	DAEKPY	6
4	243	262	RENTTAKTPQPPHHQKWPFP	20
5	264	273	TPMAQRQTS	10

Fig. 3. Surface accessibility prediction. This graph shows the probability of the domains to be exposed on surface portion and bind to antibodies. The algorithm draws a probability threshold of external sites by a horizontal line. Every graphic portion above the threshold line has high probability of being exposed on the protein surface. The circled regions indicate the synthesized peptide and thus have high probability of being on the ATAQ surface.



No.	Start Position	End Position	Peptide	Peptide Length
1	1	1	K	1
2	3	8	SCQCPA	6
3	48	56	KWDSTKQQC	9
13	222	223	NL	2
14	233	274	DAEKPYKCDCRENTTAKTPQPPHHQKWPFPTTPMAQRQTSR	42

Fig. 4. Linear epitope prediction: this algorithm analyses the primary structure of the protein and draws a probability threshold on finding linear epitopes in tertiary structure. Every graphic region above the threshold line has high probability of being a linear epitope. The circled regions indicate the synthesized peptide and have a high probability of being a linear epitope that facilitates the binding to antibodies.

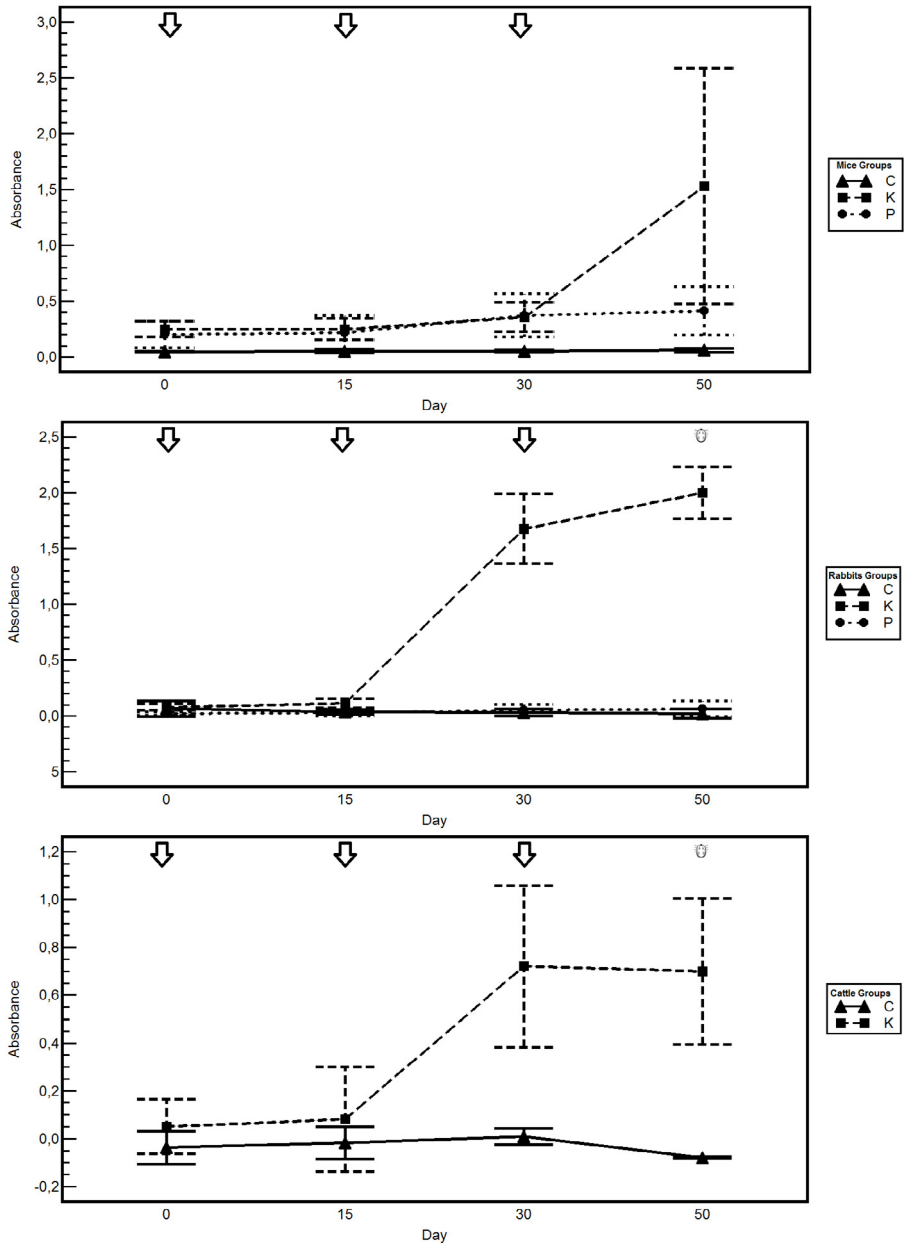


Fig. 5. Profiles of the humoral immune response in mice, rabbits and cattle, respectively, from top to bottom, vaccinated with the ATAQ projected peptide. Absorbance readings are for ELISA tests of periodically antisera samples collected from three groups of mice (six animals each) and rabbits (three animals each) and two groups for cattle (four animals each), inoculated at day 0, 15 and 30 (arrows). Infestations in rabbits and cattle occurred at 21 days after the third dose (tick symbol). Antibody titers of immunized mice, rabbits and cattle are depicted as the O.D. 490 nm of the 1:300 dilutions of serum samples. The error bars indicate one standard deviation within the groups.

A synthetic peptide derived from the ribosomal protein P0 from *R. sanguineus* conjugated with KLH was tested in rabbits against *R. sanguineus* and had an overall efficacy of 90% (Rodríguez-Mallon et al., 2012). Our results showed a lower efficacy against this tick; however, it is important to consider that this peptide was derived from *R. microplus* ATAQ. This result strongly indicated the possibility of existing ATAQ protein in *R. sanguineus* guts, once this protein was isolated from other ticks from the *Rhipicephalus* genus (Nijhof et al., 2010). Furthermore, a peptide derived from an ortholog protein could induce antibody production with the capability to crosslink to other homologues from *Rhipicephalus* species (Kopp et al., 2010).

Although our result is similar to the efficacy of 31% found with Bm86-CG antigen against the same *R. microplus* strain (Cunha et al., 2012), it is not enough to suggest this antigen as a vaccine candidate. However, our work indicates that it is worthwhile to include this antigen in vaccine formulation strategies.

In both *Rhipicephalus* species used in this study, there was no significant reduction in all parameters evaluated for both host species tested (rabbits for *R. sanguineus* and cattle for *R. microplus*), but in the recovery of detached females, we observed higher standard deviations because some animals showed more reduction in these parameters than other animals within the same group. This is consistent with what has already been reported previously in trials of other anti-tick vaccines (Andreotti et al., 2012). Vaccines could provoke consistent immune response in some animals and other not, this means that the mean of tick's parameters data could not have decreased not because the immune response wasn't enough to damage ticks, but because some animals did not have developed immune response. In this case, the high standard deviations

were due to an increase of tick recovery and larvae hatchability in both rabbits and cattle that showed IgG-binding peptides in ELISA that were infested with *R. sanguineus* and *R. micropus*, respectively. However, only *R. microplus* fed on cattle that had a consistent immune response as detected by ELISA showed statistically significant reduction.

Currently, the most used algorithms that predict bovine MHC (I or II) epitopes are based on human or murine alleles (Vordermeier et al., 2003; Nene et al., 2012; Rodriguez-Valle et al., 2013). There is a lack of information on studies that use virtual matrices based on MHC expressed by BoLA in cattle, especially in the case of MHC class II (Nene et al., 2012). Among hundreds of HLA genes that encode MHC (I or II), some are already being used to predict *R. microplus* T and B cell antigens for cattle, such as the alleles HLA-DBR1*01:01 and HLA-DBR1*03:01, which encode for MHC II that targets a humoral immune response (Rodriguez-Valle et al., 2013). On the other hand, Andreotti (2007) predicted a 29 amino acid peptide, which contains a trypsin inhibitor domain with high affinity to human MCH I (cellular immune response). However, its efficacy against *R. microplus* was only 18.4%, which helped us decide to use MCH II alleles in this trial.

In addition to the affinity of epitopes to bind to MHC-II receptors, the antigen needs a minimal length to be detected by the APCs. Once the antigen has these properties, it will be internalized by the APC through a pattern recognition receptor. After internalization, the antigen will be digested to small peptide fragments, which will be able to bind to the MHC II receptor and then be presented at the APC membrane. When the peptide is presented by an APC to CD4+ T-cells, the CD4+ T-cells will self-differentiate to T-helper II cells that recognize the peptide as an antigen and secrete Il-4 that signals to B-cells to differentiate into plasma cells. These cells will produce specific antibodies with affinity to this peptide (Germain and Margulies, 1993). On the other hand, B-cells could directly bind to the peptide antigen and start the expansion and antibody production process, speeding up the humoral response. This is the reason why we considered using a B-cell receptor prediction tool (Bepipred). Considering these features, the peptide alone is incapable of targeting a specific immune response by itself and needs some carrier to make it an antigen detectable by an APC to set up a strong and specific immune response (Friede et al., 1993; Hemmer et al., 2000).

5. Conclusion

The results of this study allow us to conclude that the peptide selected by using a bioinformatics approach is able to induce a specific immune response in the presence of carriers and adjuvants. Then, the peptide is efficiently presented to the APCs. There is certain level of efficacy against both *R. microplus* and *R. sanguineus* but not enough to protect all inoculated animals. A thorough investigation should be conducted to survey the existence of the ATAQ protein in *R. sanguineus* and its effectiveness as a vaccine against these tick. Further studies are needed to find distinct tick antigens to be used in combination, as well as several peptides of the same protein that play distinct functions. By doing so, the vaccinal efficacy can be increased, individually or collectively.

Conflicts of interest

Authors have no conflicts of interests.

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