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RA92A RECOMBINANT PROTEIN AS IMMUNOGEN TO PROTECT CATTLE AGAINST TICK CHALLENGE IN BRAZIL AND UGANDA

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Abstract

In this study, the recombinant gut protein rRa92A produced in *Pichia pastoris* yeast cells was used to immunize cattle in two experiments, one in Brazil and the other in Uganda. In both experiments, the animals were intramuscularly (IM) injected with 200 µg of recombinant protein in Brazil on days 0, 30 and 51 and in Uganda on days 0, 30. Blood samples for sera separation were collected from different days in both experiments. These samples were analyzed by ELISAs. In Brazil, ticks collected from the animals during the experimental period were analyzed for biological parameters. At Uganda, blood was collected to assess blood parameters, clinical signs were recorded and adult tick (*Rhipicephalus appendiculatus*) counts were performed. All animals of the vaccinated groups were shown to produce antibodies, and it was not possible to detect an effect on *Rhipicephalus microplus*. All the clinical parameters were considered within the normal ranges for both the experimental and control groups in Uganda. Antibody absorbance was elevated after each immunization and remained high until the end of the experiments, remaining low in the control animals. The results of stall test carried out in Brazil using *R. microplus* tick showed efficacy of 21.95%. The rRa92A immunization trial experiments in Uganda showing a decrease of 55.2% in the number of engorged adult ticks, which was statistically significant ($p<0.05$). Assessment of the immunogenicity of Ra92A produced in the *P. pastoris* expression system in bovines is reported for the first time, and the protein acted as a concealed antigen.

Keywords: Immunogenicity. Ra92A protein. *Rhipicephalus appendiculatus.* Ticks. Vaccine.

1. Introduction

Ticks are obligate ecto-parasites that transmit pathogens causing diseases that result in significant economic losses to livestock farmers in Brazil reaching an economic loss around US$3,23 billion in Brazilian cattle herds (Grisi et al. 2014), which are estimated at 17 billion of dollars globally per year (Graham and Hourrigan 1977; Playford et al. 2005).

The tick affect bovine by the effects on weight loss, damage to the hides and skin, and a drop in milk yield (Pegram et al. 1989) and cattle population are at risk of acquiring TBDs (Anon 1997). East coast fever (ECF), caused by *Theileria parva*, alone causes high mortality among naïve cattle, especially exotic breeds in Uganda. The livestock sector plays a key role in poverty reduction in Uganda (Rubaire-Akiiki et al. 2004). Currently, the major form of control is acaricides, which cause potential environmental pollution and result in tick resistance (Vudriko et al. 2016)

Anti-tick vaccines are commercially available: based on glycoprotein (Bm86), which have successfully been used to control of the *R. microplus* (Canales et al. 1997). The Bm86 vaccination has been extensively evaluated for its ability to control other tick species as well (Pipano et al. 2003; Olds et al. 2012; Merino et al. 2013). Homologous proteins to Bm86 were isolated from *R. appendiculatus* tick species and are collectively known as Ra86. There are two different homologues in ticks, Ra85A and Ra92A, and when both variants are present, one of them is transcriptionally dominant (Kamau et al. 2010). One variant of this protein, Ra92A, was explored as an anti-tick vaccine in cattle in this experimental study for its efficacy against ticks. First, this protein was used as imunogen in a stall test in Brazil to test its immunogenic potential in bovines and its efficacy against *R. microplus*, and after, it was used to immunize cattle breeds mainly kept under intensive management for beef and local cattle under free range management in Uganda under parallel arrangement confirm its efficacy to control *R. appendiculatus* infestation.

2. Material and Methods

Research design

The cattle experiments were carried in two places: at Embrapa in Campo Grande, MS, Brazil, and at the College of Veterinary Medicine, Makerere University, Uganda. The design of the experiments at Embrapa consisted of two groups of cattle divided into vaccinated and control groups with four animals in each under the stall. At Makerere, the animals were divided into two groups (vaccinated and control) of three animals that grazed freely in the University paddocks and were exposed to natural tick challenge in the paddocks.

Plasmids and *P. pastoris* transformation

Rhipicephalus appendiculatus Ra86 gut proteins were analyzed by BLAST using the blastn suite (Blast®). An ORF sequence of 2082 Bp was obtained (from start to stop codons) from GenBank with accession numbers FJ50975 and FJ850978 (Kamau et al. 2010). A partial DNA sequence from the Ra92A gene, was synthesized by GeneOne Biotechnologies (Rio de Janeiro, Brazil) optimizing for codon usage of *P. pastoris*. Sites for *EcoRI* and *XbaI* restriction enzymes were added to the 5' and 3' extremities, respectively. The synthetic sequence was cloned in the pPICZαA plasmid (Invitrogen, USA), yielding the plasmid pPICZα-Ra92A, which was propagated in *Escherichia coli* TOP10F' (Invitrogen, USA). The *P. pastoris* KM71H strain (Invitrogen, USA) was transformed by electroporation in a MicroPulser apparatus (Bio-Rad, USA) according to the manual of the EasySelect™ *Pichia* Expression Kit (Invitrogen, USA). The recombinant cells were selected on YPDS plates containing Zeocin™ (Invitrogen, USA).

Screening of expression clones

One colony was picked from the YPDS plate with Zeocin™, inoculated into BMGY and incubated. Then transferred Erlenmeyer flask containing BMGY growning to log-phase. Cell pellets were resuspended in BMMY and supplementing with 1% methanol (v/v) every 24 hours. The supernatant was recovered, and the pellet was discarded. The monoclonal antibody (mAb) anti-6×his tag (Invitrogen, USA) conjugated with peroxidase was used to detect the recombinant Ra92A 6×his-tagged protein.

Confirmation of cloning

Genomic DNA was extracted from rRa92A-expressing clones using the glass bead disruption method. The DNA extraction was worked with phenol:chloroform:isoamyl alcohol assay The DNA pellet

was recovered, and quantified using a Nano Drop 2000c Spectrophotometer (Thermo Scientific, USA). The DNA was used to confirm the recombinant clones by PCR and sequencing with 5'AOX1 and 3'AOX1 primers. To confirm the Mut^S phenotype for pPICZα-Ra92A KM71H transformants, each clone was plated on minimal dextrose medium (MD) agar plates and on minimal methanol medium (MM) agar plates as described in the EasySelect™ *Pichia* Expression Kit manual (Invitrogen, 2009).

Production of rRa92A

To express Ra92A recombinant (rRa92A) protein, one colony was isolated and inoculated in BMGY medium. Cells pellets were suspended in BMGY medium for induction and supplementated with methanol. After this induction period, the cultures were centrifuged and the supernatants were separated, treated with 1 mM PMSF (Sigma-Aldrich, USA) and frozen at -20 ºC.

Purification and quantification of recombinant protein

The expressed recombinant protein Ra92A was found in the supernatant and was detected and quantified by the standard curve method with bovine serum albumin (BSA) on 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The bands were quantified by visual analysis plus Total Lab 2.0 software (Amersham/Biosciences, United Kingdom. The expressed recombinant protein was purified using the denaturing method, where the supernatant was incubated in methanol 1:1 (v/v) to precipitate the protein by centrifugation. The pellet was then eluted by adding denaturing binding buffer and elution was then loaded onto a Ni^{2+}-charged Ni-NTA (Qiagen, Hilden, Germany) affinity colum.

SDS-PAGE and Western blotting

Purified protein was mixed with SDS-PAGE protein denaturing buffer. Electrophoresis was run in a 2D electrophoresis system (Amersham/Bioscience, United Kingdom) (Figure 2). Western blotting (WB) analysis was performed on purified rRa92A protein after 7.5% SDS-PAGE. The protein was transferred from the gel to a nitrocellulose membrane using a transfer system (Amersham/Bioscience, United Kingdom) immersed in transfer buffer.

The membrane was stained with Ponceau S solution and the strips were incubated with peroxidase-conjugated rabbit anti-bovine IgG secondary antibody (Sigma-Aldrich, USA). The strips were revealed with developing buffer with DAB solution.

The pen trial in Brazil

Controlled pen trials were conducted to evaluate the immunogenicity and protective capacity of the rRa92A protein formulated with adjuvant Montanide ISA 61 VG (Seppic, Paris, France) in adult Holstein cattle. The vaccine was constituted into doses of 2 mL containing 200 µg of the recombinant protein and 100 mM Tris-HCl in the aqueous phase. One-year-old Holstein calves, all with history of prior tick exposure, but maintained under intensive tick control with acaricides, were randomly distributed into two groups of four animals each. Four animals were injected with adjuvant alone formed the negative control. Another four animals were each injected intramuscularly with 2 mL of the formulated vaccine at day zero (D0), which was repeated after 30 days (D30). Twenty-one days after the last boost (day 51 – D51), all the animals were challenged with 15,000 larvae 14 days old of the *R. microplus* tick. The biological parameters of the ticks were observed and analyzed later. The cattle vaccine studies were conducted at Embrapa under protocols approved by the Embrapa Animal Ethics Committee (CEUA 008/2014).

Serum analysis: Brazil cattle experiment

Blood samples were taken from each animal before the first immunization at D0 and weekly. The enzyme linked immunosorbent assay (ELISA) was use do measure the level of immune response to recombinant protein. For isotyping evaluation of the humoral immune response of bovines vaccinated

with rRa92A, anti-bovine IgG1 and anti-bovine IgG2 antibodies (Bethyl Laboratories, USA) diluted 1:5,000 were used.

Efficacy assessment and statistics: Brazil pen trial

Reductions associated with immunization relative to the unvaccinated group were determined for numbers of adult female ticks, egg production, and larval hatching. Vaccine efficacy was calculated as 100 × [1– (CRT × CRO × CRF)], where CRT, CRO and CRF represent the coefficient of reduction in the immunized group relative to the control group of the total number of adult female ticks, total weight of eggs per female, and hatchability of eggs (fertility), respectively (Cunha et al. 2013).

Immunization and tick challenge experiments in Uganda

Six experimental animals were randomly selected and grouped into three in the immunized group and three in the control group. They were aged 2–5 years and consisted of Ankole-Friesian crosses, kept under free range management and tick control with acaricide. They were divided into immunized and control groups. The animals were clinically examined and dewormed using albendazole before immunization. Tick control was stopped after immunization, and the cattle were grazed in the paddocks under natural pastures. The recombinant protein Ra92A was sent from Brazil to Uganda lyophilized and reconstituted ready for use in the experimental animals at the College of Veterinary Medicine, Animal Resources and Biosecurity (CoVAB), Makerere University, Kampala, Uganda. The animals in the treatment group, three of them were each injected intramuscularly into the thigh muscles with 2 mL of vaccine at day zero (D0), which was repeated after 30 days (D30). The formulation used contained 200 µg/mL of recombinant protein (rRa92A) antigen formulated with ALhydrogel® adjuvant at a 1:1 ratio, the injection site of each animal was monitored to assess for signs of inflammation and blemishes on day two, and 5 mL of blood were taken for changes in the blood parameters. Tick control was stopped after immunization, and the cattle were grazed in the paddocks under natural pastures twenty-one days after the last boost (day 51 – D51), to be challenged with field ticks.

Clinical parameters

The clinical parameters were assessed by examination of temperature, heart rate, pulse rate, ruminal movements, respiratory rates, and swelling at the site of injection before collection of blood. The animals were examined for any clinical signs from each animal in both groups every two weeks before collection of blood. All animals were diagnosed disease free, and all physiological parameters were within normal ranges.

Collection of blood and tick counts

The clinical and hematological parameters were monitored to assess the safety of the protein used throughout the study period. The whole blood was collected on days 0, 2, 16, 23, 30 and 120 and processed for total red blood cell (RBC) and white blood cell (WBC) counts using a hemocytometer and packed cell volume (PCV) for hemoglobin. Thin blood smears were made to determine RBC indices and WBC differential counts. Serum was processed and analyzed for its kinetics of antibody production using an indirect ELISA. However, tick counts were carried out at days 85, 100, 115 and 141 post first immunization, when ticks with a size larger than 5mm long fixed to the bovine skin were counted.

Statistical analysis

Means of antibody levels (absorbance and percentage) were determined for each group by day and analyzed using 2-factor analysis of variance (ANOVA), and means were compared using the F-test to determine the significance of any observed differences between groups. The differences were considered significant when $p<0.05$. Data on female reproductive parameters were analyzed using a t-test. Analyses were performed using MedCalc®, version 10.3.0.0.

3. Results

The synthesized gene was cloned into the pPICZα expression vector to form the recombinant plasmid, named pPICZalpha-Ra92A. This construct was successfully transformed into the *P. pastoris* KM71H strain. Then, we obtained 8 clones expressing the protein (figure 1). The best rRa92A-expressing clone (KM-Ra92A 5) was used to produce the recombinant protein for all the experiments. The recombinant protein was successfully purified by nickel affinity chromatography (figure 2, lane 1). Vaccine formulations with Ra92A induced an immune response, which was confirmed by Western blot analysis (figure 2, lanes 2-5) and ELISA (figure 3 A). The vaccinated animals responded to the immunization protocol and showed a specific immune response against rRa92A by day 23, while IgG1 and IgG2 production peaked up to day 51, levelling to a plateau with similar absorbencies on days 51 and 85 (figure 3 B and C).

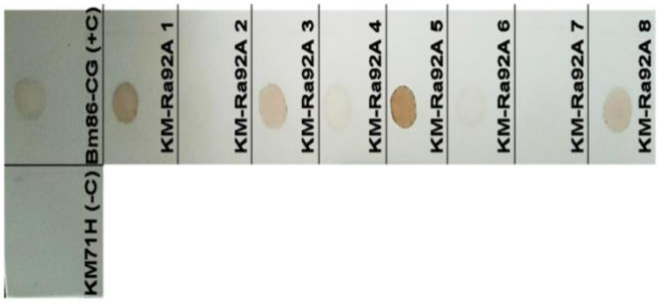

Figure 1. Dot blot of the supernatant from *Pichia pastoris* clones expressing rRa92A. Each supernatant was applied to the membrane and revealed with Mab anti-His (Invitrogen™, USA).

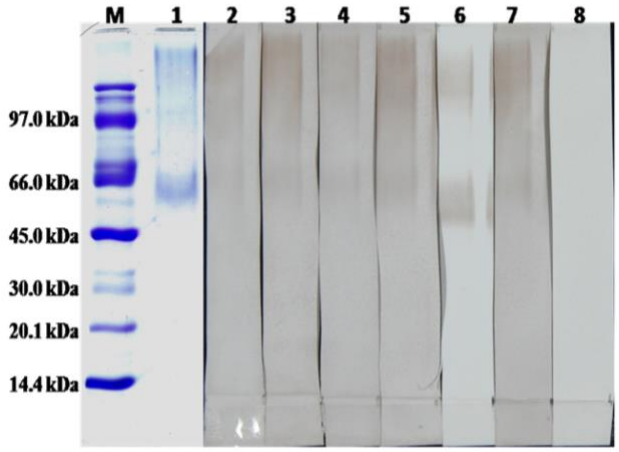

Figure 2. SDS-PAGE at lanes M and 1; and Western blot at lanes 2 to 8. M) molecular weight markers (GE Healthcare, UK); 1-8) loaded with of purified rRa92A. Lanes 2 to 8 were revealed with sera from vaccinated animals. Lane 6 was revealed with MAb anti-His. Lane 7 was revealed with a pool of sera from animals vaccinated with rBm86-CG. Lane 8 was not incubated with any serum or MAb.

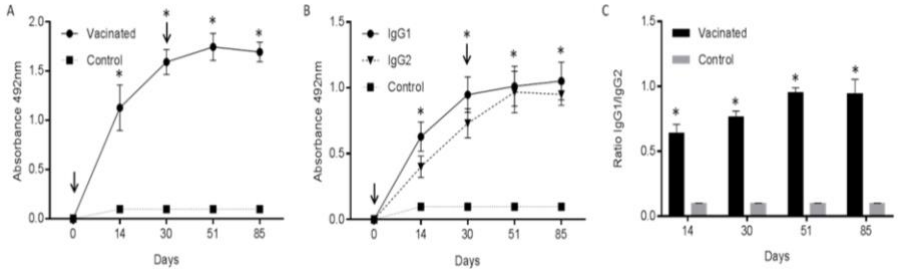

Figure 3. (**A**) Profile of the expression levels of total IgG. (**B**) Dynamics of the expression of IgG1 and IgG2 in cattle vaccinated with rRa92A. The data represent the mean (± standard error of the mean) of the absorbance values obtained by the indirect ELISA. The arrows represent the days of inoculations. (**C**)

IgG2/IgG1 ratio. Data represent the mean (± standard error of the mean) of the IgG2/IgG1 ratio of bovine vaccinated. Asterisks (*) represent significant (p<0.05) differences between the mean of the vaccinated group and the control group in the F test associated with analysis of variance (ANOVA).

The results of stall test carried out in Brazil using *R. microplus* tick are shown in the Table 1. The percentage of efficacy observed in the Brazilian pen trial was 21.95%. Among the differences between vaccinated and control groups in all the biological parameters, none was statistically significant.

Table 1. Efficacy of vaccine containing *R. appendiculatus* Ra92A recombinant gut protein protection against *R. microplus* ticks infesting cattle and their effect on female reproductive parameters.

Animal	Tick total number		Tick mean weigt (mg)		Egg mean weight (mg)		Larval hatchability (%)	
	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control
1	115	137	253	274	132	139	88,60	92,10
2	159	382	232	267	138	136	85,10	92,50
3	501	278	271	276	134	137	89,40	99,50
4	130	263	248	262	136	142	90,10	90,70
Mean ± SD[a]	226 ± 92	265 ± 50	251 ± 8	270 ± 3	135 ± 1.3	139 ± 1.3	88,3 ± 1.1	93,7 ± 2.0
t-Test	p = 0.7244		p = 0.0734		p = 0.1071		p = 0.0543	
% of reduction[b]	DT = 14.72		DW = 7.04		DO = 2.88		DF = 5.76	
Efficacy = 100× [1-(226/265×135/139×88.3/93.7)] = 21,95%								

[a] Arithmetic mean ± standard deviation; p-values of t-test for independent samples are shown. [b]Percent reduction was calculated in relation to the control unvaccinated group: DT, adult female ticks; DW, tick weight; DO, egg laying capacity; DF, fertility. Efficacy (%) = 100 [l − (CRT × CRO × CRF)]; where CRT: coefficient of reduction in the number of adult female ticks, CRO: coefficient of reduction in the egg laying capacity, CRF: coefficient of reduction in fertility.

The rRa92A immunization trial experiments in Uganda monitored the clinical and hematological parameters, which assessed the safety of the recombinant protein, plus immunogenicity, efficacy and protection against the local ticks found on the university paddocks. There were no clinical signs observed related with temperature, heart rate and respiratory rate in the animals immunized with the gut protein and there was no adverse reaction at the site of injection.

Total red blood cell counts for all animals in both groups remained within the normal range throughout the period of the study. However, there was a statistically significant difference in total red blood cell counts between the two groups (p<0.05). There was no significant difference in the values for packed cell volume (PCV), hemoglobin (Hb), mean corpuscular volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) between the two groups of the animals in the experiment (all p>0.05) (Table 2).

Table 2. Mean values for red blood cell parameters in the vaccinated and control groups.

Parameter	Vaccinated	Control	p-value	Normal range
TOTAL RBC COUNT (10^6/µL)	6.59 ± 0.047*	6.91 ± 0.119*	0.020	5–10
PCV (%)	24.5 ± 0.336	25 ± 0.404	0.348	24–46
Hb (g/dL)	7.81 ± 0.124	7.84 ± 0.115	0.844	8–15
MCV (fL)	37.167±0.553	36.34±0.780	0.393	40–60
MCH (pg)	11.85 ± 0.220	11.42±0.272	0.225	11–17
MCHC (%)	31.92 ± 0.470	31.65±0.773	0.765	30–36

*Arithmetic mean ± standard error; p-values of t-tests for independent samples are shown. PCV: values for packed cell volume; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean cell hemoglobin; MCHC: mean cell hemoglobin concentration.

There was a significant difference in the total leukocyte count between animals in the experimental and the control groups (p<0.05). Additionally, there was a significant difference in the relative and absolute values of the lymphocytes between the two groups. However, there were no significant differences in the values for band neutrophils, segmented neutrophils, eosinophils, monocytes and basophils (Table 3).

Table 3. Mean values for white blood cell counts for the vaccinated and control groups.

Parameter	Vaccinated Group		Control Group		p-value	Normal Ranges	
Total WBC	8.59 ± 0.310 x 10^3/µL		7.87 ± 0.078 x 10^3/µL		0.030	4-12 x 10^3/µL	
Differential Counts	Relative (%)	Absolute (10^3)	Relative (%)	Absolute (10^3)		Relative (%)	Absolute (10^3)
Band Neutrophils	3.11± 0.312	0.232	2.17± 0.256	0.236	0.026	0–2	0–0.1
Segmented Neutrophils	28.6 ± 0.746	2.379	26.56 ±0.611	2.179	0.040	15–45	0.6–4.0
Eosinophils	2.83 ± 0.202	0.206	3.28 ±0.211	0.275	0.137	2–20	0–2.4
Basophils	0.5 ± 0.121	0	0.722 ± 0.135	0	0.230	0–2	0–0.3
Monocytes	3.72 ± 0.266	0.232	3.33 ± 0.268	0.267	0.310	2–7	0.1–0.8
Lymphocytes	61.056 ± 1.13	5.54	63.944 ± 0.756	4.91	0.041	45.75	2.5–7.5

*Arithmetic mean ± standard error; p-values of t-test for independent samples are shown.

For the control group animal, the anti-Ra92A showed no antibody reaction. These animals were under intensive tick control before the experiment, in contrast to the immunized animals, which developed specific humoral immune response characterized by anti-rRa92A IgG levels. There was a rise in antibody titer detected from day 23 in the animals administered the recombinant protein. This change increased with the boost, and a strong immune response was still visible even in the sera that were taken day 120 post immunization (Figure 5).

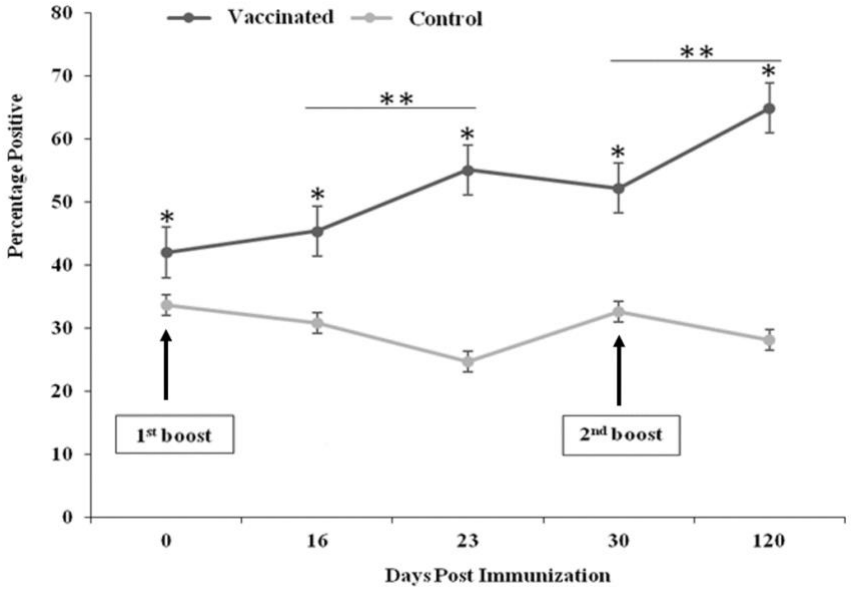

Figure 4. Percentage positivity between the immunized and control cattle. Graphic showing the antibody titer between immunized and control cattle over a period of 121 days. There was a significant difference with a p-value<0.05 between the two groups of animals on all days (*). Double asterisks (**) indicate significant differences between two means in the same group (p<0.05). Statistics were calculated by ANOVA in association with the F test. Using the rRa92A as the coating antigen in iELISA brings high O.D values in serum from non-immunized (Control) cattle or in the pre-immune sera as a result of background cross reacting antibodies. These high background values were also observed by Olds et al. (2012) when they immunized cattle with Ra86 variant proteins (Ra85A and Ra92A).

There was a significant difference in the number of engorged adult ticks between the vaccinated and control groups on days 85 pi and 100 pi (p< 0.05), whereas no significant difference (P>0.05) was observed on days 114 pi and 148 pi (Figure 5). Decreases of 33.7% and 71.1% were observed in the number of engorged ticks on the vaccinated animals compared to the control animals, respectively. The mean decrease of these two days was 55.2%.

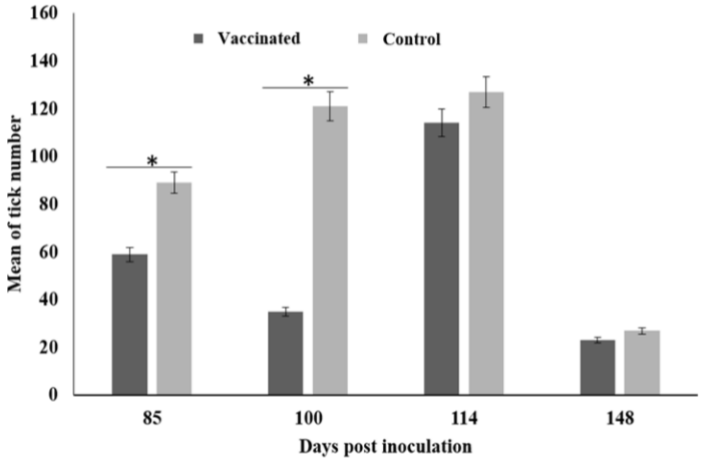

Figure 5. Graphic showing the mean ± standard deviation of adult tick number observed on the animals of the vaccinated and control groups at days 85, 100, 114 and 148 post-inoculation (pi).

4. Discussion

According to Kamau et al. (2010), the Ra92A protein is a homologue of the Bm86 gut protein. This protein was expressed in *P. pastoris* and used in cattle immunization under challenge with *R. microplus* and *R. appendiculatus* tick species. The rRa92A protein immunization of cattle in the experiments carried out in Uganda and Brazil elicited high antibody production, which agrees with previous work by Saimo et al. (2011) and Olds et al. (2012). The work carried out in Brazil where cattle were immunized with rRa92A showed limited efficacy for *R. microplus* tick protection despite 74% homology in the amino acid sequence with the Bm86 protein. The animals developed antibodies against rRa92A protein indicating that the recombinant protein was immunogenic and induced IgG, IgG1 and IgG2 antibodies. However, these had no statistical difference in the protection against *R. microplus* the results agree with the observed results found by other scientists when cattle were immunized with Bm86 recombinant protein (Vargas et al. 2010). This result could mean that Ra92A does not share antigenic determinants with Bm86 or that the antigenic peptides do not fall within the protective region or are differentially presented to the immune system following vaccination with recombinant Ra92A protein. It could also be that the feeding habits of the two ticks differ in intensity since effect on ticks depends on the antibody quantity taken in by the ticks therefore the amount of the blood meal in the different ticks could be different. Alternatively, Ra86 antigen could be differently located in the two tick species. Since an inverse correlation between vaccine efficacy and sequence variation in Bm86 exists (García-García et al. 1998), it is not surprising that an alignment of 74% sequence identity between Ra92A and Bm86 is greater than 2.8% variation and therefore results in a lowered efficacy. However, there was a significant decrease in the number of engorged *R. appendiculatus* ticks in the rRa92A-immunized animals in the experiments carried out in Uganda.

IgG2 has a higher opsonizing activity than IgG1 where the function is to increase phagocytosis by neutrophils and macrophages (Estes et al. 1995; Mcguire et al. 1979) and can initiate the classical complement pathway in bovine infections with extracellular agents (Bastida-Corcuera et al. 1999). After the second vaccination boost, IgG2 levels increased and were found to be high in relation to IgG1, indicating a switch to the Th1 immune response. The stimulation of high levels of IgG2 is desirable for a tick vaccine because this is more efficient in the opsonization of antigens in the cell wall of the digestive (intestinal) cells, and it can lyse the cells by activating complement through the classical route.

The increase in the IgG2/IgG1 ratio over time (close to 1.0) is due to the relative increase in IgG2 production, which suggests that rRa92A-inoculated bovines initially developed a balanced Th2/Th1 immune response that was modulated over the time, tending to Th1.

This finding shows that Ra92A protein did not induce adverse effects on erythrocytes and are not expected when this protein is administered to cattle (Fraser 1991). The total leukocyte count for animals in both groups remained in the normal ranges for cattle (Table 3). However, there were significant differences in relative and absolute values for the total leucocyte count, band neutrophils, segmented neutrophils and

lymphocytes between the two groups. This can be explained by the fact that this protein is an antigen capable of eliciting an immune reaction in cattle. During an immune reaction, more lymphocytes are produced in response to the antigen. B-lymphocytes are precursors of plasma cells that produce antibodies for humoral immunity (Fraser 1991). However, since the values did not go beyond the normal ranges, if there was any inflammation, it was mild and could therefore not be considered an adverse reaction (Gründer 2006).

Ra92A is a concealed antigen (Andreotti et al. 2012), indicating it is normally hidden from the host's immune system. However, immunoglobulins from the bovine host can interact with a protein and could be a candidate concealed antigen for use in a cattle tick vaccine (Guerrero et al. 2012). Antibody response is an important indicator of whether a vaccine works (Maizels et al. 1999; Harris and Gause 2011) and in this work the vaccinated group were positive throughout the experimental period, indicating that Ra92A protein elicited an immune response in animals from the experimental groups.

5. Conclusions

These results show that the tick-gut protein Ra92A produced an immune response in immunized cattle whereas there was no response in cattle not immunized (control) in both experiments in Brazil and Uganda. However, after boosting, the percentage positivity increased to 46.3% in the first week and 60.9% after 2 weeks. This finding shows that the tick-gut protein Ra92A requires a booster to evoke a strong immune response in the animal until 3 months after boosting against *R. appendiculatus*. Although the tick species that were attached were of different strains, the protein is homologous with Bm86 which is found in *R. microplus* ticks found in Brazil, therefore identification of proteins that can offer protection to more ticks species is of great interest for anti-tick vaccine development initiatives.

Authors' Contributions: CUNHA, R.C.: conception and design, acquisition of data, analysis and interpretation of data, drafting the article; SAIMO-KAHWA, M.: conception and design, acquisition of data, analysis and interpretation of data, drafting the article; GARCIA, M.V.: conception and design, acquisition of data, analysis and interpretation of data, drafting the article; SANTOS, F.D.S.: acquisition of data, analysis and interpretation of data; SAMUEL, E.: acquisition of data, analysis and interpretation of data; ANN, N.: acquisition of data, analysis and interpretation of data; IKWAP, K.: acquisition of data, analysis and interpretation of data; AKWONGO, C.J.: acquisition of data, analysis and interpretation of data; LEITE, F.P.L.: conception and design, analysis and interpretation of data, drafting the article; ANDREOTTI, R.: conception and design, acquisition of data, drafting the article. All authors have read and approved the final version of the manuscript.

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Ethics Approval: Approved by Animal Ethics Committee of Embrapa. Number: 008/2014.

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