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Clearance of *Tritrichomonas foetus* in experimentally infected heifers protected with vaccines based on killed-*T. foetus* with different adjuvants

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ABSTRACT

Tritrichomonas foetus is a flagellated protozoan that causes a sexually transmitted disease in cattle. Trichomonosis is characterized by early abortions, subfertility and a significant decrease in productivity. Vaccine preparations containing whole *T. foetus* can reduce the time of residence of the pathogen in the host cervix after experimental infection. Here, *T. foetus* vaccines prepared with different adjuvants were tested, in parallel with a commercial vaccine, for their efficacy to clear the infection. The median time for clearance of infection was 69 days in non-immunized animals, 55 days in animals treated with aluminum hydroxide, 41 days with oil-in-water or saponin based vaccines or with a commercial vaccine and 27 days in animals treated with saponin plus aluminum hydroxide. A slight increase in the risk of *T. foetus* clearance from the genital tract was found with the saponin based vaccine (hazard ratio, 2.52; 95% confidence interval, 1.03–6.17) or the commercial vaccine (hazard ratio, 2.61; 95% confidence interval, 1.07–6.38). A significant increase in the risk of *T. foetus* clearance was found with the combination of saponin plus aluminum hydroxide based vaccine (hazard ratio, 5.12; 95% confidence interval, 2.04–12.83).

1. Introduction

Bovine trichomoniasis, or trichomonosis, is a sexually transmitted disease caused by the flagellate protozoan *Tritrichomonas foetus*. The infection is asymptomatic in bulls and linked to the development of a chronic condition, without affecting the quality of the semen or the libido [22]. In cows, *T. foetus* causes irregular heat cycles, early abortions, pyometra and temporary subfertility [23]. It is usually detected by extended breeding periods and lower pregnancy rates. The damages predicted through a model of the disease show a reduction of 14% in calf annual crop for a *T. foetus* prevalence of 20% in a herd [25].

Although trichomonosis is worldwide distributed, the incidence of the disease has decreased or even has been eradicated from some regions where artificial insemination is widely practiced. However, in countries with extensive farming systems, the artificial insemination is not a realistic choice. These countries have implemented annual reports with the intention to control the affection [19]. The practiced systematic approach of bovine trichomonosis control relies upon identification of infected bulls followed by their removal from the herd. The infection is recognized by microscopic test of cultures inoculated with preputial samples. The presence of *T. foetus* can be confirmed through polymerase chain reaction (PCR) followed by agarose gel migration, by quantitative PCR or by loop mediated isothermal amplification (LAMP) [14,21,17,20]. Several states in USA, Australia and Argentina have enforced rules and restrictions to curtail the disease. Testing and culling is an effective practice to control the prevalence of the trichomonosis; however, to increase the system efficiency, new management procedures would be desirable [27,6].

The commercially available vaccines in Argentina (Tricovac) and USA (TrichGuard®) are based on killed *T. foetus* cells combined with proprietary adjuvants. Experimental vaccines based on killed *T. foetus* cells have shown to reduce the number of infected cows by shortening the period of genital infection and by increasing the percentage of pregnancy in comparison to non-vaccinated animals [16,2,10]. Other preparations containing fractions of *T. foetus* cells such as membranes or glycoproteins were also able to increase the antibody levels and reduce the time of infection in

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cows [3,1,9]. To our knowledge these vaccines were not shown to prevent the colonization of the genital tract.

The adjuvants enhance the immune response, by eliciting the antigen release or driving targeted responses as the activation of specific immune pathways [4,24]. Aluminum hydroxide gels or oil-in-water emulsions are among the most employed adjuvants. These enhance the immune response through a slow release of the antigen. Aluminum salts form complexes that retain antigen aggregates promoting the recruitment of antigen presenting cells [4]. Emulsions with non-metabolizable oils (mineral oil) are thought to form a deposit in the injection site that promotes Th2 mediated cell stimulation and plasma cell response [24]. Saponins, such as Quil-A® that contains an aqueous fraction of *Quillaja saponaria*, promote immune responses via T-dependent as well as T-independent pathways [13]. Vaccines for trichomonosis have been designed with killed-*T. foetus* cells as water in oil emulsions and incomplete Freund's adjuvant plus dextran sulphate [16,2,9,10]. There is not enough evidence supporting that a better response can endorse one or the other.

The aim of this study was to compare the efficacy of different adjuvants in *T. foetus* vaccine preparations. Experimental vaccines were tested in parallel with a commercial vaccine. Vaccinated heifers were experimentally infected and assessed for clearance of *T. foetus*.

2. Materials and methods

2.1. Foetus culture and vaccine preparation

The strain B1 of *T. foetus* was originally isolated from a cow with pyometra [9,10]. The strain is maintained in liquid nitrogen at the Animal Health Facility from the Instituto Nacional de Tecnología Agropecuaria (Balcarce, Argentina) and it is available under request. The cells were cultured for 24–48 h at 37 °C in liver infusion broth containing 1 g/L streptomycin, 1000 IU/L ampicillin, 1.2 mg/L lincomycin, 12.5 mg/L amphotericin and 10% v/v heat inactivated horse serum [5,9] A batch was obtained from approximately 12 L of culture incubated at 37 °C until mid-log phase (24–36 h). The cell suspension was centrifuged (1400*g*, 15 min) and the pellet was washed twice with phosphate buffered saline buffer (PBS). Parasites were inactivated with 0.1% v/v formaldehyde in PBS [7]. Each vaccine dose (5 ml) contained 10^8 cells/ml of *T. foetus*. The sterile condition of each batch was assessed through aerobic culture on blood agar plates.

The adjuvant preparations (summarized in Table 1) were performed as follow:

Saponin (S): stock solution (20 mg/ml) of saponin Quil-A (Brenntag Lab, Denmark) was prepared according to the manufacturer instructions and stored at −20 °C. Quil-A was mixed with the cell suspension to a final concentration of 0.4 mg/ml.

Table 1
Heifers randomly assorted to experimental groups were vaccinated with killed-*T. foetus* cells containing the indicated adjuvants. Cn: non vaccinated and non challenged, Ci: non vaccinated, Tv: Commercial vaccine (Tricovac, Laboratorio Tandil, Argentina), O: oil-in-water adjuvant, S: saponin adjuvant, Al: aluminum hydroxide adjuvant, SAl: saponin + aluminum hydroxide adjuvant.

Group	Heifers (n)	Adjuvant
Cn	11	No vaccine – No challenge
Ci	11	No vaccine – Vaginal challenge
Tv	11	Commercial vaccine (Tricovac)
O	10	50% v/v Montanide ISA 206 VG
Al	10	6% w/v aluminum hydroxide
S	11	0.4 mg/ml Quil-A
SAl	11	0.4 mg/ml Quil-A/6% w/v aluminum hydroxide

Aluminum hydroxide (Al): the antigen was mixed with the aluminum hydroxide gel as indicated (Tecnovax SA, Argentina). Briefly aluminum hydroxide was added dropwise to the antigen suspension under continuous stirring at 4 °C, until reaching a final concentration of 6% w/v. Stirring was pursued for 12 h at 4 °C.

Saponin + aluminum hydroxide (SAl): Quil-A was mixed with the cell suspension to a final concentration of 0.4 mg/ml. Aluminum hydroxide was added dropwise to the cooled (4 °C) mixture until a 6% w/v final concentration was reached. Stirring was pursued for 12 h at 4 °C.

Mineral Oil (O): Montanide (Seppic, France) was vigorously stirred at 30 °C and the antigen was added dropwise to reach 50% v/v. The final mixture was stirred yet for 10 min at 30 °C and cooled to 15 °C. The emulsion stability was evaluated by suspension of a drop on the surface of an aqueous solution [18].

2.2. Cattle and experimental design

The experimental animals consisted of 74 four Aberdeen Angus virgin heifers (with no sexual exposure to bulls since weaning) 15–18 months old, with cyclic ovarian activity and a body condition 6 and 7 (in a scale 1–9 according to [11] were used. The animals were kept under conditions of extensive grazing in a herd free of brucellosis, campylobacteriosis and trichomonosis for more than ten years. The presence of *T. foetus* is tested every year through culture of two consecutive scrapping samples from bulls in reproductive age. The experimental females were controlled for infection through culture of cervical fluid samples taken 3 and 6 weeks before the trial and just before experimental infection.

The heifers were randomly assigned to experimental groups and were immunized through subcutaneous (SC) route with the *T. foetus* B1 preparations containing different adjuvants (S, Al, SAl and O) or with a commercial vaccine (Tv, Tricovac, Laboratorio Biológico de Tandil SRL, Argentina). Two other groups consisted of not immunized animals (C- and Ci). The number of animals by group, control groups and procedures are summarized in Table 1. The immunization procedure consisted of two SC injections in the neck and a third dose delivered with a Cassou pipette in the lumen of the vagina [9]. Immunizations were performed with intervals of 20 days.

Previous to infection the animals were synchronized with two intramuscular doses (500 μg) of prostaglandin F2 analogue (Estrumate, Schering-Plough, Germany) with an interval of 11 days. The challenge was then carried out by using a Cassou pipette to deliver 2 mL of medium containing 1.5×10^6 *T. foetus* B1 motile cells/mL (>99% viability) into the fornix. Heifers were evaluated for clinical signs and inflammatory response every second week until the end of the trial at day 98 post infection (pi).

Heifers used in this work were handled by trained personnel, according to standards of good practices and conditions approved by the Animal Ethics Committee of INTA.

2.3. Sample collections

Blood samples were obtained by jugular venipuncture. Serum was obtained after blood centrifugation at 2500*g* for 15 min and stored at −20 °C until determination of anti-*T. foetus* IgG antibodies. Cervical vaginal mucus (CVM) samples were taken every 14 days from the anterior portion of the vagina and cervix using a Cassou pipette. Approximately 1 ml of each collected sample was seeded in transport and culture medium, incubated at 37 °C and daily observed under optical microscope for 7 days. Aliquots of CVM (1 ml) were diluted with 2 ml PBS and stored at −20 °C until quantification of anti-*T. foetus* IgA antibodies.

2.4. Quantification of anti-T. foetus antibodies

The concentration of anti-*T. foetus* immunoglobulins was assessed by indirect ELISA assay as previously indicated [9,10]. Briefly, plates (96-well, Immulon® 1B, Labsystems, USA) were sensitized with 4×10^6 B1 cells/well in 50 μl of PBS. The plates were incubated 4 h at 22 °C, drained on paper towels and fixed by adding 50 μl of 96% ethanol per well. After draining the ethanol each plate was incubated at 22 °C overnight and stored at -20 °C until use. Each well was blocked with 100 μl of PBS containing 0.05% v/v Tween 20 and 0.2% w/v gelatin (PBS-TG) for 2 h at 37 °C. Sera were tested in duplicate at 1:1000 dilution in PBS-TG for 1 h at 22 °C. The plates were washed 3 times with PBS-T and incubated with a rabbit anti-bovine IgG1-2 conjugated to horseradish peroxidase (Sigma, St. Louis, Mo, USA). Peroxidase activity was measured after washing with PBS-T and PBS, respectively. One hundred μl of substrate (40 mM ABTS [2,2′-azino-di (3-ethylbenzthiazoline sulphonate)], containing 4 mM H_2O_2 in 50 mM citric acid, pH 4.5) was added (Sigma, St. Louis, Mo, USA). Absorbance at 405 nm was measured after 40 min of incubation at room temperature using a microtiter plate reader (Labsystems, Helsinki, Finland).

Samples of CVM (duplicate) were sonicated in an ice bath with a team Sonics Vibra Cell (Newtown, USA) at 80% power for 20 s and then diluted 1:50 in PBS-TG. The presence of IgA was revealed through the binding of a rabbit anti-bovine IgA conjugated to horseradish peroxidase (Bethyl Lab., Texas, USA). Sera or mucus from animals hyperimmunized against *T. foetus* were used as positive controls [9]. Samples from heifers without contact with the parasite were used as negative controls. The samples were tested in duplicates, and positive control and negative control were included in each plate.

2.5. Statistic analysis

Statistical analysis was performed with the R software package (cran.r-project.org). Differences in immunoglobulin levels among the treatment groups were evaluated with Kruskal-Wallis test with post-hoc analysis of significant differences with the Tukey-Kramer (Nemenyi) test. Cumulative times to reach remissions were calculated by the Kaplan-Meier method. The statistical significance of the obtained differences was tested with the log rank test with rho equal to 0 or 1 and hazard ratios were calculated from a Cox proportional model. Remission times and immunoglobulin concentrations were analyzed using Spearman rank correlation coefficient.

3. Results

3.1. Clinical signs

No clinical signs of ill-health were observed in the treated animals. Notwithstanding all the heifers vaccinated with the oil-in-water adjuvant (group O) presented a granulomatous inflammation at the point of injection. These local reactions were firm in consistency and variable in size, reaching 2 cm in diameter after 2–3 weeks of the first dose injection. The inflammation persisted and increased in size after the second dose inoculation, reaching 2–5 cm in diameter. The lesions disappeared at around day 60 of the trial.

3.2. Humoral immune response

Untreated animals, i.e. the group without contact to the parasite, showed basal concentrations of IgG throughout the trial (Fig. 1). Meanwhile serum from the animals of the Ci group showed basal IgG levels until the day of the experimental infection and thereafter a slow and steady increase in concentration (Fig. 1). In vaccinated animals IgG levels increased after the first vaccine application regardless the used adjuvant. Oil-in-water and saponin based vaccines (groups O, S and SAl) showed abrupt slopes in IgG concentration and reach a plateau after 2 weeks, while in the Al group IgG concentration raised slowly and did not reach the values of the precedent groups (Fig. 1). By the time of experimental infection the Al group did not reach a significant difference ($p = 0.6086$) in comparison to the control group (Ci) while groups O, S, Tv and SAl showed significant differences in IgG concentration ($p = 0.0002$, $p = 0.0006$ $p = 0.0008$ and $p = 0.005$ respectively).

3.3. Local immune response

IgA levels remained at baseline levels in all the tested groups after vaccination. Only the challenge with live *T. foetus* determined a sharp increment in the local concentration of IgA in all groups (Fig. 1). Such increase in IgA reached the maximum levels after two weeks post-infection independently of the treatment assigned. Although IgA concentration in animals belonging to groups O, S and SAl showed higher IgA values than Ci animals after infection, the differences were no significant.

3.4. T. foetus clearance

Regardless of the immune status of the host the artificial infection of progesterone treated heifers with *T. foetus* was followed by an effective colonization of the cervical tract. Positive cultures were obtained from every infected animal two weeks after the challenge. Since then the persistence of the infection was variable among the assayed groups. The procedure used to ascertain if an animal was free of *T. foetus* was similar to that employed in the annual diagnostic test performed in every bull in the region (i.e. "a negative culture followed by a positive one was considered positive"). The culture results are shown in Table 2. All the animals in the Ci group remained infected by day 42 pi and then showed regularly negative cultures. By the end of the trial at day 105 pi 2 out of 11 animals in the Ci group were still infected (Fig. 1). In every vaccinated group negative cultures were observed in samples from day 27 after infection. Kaplan-Meier curves shown in Fig. 1 depict the rate of infection clearance. The median time for clearance among unvaccinated heiffers (Ci) was 69 days while immunized animals showed median values of 55 days in the Al group, 41 days in the TV, O and S groups and 27 days in the SAl group. A generalized Wilcoxon test showed a p 0.00594 meaning that, at least, one experimental procedure was significantly different from the control. Therefore, the vaccinated groups were tested individually against the Ci group. The Al group did not reach a significant difference (p 0.19), while the Tv (p 0.007), O (p 0.01) and S (p 0.003) groups appeared effective in the clearance rate of the parasite. Furthermore, the combination of saponin plus aluminum hydroxide (SAl group) gave an enhanced response ($p \sim 0.0001$). Hazard ratios were then used to measure the rate of clearance of infection by unit of time in every treated group as the reference group. As seen in Fig. 2 significant values for the rate of decay of infection per unit of time were found in groups treated with the commercial vaccine or with a vaccine containing saponin as adjuvant. Hazard ratios in those groups were near 2.5 but the low limit of confidence intervals was still close to the reference values (HR = 2.61, CI 95% = 1.07–6.38 for Tv group and HR = 2.52, CI 95% = 1.03–6.17 for S group). A more significant difference was observed in the group with saponin plus aluminum hydroxide with a clearance rate 5.12 (CI 95% = 2.04–12.83) times the clearance observed in the control group.

As the antibody levels are expected to be directly related to the clearance rate of *T. foetus* a correlation analysis was performed

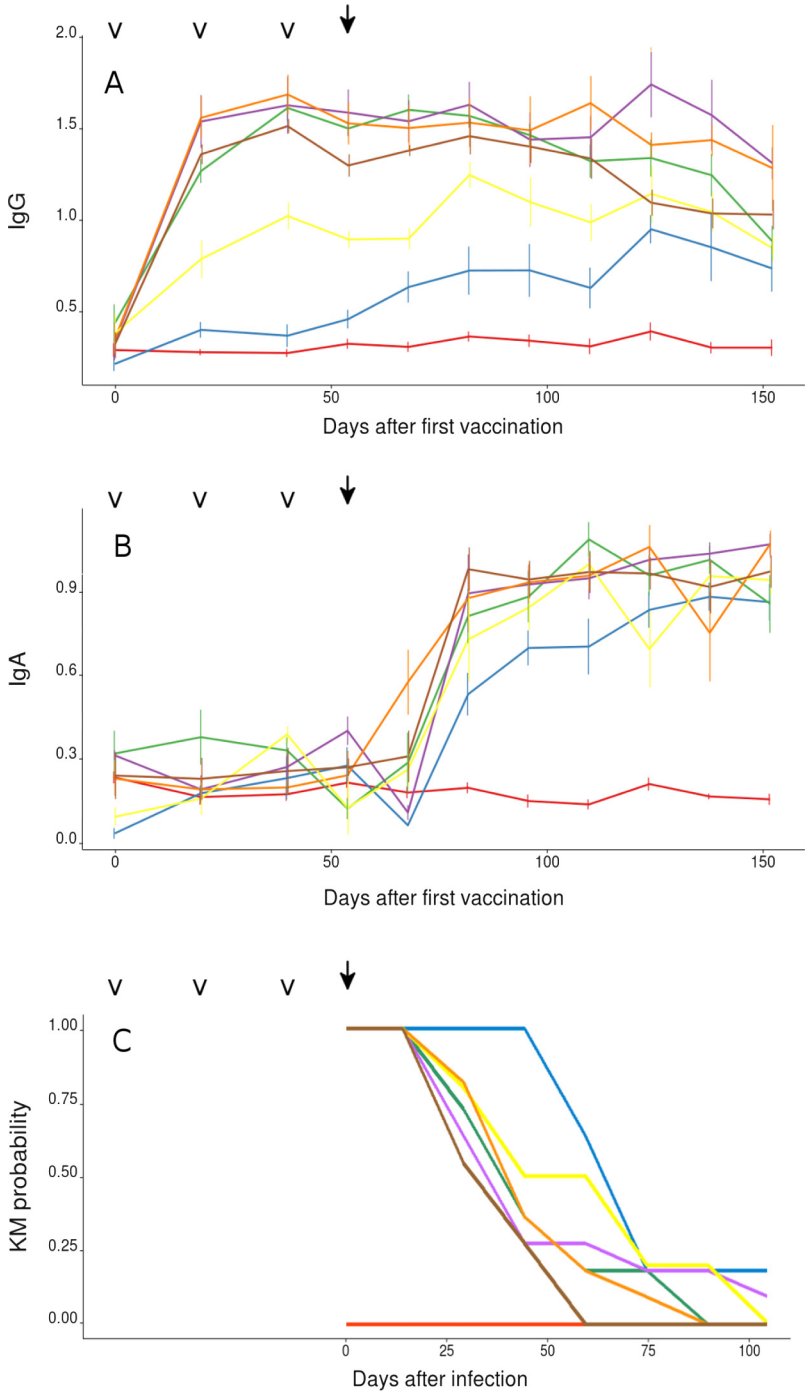

Fig. 1. Immune response and *T. foetus* clearance in vaccinated heifers. The concentration of IgG in serum (A) and IgA in cervical mucus (B) at different time points are shown as mean absorbance (405 nm) ± standard error values. Kaplan Meier clearance probabilities are plotted at the bottom (C). The immunization days are indicated (V) in the top of each plot as well as he day of experimental infection (arrow). C: non-vaccinated/non-infected; Ci: non-vaccinated, Tv: commercial vaccine, O: oil adjuvant, Al: Aluminum hydroxide adjuvant, S: Saponin, SAl: Saponin plus Aluminum Hydroxide adjuvant.

between concentration values obtained the infection day and mean time for clearance in every group. The Spearman rank test did not showed a significant correlation between the parasite clearance and the level of humoral IgG (ρ 0.4) or local IgA (ρ 0.1).

4. Discussion

Previous studies have shown that intramuscular or subcutaneous applications of whole cells of *T. foetus* does not prevent the experimental or naturally induced colonization of the bovine vaginal tract [7,16,15,3,2,10,8]. However vaccination can reduce the time of residence of the pathogen in the host cervix and may probably prevent abortion. Besides it was estimated that genital or placental injuries caused by *T. foetus* would be related to a minimum residence time of 50 days and fetal losses would occur after 70 days of infection [23,26]. In this work, animals treated with an experimental vaccine containing aluminum hydroxide reached the threshold requirement of clearance at the limit of day 60 post challenge. To our knowledge, this adjuvant has not been used previously and our results do not encourage their use. On the other

Table 2
Measure of infection with *T. foetus*. Positive cultures in every experimental group (left column) were assessed every two weeks with the exception of the last measure that was taken after three weeks. Ci: unvaccinated, Tv: commercial vaccine, O: oil adjuvant, Al: Aluminum hydroxide adjuvant, S: Saponin, SAl: Saponin plus Aluminum hydroxide adjuvant, C-: infection control.

	Days after infection							
	0[a]	14	28	42	56	70	84	105
Ci	0	12	10[b]	11	7	2	1	2
Tv	0	11	8	4	2	2	0	0
O	0	11	7	3	3	0[b]	2	1
Q	0	11	9	4	2	1	0	0
Al	0	10	8	4[b]	5	2	2	0
QAl	0	11	6	3	0	0	0	0
C-	0	0	0	0	0	0	0	0

[a] Culture from day 0 was performed in samples taken just before the inoculation.
[b] shows negative cultures followed by positive tests (eg. 10 followed by 11 in the control group) in the same animal, that were interpreted as positive tests.

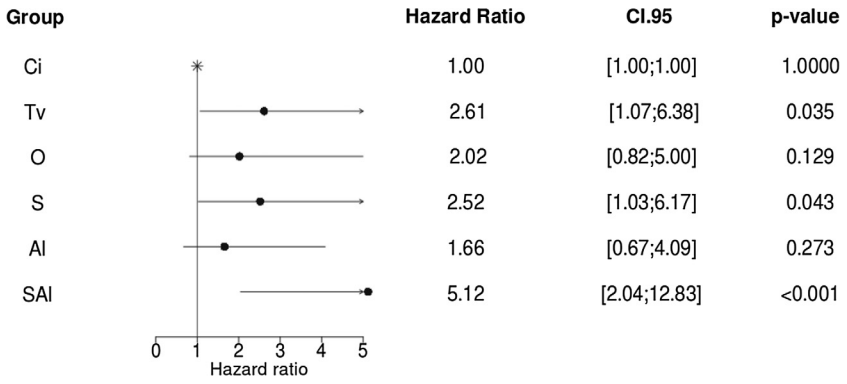

Fig. 2. Association between immunization and the rate of clearance of *T. foetus*. Forest plot for unadjusted Cox model. Circles indicate unadjusted hazard ratios for a given immunization treatment and horizontal lines indicate 95% confidence intervals. Hazard ratios, confidence intervals (CI.95) and *p*-values for experimental groups Ci: unvaccinated, Tv: commercial vaccine, O: oil-in-water adjuvant, S: saponin adjuvant, Al: aluminum hydroxide adjuvant, SAl: saponin + aluminum hydroxide adjuvant.

hand, an oil-in-water or a saponin based vaccine used in this work led to a clearance of infection of 75–80% of the treated animals in the window period of 50 days. This result is similar to that found with the commercial vaccine used here and is in agreement with previous reports [9,10]. Furthermore a vaccine containing aluminum hydroxide plus saponin reduced the time of clearance of the genital infection with higher efficacy than preparations with a single adjuvant (median of 27 days). The enhanced response obtained with mixed adjuvants can thus encourage further research to find better formulations.

The concentration of serum antibodies and local mucosal antibodies was evaluated as a measure of the response achieved by each vaccine preparation. In the control group (Ci) the concentration of specific IgG antibodies increased slowly since the beginning of infection and its concentration reached the maximum levels when most cows have already cleared the infection. On the other hand, circulating antibodies undergo a sudden increase soon after immunization in groups S, O, SAl. Although the titers of circulating antibodies seemed to be related to the clearance rate of the parasite, we were not able to find a statistical relationship.

Previous studies have suggested that systemic immunization would lead to a gradual increase of immune cells in the vaginal epithelium [3,12]. In this work, significant changes in the IgA concentrations were only observed after exposure to alive cells of the parasite and no difference were found among treatments after experimental infection.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.12.030. These data include Google maps of the most important areas described in this article.

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