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Heifers immunized with whole-cell and membrane vaccines against *Tritrichomonas foetus* and naturally challenged with an infected bull

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Abstract

The performance of a whole-cell vaccine and the other vaccine with cellular membranes of *Tritrichomonas foetus* applied to heifers naturally challenged by mating with an infected bull was determined. Forty heifers were divided into three groups: a control group ($n = 16$) without immunizing, another group ($n = 12$) immunized with whole cells (10^8/dose) and a third group ($n = 12$) immunized with cellular membranes (300 μg of membranes/dose protein). The females were subcutaneously vaccinated at 3-week on two occasions and received a third intravaginal booster dose. After 3 weeks of the last vaccinal doses, the heifers were served by a *T. foetus* infected bull over 90-day period. The mean duration of infection for membrane-vaccinated heifers was 60 days ±25, compared with 63 days ±35.8 of infection for whole-cell-vaccinated heifers and 79 days ±41.3 for control heifers. Calving rates were 6/12 for membrane-vaccinated heifers, 3/12 for whole-cell-vaccinated animals, and 2/16 for control animals. Fetal mortality rates were 3/12 for membrane-vaccinated animals, 4/12 for those vaccinated with whole cells and 10/16 for control animals. These reproductive parameters were significantly different ($P < 0.05$) between heifers vaccinated with membranes and control heifers. The hemolytic test and enzyme-linked immunoabsorbent assay (ELISA) with *T. foetus* antigen showed that serum immunoglobulins peaked before and during the breeding period. The heifers vaccinated with membranes developed an important response during the critical period of fetal loss, second and third month of the breeding time, and another month after the same period. The ELISA method was more sensitive and more reliable than the hemolytic test for the evaluation of the systemic immune response in females infected and/or vaccinated with *T. foetus*.

Keywords: *Tritrichomonas foetus*; Vaccine; Bovine; Protozoa

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1. Introduction

Bovine trichomonosis is a disease of sexual transmission caused by *Tritrichomonas foetus* (*T. foetus*) (Skirrow and BonDurant, 1988). In bulls, the infection by *T. foetus* is chronic with the male asymptomatic carrier of the disease (Clark et al., 1983). In the bovine female, *T. foetus* persists in the genital secretions from 13 to 28 weeks (Skirrow and BonDurant, 1990a) causing early embryonic death, transient infertility, uterine discharge, pyometra, and sporadic abortions (Parsonson et al., 1976). Fetal losses for *T. foetus* infectious in the female occur from approximately day 63–70 after infection, when there is also an important immune and inflammatory response (Parsonson et al., 1976; Anderson et al., 1996). Lesions in the fetus are observed after 80–90 days post-infection (Parsonson et al., 1976; Rhyan et al., 1988).

The lack of an effective therapy against trichomonosis has encouraged the research into the development of vaccines (Skirrow and BonDurant, 1988). Therefore, a vaccine was made with inactivated whole cells of *T. foetus* and this vaccine systemically applied to heifers reduced the number of infected females, the reproductive losses, and the duration of the genital infection (Kvasnicka et al., 1992; Hudson et al., 1993b; Gault et al., 1995). However, there were no advantages to the use of whole-cell vaccines, either in cows (Herr et al., 1991) or in bulls older than 5 years (Clark et al., 1984). On the other hand, a vaccine made with glycoproteic fractions of *T. foetus* membranes produced acceptable results on bulls (Clark et al., 1984). The use of membrane vaccines in bovine females produced different results Campero et al. (1999) observed a shorter time of infection in the vaccinated females, whereas Hudson et al. (1993a) mentioned a better performance of a whole-cell vaccine compared with another vaccine based on *T. foetus* fractions.

In spite of the advances made, there is not enough information on the performance of vaccines against bovine trichomonosis under natural challenge conditions. Moreover, a characteristic of the breeding herds in the Argentinian humid pampas and other areas of extensive cattle raising is that, where service with bulls for 90-day periods, a significant and durable immune response from the females is required. The purpose of this work was to evaluate two types of vaccines against *T. foetus* in bovine females naturally challenged by mating with an infected bull: one formalized with whole cells and the other one with cellular membranes of *T. foetus*.

2. Materials and methods

2.1. Vaccine preparation

2.1.1. Organism

A *T. foetus* B1 strain originally obtained from a cow with pyometra (Campero et al., 1998, 1999) was employed and an axenic clone was obtained by isolation in trypticase-yeast extract-maltose agar without antibiotics (Jensen, 1983). *T. foetus* was grown on a liver infusion medium containing 15% tryptone and 10% inactivated horse serum (Campero et al., 1993; Cobo et al., 2001).

2.1.2. Whole-cell vaccine

The whole-cell vaccine was prepared according to Clark et al. (1983). The cells of *T. foetus* were collected from a 24 to 48 h culture (Campero et al., 1993) and washed (3×) with phosphate-buffered-saline (PBS) pH 7.2 by centrifugation (10,000 rpm, 7500 × *g*) for 15 min and suspended in PBS pH 7.2 and formalin added to 1% (v/v).

2.1.3. Membrane vaccine

Membrane vaccines were prepared according to Clark et al. (1984). *T. foetus* cells were collected from a 24 to 48 h culture (Campero et al., 1993), washed (3×) with PBS pH 7.2 by centrifugation (10,000 rpm, 7500 × *g*) for 15 min and suspended in PBS containing 1 mM $MgCl_2 \cdot 6H_2O$ (PBS-ClMg) and 1% phenyl-methylsulphonyl fluoride (7626 Sigma) (88 mg/ml methanol) (homogenizing buffer) (Campero et al., 1998). The suspension was subjected to 5 min of sonication (5×) on ice in an ultrasonicator (Sonifier 250 Branson Sonic Power, USA) (Clark et al., 1984; Campero et al., 1998). The sonicate was centrifuged (4000 rpm, 1100 × *g* for 10 min) and the supernatant was layered onto 10 ml of 41% (v/v) sucrose in homogenizing buffer before centrifuging at 35,000 rpm (30,000 × *g*) for 150 min at 6 °C (Centrikon T-1170 with rotor TFT 45-94). The membranes were collected from the interface between the buffer and the sucrose solution (Clark et al., 1984). Protein content (15 μg/ml) was measured by Micro BCA Protein Assay Pierce Lab 23535.

2.1.4. Control solution

A PBS-ClMg solution as aqueous phase was made as inoculum for the control group. To all the different preparations 0.5% (v/v) of gentamicin sulfate (7507 Sigma) (5 mg/ml) and 1/10,000 (w/v) thimerosal (5125 Sigma) were added. The vaccines and control solution were mixed in 50:50 (v/v) ratio with an oil adjuvant consisting of Marcol 52, Arlacel C and Tween 80 (Campero et al., 1998, 1999). The membrane vaccine contained 300 μg of membrane protein per 6 ml dose, the whole-cell vaccine 1×10^8 cells per 5 ml dose and the control solution 2.5 ml PBS per 5 ml dose.

2.2. Experimental animals

2.2.1. Immunization

Forty post-pubertal Aberdeen Angus, Hereford, and crossbreed virgin heifers, 18–24-month-old, were divided into three groups and immunized three times at 3-week intervals. All heifers received two subcutaneous doses followed by an intravaginal boosting with an artificial insemination Cassou pipette. The control group (C) ($n = 16$) was given a control solution. Group (W) ($n = 12$) was immunized with whole cells. Group (M) ($n = 12$) was immunized twice with *T. foetus* membranes and an intravaginal booster with whole cells was administrated.

2.2.2. Challenge

Three weeks after the last immunization all heifers were exposed to infection with *T. foetus* by mating them with a naturally infected bull over a 90-day period. The bull was 6-year-old, assigned to a satisfactory breeding status on the basis of physical soundness, scrotal circumference, and semen examination. The bull had a *T. foetus*-positive culture

for 5 weeks prior to the breeding time with the heifers. Moreover, the bull had negative culture for *Campylobacter fetus* and *Haemophilus somnus* of the reproductive tract and negative for Brucellosis by the serology test. To further ensure infectivity during breeding time, the bull was infused into the preputial cavity with 1.5–2 $\times$ 10^6 cells of *T. foetus* fortnightly.

2.2.3. Collection and examination of samples

Vaginal mucus and serum samples from heifers were collected weekly, before and during the breeding period and until 83 days after its end (day 237 post the first dose (pfd)). Preputial samples of the bull were obtained and cultured weekly to determine if it remained *T. foetus*-infected throughout the 90-day breeding period. Vaginal mucus from heifers and preputial smegma from bull specimens were collected into an insemination Cassou pipette and cultured onto liver infusion medium with antibiotics at 37 °C and daily examined during a week at a microscope after collection (Campero et al., 1993; Cobo et al., 2001).

2.2.4. Pregnancy tests

Conception data of all heifers were obtained by transrectal ultrasonography on day 44 after the start of the breeding period, at the end of this, and 49 days after the end of the breeding period. Subsequent pregnancy detection was determined by rectal palpation every 2 weeks for detection of reproductive losses until day 237 pfd.

2.3. Antibody assay

2.3.1. ELISA

Systemic antibodies to *T. foetus* in bovine serum were quantitated by the enzyme-linked immunoabsorbent assay (ELISA) as previously described by Skirrow and BonDurant (1990b) with minor modifications. Serum was diluted 1:1000 in PBS plus 0.05% (v/v) Tween 20 and gelatin (Difco Laboratories, Detroit, MI) (PBS Tw–g) before a 30 min incubation at 37 °C in 96-well microtiter plates (Immunoblot, Dynatech, VA). Previously, the plates were coated with whole *T. foetus* organisms (50 μl at 10^6/ml in PBS) and blocked with 3% gelatin (Difco Laboratories, Detroit, MI) in PBS. All samples were done in duplicate. Peroxidase-labeled rabbit anti-bovine whole immunoglobulins (Sigma, St. Louis, MO) at 1/5000 in PBS Tw–g were added and incubated 30 min at 37 °C. For color development, 2,2′-azino-die(3-etilbenzotiazolin sulfonate) (ABTS) (Sigma, St. Louis, MO) was used. A stock solution of 40 Mm ABTS (21.94 mg/ml bidistilled water) was diluted 1/4 in 0.05 M citric acid pH 4.5 plus 0.006% hydrogen peroxide (Cobo et al., 2001). After 4 min, the reaction was stopped with 50 μl of 2 M sulfuric acid and the optical densities (OD) were read on an ELISA microplate reader (Multiskan EX, Labsystems, Helsinki, Finland) at 205 nm (Cobo et al., 2001). OD in the ELISA were corrected for variation among plates by absorbance of a positive control sample (serum from a heifer subcutaneously immunized 14 times every week with formalized *T. foetus* whole cells and *T. foetus* membranes) and negative control sample (serum from non-infected and non-exposed virgin heifers). Control samples were included on each plate (Cobo et al., 2001). The following formula was used (Hum et al., 1991):

$$\text{ELISA values (EV)} = \frac{\text{mean sample OD} - \text{mean OD of negative control on the same plate}}{\text{mean OD of positive control on the same plate} - \text{mean OD of negative control on the same plate}} \times 100$$

2.3.2. Hemolytic assay

Systemic antibodies against *T. foetus* in bovine serum were quantitated by the hemolytic assay as previously described (BonDurant et al., 1996). Briefly, 10% fresh bovine erythrocytes were added to pH adjusted *T. foetus*-conditioned Diamond's medium supernatants and the mixture was incubated overnight at room temperature on a mixing rotator After washings with PBS (2×), the erythrocytes were resuspended in 1% PBS fetal calf serum at a concentration of 1% erythrocytes. Suspension of 25 μl was added to 25 μl of doubling dilutions in PBS of serum samples in U-shaped 96-well microtiter plates (Immunoblot, Dynatech, VA). Positive and negative control samples as described for ELISA were included in each plate. Microtiter plates were shaken and incubated for 60 min at 4 °C. Guinea-pig complement of 50 μl, diluted 1:10 in 1% PBS fetal calf serum, was added to each well, and the plates were incubated at 23 °C for 60 min, shaken, and further incubated for 90 min. Then, plates were visually examined for hemolysis and the serum hemolytic titer was defined as the last dilution with lysis of erythrocytes. Because these titers were not drawn from a normally distributed population, one unit was added to each titer and that value was transformed into its natural logarithm (Siegel, 1956).

2.4. Statistical analysis

Statistical differences were accepted as statistically significant at the 95% confidence limit ($P < 0.05\%$). Data about the clearance of trichomonads from the vagina of immunized and control heifers were analyzed using chi-square test (χ^2) for independent samples. The mean duration of infection in heifers was analyzed using the Student's *t*-distribution. Pregnancy and reproductive losses were analyzed using Fisher's exact probability test (Siegel, 1956). For ELISA, corrected absorbances were compared between groups by using MIXED SAS for dependent samples (Littell et al., 1998; Cobo et al., 2001). For the hemolytic test, transformed titers were analyzed using MIXED SAS for parametric and dependent samples and their interactions by contrasts (Littell et al., 1998). On the other hand, transformed titers were analyzed using Kruskall Wallis for non-parametric and independent samples and their means by Duncan's test (Campero et al., 1999).

2.5. Correlation between ELISA and the hemolytic test

The serum samples analyzed by ELISA and the hemolytic test were used to measure correlation between both methods. In the parametric case, correlation was analyzed using the Pearson's correlation coefficient (r) (−1 to 1), but in the non-parametric case the Spearman's rank correlation coefficient (cS) (−1 to 1) was used (Siegel, 1956). Global correlation and correlation for low, medium, high, and extra-high antibody levels were determined. For correlation at different antibody levels, the data were divided arbitrarily but equitably. For

low antibody levels, 386 samples of lower values for ELISA (EV 25.49–0.33) and 386 samples of lower values (HT) for the hemolytic test (HT 0) were selected. For medium antibody levels, 386 samples of medium values for ELISA (EV 0.33–9.69) and the hemolytic test (HT 0–2.19) were selected. For high antibody levels, 388 samples of higher values for ELISA (EV 9.7–106.7) and the hemolytic test (HT 2.19–4.85) were chosen. For extra-high antibody levels, 66 samples of maximum values for ELISA (EV 36.36–106.7) and the hemolytic test (HT 4.85) were selected.

3. Results

3.1. Immunization of heifers and infection of the bull

Neither adverse general reactions nor clinical signs were caused by vaccination of the heifers. Preputial smegma samples from the bull had *T. foetus*-positive cultures during the breeding period.

3.2. Infection of heifers

Pre-breeding samples of the heifers had *T. foetus*-negative cultures. After that, culture results of immunized or control cattle showed a higher percentage of infection starting from the second month of service (97–125 days pfd), where 81% (13/16) females of group (C) and 83% (10/12) females of the vaccinated groups (W) (M) were infected (Fig. 1). The mean duration of infection in vaccinated and control heifers for (M), (W), and (C) groups was 60 days (±25), 63 days (±35.8), and 79 days (±41.3), respectively. Although there were no significant differences among the three groups ($P > 0.05$), there was a tendency towards a shorter infection to group (M) compared with group (C) ($P = 0.09$). Vaccinated females began to recover from infection from the third month of service onwards, while control females remained infected or recovered more slowly until 4 weeks after service (Fig. 1). Also, the infection index was significantly lower ($P < 0.05$) in groups (W) and (M) with regard to group (C) from the last week of service until 3 weeks after its conclusion (154, 167 and 174 days pfd). (One female, after 83 days of the end of service (237 days pfd), stills remained infected to group (C)) (Fig. 1).

Among the vaccinated groups the infection percentage in females in the different samplings did not differ ($P > 0.05$), except 174 days pfd when the infection index was significantly lower ($P < 0.05$) to group (M).

On the other hand, only four females (Nos. 13 and 16 of group C, and 24 and 25 of group W) were not infected with *T. foetus*, became pregnant and gave birth normally.

3.3. Reproductive performance

The percentage of pregnant females at 160 days pfd, when service ended, was 75% in groups (C) (12/16) and (M) (9/12) and 58% in group (W) (7/12) (Table 1). The percentage of females that maintained pregnancy and gave birth normally was 50% (6/12) in group (M), 25% (3/12) in group (W), and 12% (2/16) in group (C). Therefore, the percentage of

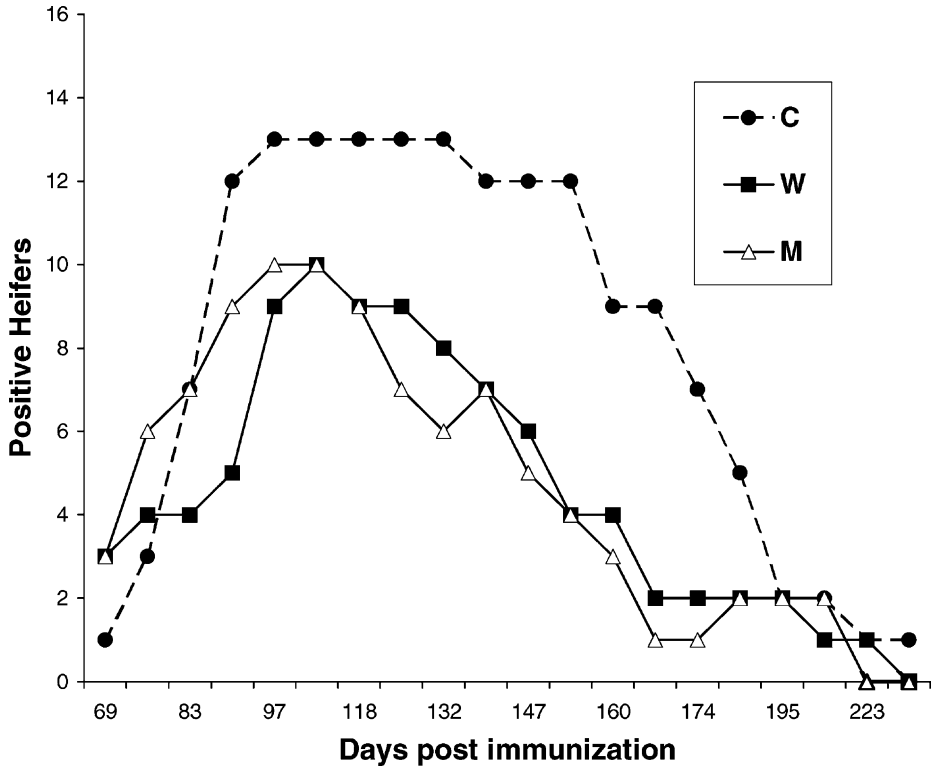

Fig. 1. Weekly vaginal *T. foetus* culture results of immunized and control heifers during and after breeding period. Group C heifers were non-immunized controls; group W heifers were immunized subcutaneously twice and once intravaginally with 10^8 formalized whole cells; and group M heifers were immunized subcutaneously twice with membranes (300 μg) of *T. foetus* and once intravaginally with 10^8 formalized whole cells. Animals were immunized at days 0, 20 and 41, and challenged by breeding with an infected bull since 62–154 days after first immunization.

Table 1
Pregnancy, calving and abortion rates in vaccinated and control heifers[a]

Group	No. of heifers pregnant at the final of breeding time	No. of heifers gave birth to calves	No. of aborted heifers	No of barren heifers
C ($n = 16$)	12	2 (a)	10 (a)	4
W ($n = 12$)	7	3 (ab)	4 (ab)	5
M ($n = 12$)	9	6 (b)	3 (b)	3

[a] Group C heifers were unimmunized controls; group W heifers were immunized subcutaneously twice and once intravaginally with 10^8 formalized whole cells; and group M heifers were immunized subcutaneously twice with membranes (300 μg) of *T. foetus* and once intravaginally with 10^8 formalized whole cells. At the final breeding period was 160 days after first immunization. Different letters refer $P < F'$ 0.05.

females that suffered reproductive losses was 83% (10/12) in group (C), 57% (4/7) in group (W), and 33% (3/9) in group (M). Reproductive patterns differed significantly ($P < 0.05$) between groups (C) and (M) (Table 1).

3.4. ELISA

Levels of serum antibodies (EV) were higher in the immunized groups (W) and (M) than group (C) from 20 up to 237 days pfd, establishing significant differences ($P < 0.05$) from day 27 until day 160 pfd and again starting from day 209 pfd until the end of the experiment. Maximum EV were registered in group (W) at 27 and 34 days pfd (EV 33.06 and 39.94, respectively) and in group (M) at 140 and 147 days pfd (EV 32.61 and 29.79, respectively) (Fig. 2). The application of the second vaccinal dose increased the EV in groups (W) (29.7 units) and (M) (17.2 units) after 2 weeks (34 days pfd) (Fig. 2). However, neither in group (W) (3.4 units) nor in group (M) (2.78 units) (Fig. 2) did the application of the intravaginal booster produced important increments of the EV after 2 weeks of the application (55 days pfd). In group (C) the EV maintained a basal level during the whole experiment, except for a slight increase during service (between 125 and 147 days pfd) when it reached values of 6.34 with a mean of 5.63 (Fig. 2).

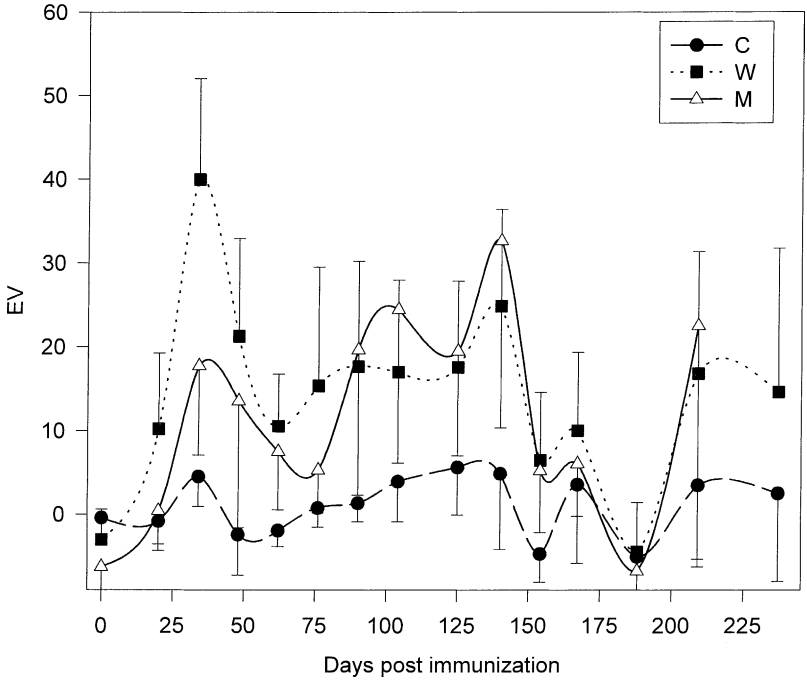

Fig. 2. Serum antibody responses to *T. foetus* measured by ELISA. EV: ELISA values. Group C heifers were non-immunized controls; group W heifers were immunized subcutaneously twice and once intravaginally with whole cells; group M heifers were immunized subcutaneously twice with membranes and once intravaginally with whole cells.

During service, mean EV were highest in group (M) (16.23 with a range between 5.23 and 32.61), followed by group (W) (15.4 range 4.56–24.77) and finally in group (C) (1.57 range −4.72 to 6.34) (Fig. 2).

During the reproductive loss period (from 60 days starting from the beginning of service until 60 days after its conclusion (118–209 days pfd)), mean EV were higher in group (M) (13.07 range −6.76 to 32.61), than group (W) (11.3 range −4.46 to 24.77) and group (C) (1.84 range −5.07 to 6.34) (Fig. 2).

When the performance between both vaccines was compared, it was determined that group (W) obtained higher EV after day 12 pfd and during the first month of service, establishing significant differences ($P < 0.05$) between 12 and 34 days pfd and at 76 and 83 days pfd (Fig. 2). However, during the second and third month of service the highest EV corresponded to group (M), establishing significant differences ($P < 0.05$) at day 147 pfd (Fig. 2). Later, no significant differences between both vaccines were observed (Fig. 2).

3.5. Hemolytic test

Levels of serum antibodies (HT) were higher in the immunized groups (W) and (M) compared to group (C) from 20 up to 237 days pfd, establishing significant differences ($P < 0.05$), at least by Mixed SAS or Kruskall-Wallis, from 27 until 167 days pfd and at 188, 223 and 237 days pfd (Fig. 3). Maximum HT were registered in group (M) at 34 and 118 days pfd (4.13 and 4.36, respectively) and in group (W) at 27 and 69 days pfd (3.71 and 3.67, respectively) (Fig. 3). The application of the second vaccinal dose increased HT in groups (W) (2.68 points) and (M) (3.54 points) after 2 weeks (34 days pfd) (Fig. 3). However, neither in group (W) (0.69 units) nor in group (M) (−0.97 units) (Fig. 3) did the application of an intravaginal booster after 2 weeks of the application (55 days pfd) increased HT. In group (C) the HT remained at a basal level during the whole experiment (values <0.7), except for a slight increase during service (between 118 and 160 days pfd) when it reached values of 1.41, with a mean of 0.99 (Fig. 3).

During service, mean HT were similar between groups (W) and (M) (2.77 range between 1.23–3.67 and 2.69 range 0.89–4.36, respectively), although higher than group (C) (0.51 range 0–1.41) (Fig. 3).

During the critical period of reproductive losses, mean HT were higher in group (M) (2.01 range 0.45–4.07), followed by group (W) (1.99 range 0.18–3.13) and finally by group (C) (0.56 range 0.1–1.36) (Fig. 3).

When the performance between both vaccines was compared, group (M) surpassed group (W) significantly ($P < 0.05$) at 34 days pfd (Fig. 3). Then, group (W) obtained higher HT from day 48 pfd until the second month of service, establishing significant differences ($P < 0.05$) by both statistical methods at 48, 62, 69 and 76 days pfd (Fig. 3). However, during the second and third month of service the highest HT corresponded to group (M), except at day 140 pfd, establishing significant differences ($P < 0.05$) at 118 and 132 days pfd (Fig. 3). Later, a superiority pattern between both vaccines was not established (Table 1).

The Mixed SAS and Kruskall Wallis statistical methods applied to the hemolytic test arrived at similar results, coinciding in almost all samplings except for days 34, 167, 188 and 223 pfd.

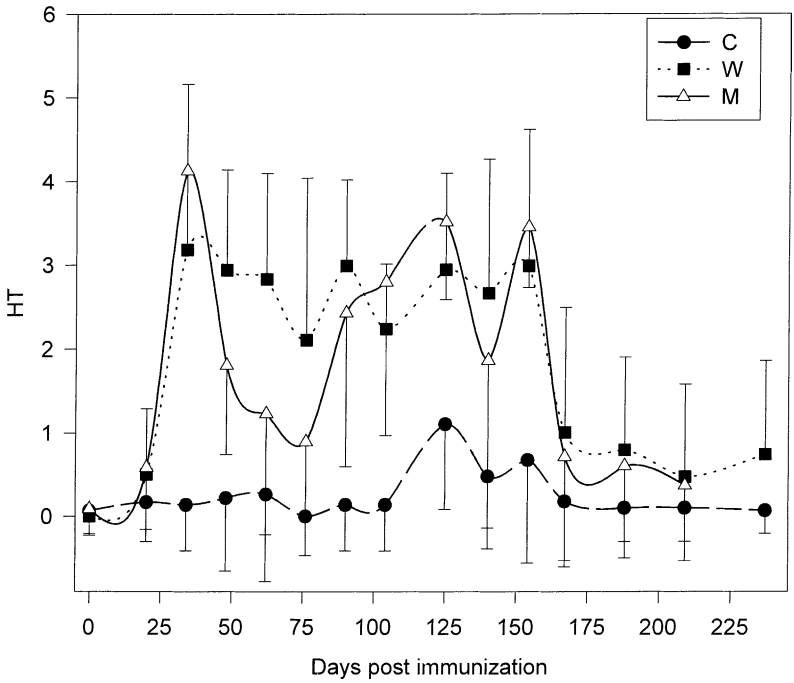

Fig. 3. Serum antibody responses to *T. foetus* measured by hemolytic test. HT: hemolytic test titers. Group C heifers were non-immunized controls; group W heifers were immunized subcutaneously twice and once intravaginally with whole cells; and group M heifers were immunized subcutaneously twice with membranes and once intravaginally with whole cells.

3.6. Correlation between ELISA and the hemolytic test

Data obtained by the ELISA and hemolytic tests had high global correlation (*r* 0.89, cS 0.91) and so did medium and high levels of serum antibodies (*r* 0.9, cS 0.85 and *r* 0.87, cS 0.97, respectively). However, the correlation was regular facing low and very high levels of serum antibodies (*r* 0, cS 0.5 and *r* 0, cS 0.5, respectively). Both tests had a kinetics of serum antibodies similar for the three groups throughout the experiment, except for some punctual samplings (20, 34, 41, 90, 140, 154, 160, 174, 209 and 223 days pfd). Also, the lineal regression of the data obtained by both tests in the three groups described a similar kinetics, where group (W) presented the highest values up to 120–150 days pfd and then, group (M) presented the highest values up to day 237 pfd (Fig. 4).

4. Discussion

In this work, the high percentages of vaccinated and control females infected during the first half of service coincides with that mentioned by Parsonson et al. (1976), and indicates an important exposure to the agent and a great susceptibility of the heifers to *T. foetus*.

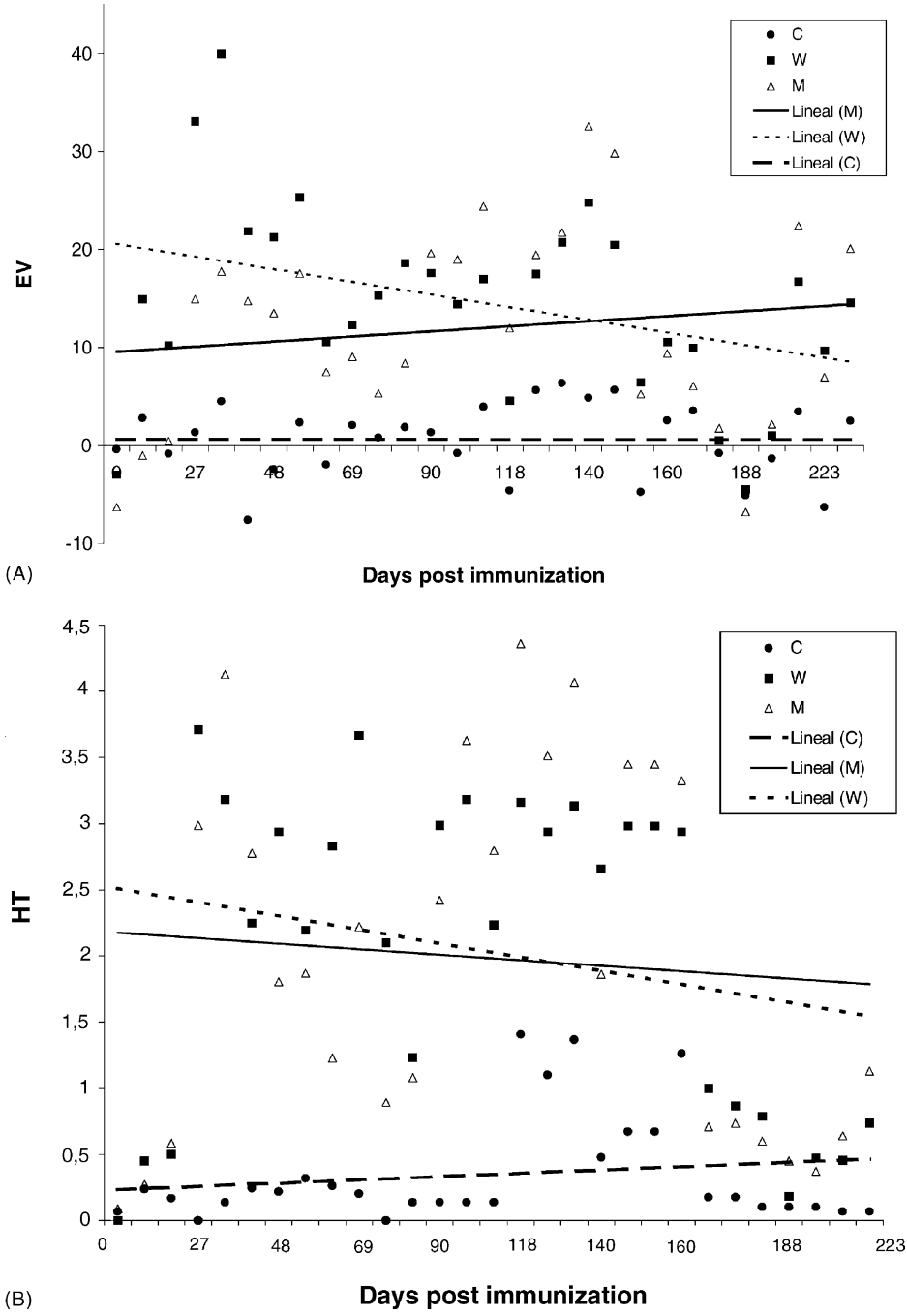

Fig. 4. Serum antibody responses to *T. foetus* measured by ELISA (A) and hemolytic test (B). EV: ELISA values. HT: hemolytic test titers. Points indicate mean percentage and lines indicate the lineal regression for each group.

In this experience, coinciding with the works of Kvasnicka et al. (1992), the intrapreputial reinoculation with *T. foetus* determined a higher exposure level to the disease in the females. In natural service, the multiple coitus by the bull can cause a decrease in the amount of protozoa in the preputial cavity. The detection of females served by the bull that were not infected and gave birth (10%) indicates the presence of animals naturally resistant to the disease, as it was already mentioned (Campero and Palladino, 1983). Such individual resistance could be due to the fact that certain bovine genetic lines would elaborate IgG2 resistant to the cysteine protease of *T. foetus*, granting resistance to infection and better response to immunoprophylaxis (Bastida-Corcuera et al., 2000).

The detection of serum antibodies in females without vaccination before and during service indicates the presence of antigens common to *T. foetus* and other agents of the normal vaginal flora and a systemic stimulus generated by the challenge with the infected bull (Skirrow and BonDurant, 1990b). Other authors determined an increase in systemic IgG1 and IG2 and in IgA and IgG1 in vagina and uterus in animals naturally infected (Skirrow and BonDurant, 1990b; Gault et al., 1995; Ikeda et al., 1995; BonDurant et al., 1996). The genital immune response of IgA usually persists up to 24 weeks in the genital secretions, while the IgG decreases in the serum and genital secretions after 6–8 weeks following challenge (Ikeda et al., 1995; Corbeil et al., 1998). However, the immune response in females naturally infected is usually late and rarely prevents reproductive losses (Gault et al., 1995).

This study proved that the natural immune response was not enough to get over the infection with *T. foetus*, detecting an infected female in the control group up to 83 days after the end of service. Coincidentally, naturally infected females were detected until 175 days after challenge (Skirrow and BonDurant, 1990a,b). In addition, IgA and IgG would play an important role in the genital immunity of bovine females against *T. foetus* (Corbeil et al., 2001). Because in vitro IgA shows an immobilizing ability over *T. foetus* (Ikeda et al., 1995), and IgG1 prevention of the adherence of *T. foetus* to the bovine vaginal epithelium, immovability the protozoan, activation of the cascade of the complement, and stimulation of phagocytosis by monocytes (Hodgson et al., 1990; Burgess and McDonald, 1992; Corbeil et al., 1998).

The vaccines used in this study and those used in previous experiences did not avoid the colonization of the reproductive tract of the vaccinated females (Kvasnicka et al., 1992; BonDurant et al., 1993; Gault et al., 1995; Corbeil et al., 2001). However, the duration of the genital infection was shorter in the vaccinated females, even more in those vaccinated with membrane, compared with non-vaccinated females. The longest duration of the genital infection in control females with regard to vaccinated females (difference >2 weeks) was similar to that observed previously under conditions of natural challenge (Kvasnicka et al., 1992). However, when using intravaginal challenge with *T. foetus*, the difference in the duration of infection between vaccinated and non-vaccinated females was of 4–7 weeks (BonDurant et al., 1993; Gault et al., 1995; Campero et al., 1999).

In this work, the vaccinated females remained infected for a shorter period of time (average 9 weeks) with respect to control heifers. However, this permanence was greater when comparing it with females vaccinated in other experiments with natural or experimental challenged (infection remained from 3 to 4 weeks to a maximum of 7 weeks) (Kvasnicka et al., 1992; Herr et al., 1991; BonDurant et al., 1993; Hudson et al., 1993b; Gault et al., 1995; Corbeil et al., 2001). The causes of a longer genital infection observed in this work could be because of an insufficient amount of vaccinal antigen (low number of protozoa

per dose in the whole-cell vaccine or insufficient protein concentration per dose in the membrane vaccine), the type of adjuvant used, or the over challenge of the reinfected bull.

In this study and after 4 weeks post-service, a lower percentage (31%) of infected control females was observed compared with the works of Kvasnicka et al. (1992) and Gault et al. (1995), who using a service with infected bulls during 45 and 14 days, respectively, detected 80% of non-vaccinated infected females at that moment. Probably, the prolonged duration of the service used in this experiment (90 days) allowed the development of a natural immune response able to liberate the genital infection in the heifers. Moreover, it is possible that several females had repeated estrus towards the end of service, exceeding the copulatory capacity of the bull, or after the end of service. In such circumstances it is possible that they had not been served. The time of liberation of the infection did not differ among females vaccinated with whole cells or with cellular membranes, in opposition to the findings of Hudson et al. (1993a), who observed a shorter time of infection using whole-cell vaccine. However, the use of different protocols of elaboration of the membrane vaccine could influence the results obtained in this study.

The use of the vaccines in this experiment caused less reproductive losses with the cellular membrane vaccine being the more efficient. Moreover, the index of final calving (25% for the whole-cell vaccine and 50% for the membrane vaccine) exceeded the values observed by Herr et al. (1991), who using a whole-cell vaccine only achieved 8.3% of calving. On the other hand, our reproductive performance was lower than that achieved by Kvasnicka et al. (1992) and Hudson et al. (1993b), who obtained total calving indexes of 66.6 and 55.5%, respectively, in the groups vaccinated with whole cells. It is possible that the low indexes of our experience could be due to the use of a single bull for the service and the periodic intrapreputial reinoculation with *T. foetus*.

The serum immune response in the vaccinated females for both immunogens was of the systemic type, characterized by an important increase in serum antibodies after 2 weeks following the second systemic vaccinal dose. In previous works, where bovine females were immunized with whole-cells and membrane subunits, an increase in the serum and vaginal level of IgG1 isotypes was proven. Probably, the preponderance of IgG1 is due to the capacity of *T. foetus* to degrade IgG2 and to the transudation of serum IgG1 towards vaginal secretions (BonDurant et al., 1993; Gault et al., 1995; Corbeil et al., 1998, 2001; Bastida-Corcuera et al., 2000). In turn, the type of adjuvant conditions the immune response and while the use of Freund's incomplete oleaginous adjuvant in systemic immunizations increased the levels of genital IgG and IgA (BonDurant et al., 1993), the systemic immunization with Quil A adjuvant determined a genital response exclusively of IgG1 (Corbeil et al., 1998, 2001).

Local immunization of heifers in the vaginal mucosa with oleaginous adjuvant did not induce an increase in the levels of serum antibodies, contrary to Campero et al. (1998), who inoculated the antigen in the vaginal submucosa and obtained an increase in the serum levels detected by the hemolytic test. However, our finding coincides with Corbeil et al. (2001), who immunizing heifers intravaginally and nasally with Tf 1.17 antigen in Quil A adjuvant determined an important increase of IgA in uterine and vaginal secretions, although it did not determine a systemic response (Corbeil et al., 2001).

In this work, the kinetics of systemic antibodies was different for each one of the immunogens evaluated. While the whole-cell vaccine stimulated an important immune response starting from the second vaccinal dose and during the first month of service, the

membrane vaccine generated not only a "booster" effect after the second vaccinal dose but also an important systemic immune response during the second and third month of service that was prolonged until a month after it. However, when bovine females were vaccinated with *T. foetus* membranes with the same adjuvant but with more protein concentration per dose, the maximum values of serum antibodies to the hemolytic test were observed at 15–50 days after the last systemic dose (Campero et al., 1998, 1999). It is possible that the use of the hemolytic test determines variations in the different experiences due to uncertain irregularities or deficient quality of the reagents inherent to the test (BonDurant et al., 1996; Campero et al., 1999). Also, in this experiment it is possible that a lower antigenic protein concentration or the continuous challenge by service with the infected bull could have conditioned the immune response in time and form.

The high systemic immune response generated by the membrane vaccine during the second half of the 90-day period of natural service and 1 month after it should protect against the reproductive losses for the following reason. The reproductive losses occur between days 70 and 90 after infection (Parsonson et al., 1976; Anderson et al., 1996). In this work most heifers were served and therefore infected during the first month of service. Due to this, the period of reproductive losses would take place during the second half of the 90-day period of natural service and 1 month after it, coinciding with the high immune response. This immune response should allow a precocious liberation of the disease, before embryonic and early pregnancy mortality.

Previously, the vaccine with cellular membranes of *T. foetus* was effective in heifers, where it generated high levels of antibodies during 200 days when it was applied subcutaneously and vaginally (Hudson et al., 1993b; Campero et al., 1999) and also when it was used in bulls younger than 5.5 years, where it had a preventive and healing effect (Clark et al., 1983, 1984).

In this work, both serum tests described a similar kinetics of serum antibodies to *T. foetus*, demonstrated by high indexes of global correlation and in front of intermediate values. However, the ELISA test was more practical and more efficient to determine the humoral immune response due to greater repetitiveness of results, the use of a smaller volume of reagents, and the possibility of working with parametric data that allow the use of more exact statistical programs. Despite the usefulness of the hemolytic test to detect the serologic response in naturally and/or experimentally infected females (BonDurant et al., 1996), and in females vaccinated with different immunogens (Campero et al., 1998), the correlation between this test and the ELISA test was low in the presence of extreme values. Probably, the short scale of the hemolytic method did not always allow explaining serum variations and, under our laboratory conditions, we observed phenomena of unspecific agglutination in the serums, as it was already mentioned (BonDurant et al., 1996; Campero et al., 1998). Finally, if the hemolytic test is used, the equality of results obtained by Mixed SAS or Kruskall-Wallis allows suggesting the use of the first program to work on parametric data and to allow the time as variable.

5. Conclusions

The lack of characterization of Ig serum isotypes and the non-determination of vaginal antibodies in this work deprives us of information. However, the vaccination plan (two

parenteral vaccinal doses and a third vaginal booster dose in oleaginous adjuvant) used by us was similar to that used in previous works (BonDurant et al., 1993; Corbeil et al., 1998, 2001). Moreover, it allows us to presume an increase in serum antibodies, mainly IgG1, transudation of this immunoglobulin towards genital secretions, and an increase in genital IgA due to the booster in the superficial vaginal mucosa.

Finally, the vaccine elaborated with *T. foetus* membrane induced a better systemic immune response during service and determined fewer fetal losses. Also, heifers immunized with such antigen had lower mean persistence of genital infection, higher pregnancy indexes, and less reproductive losses, with significant differences with regard to non-vaccinated females.

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