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Immunization in heifers with dual vaccines containing *Tritrichomonas foetus* and *Campylobacter fetus* antigens using systemic and mucosal routes

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Abstract

Vaccines against both bovine venereal campylobacteriosis and trichomonosis were tested. Heifers were assigned to three groups. Groups 1 ($n = 21$ heifers) and group 2 ($n = 20$) received a commercial or experimental vaccine, respectively, containing both *Campylobacter fetus* and *Tritrichomonas foetus* antigens. Group 3 ($n = 21$) received adjuvant alone. Preparations were injected SQ in groups 1 and 3 at days −60 and −30 (day 0 was considered the first day of a 90-day breeding period), and in group 2 SQ at days −30 and +11 and into the vaginal submucosa at day −9. Heifers were exposed to two pathogen-infected bulls for 90 days (from day 0 to day +90); furthermore, half of the heifers in each group were challenged at day +39 by an intravaginal instillation of *C. fetus venerealis* and *T. foetus*. Pregnancy diagnosis, vaginal culture, and determination of systemic IgG for both organisms were performed. Compared to controls, vaccinated heifers resisted or quickly cleared both pathogens, had a higher pregnancy rate and a higher systemic immune response during and after the breeding period. Overall, the experimental vaccine was superior to the commercial vaccine (groups 2 and 1, respectively). In conclusion, an experimental vaccine containing both *C. fetus* and *T. foetus* antigens,

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given both SQ and intravaginal immediately before breeding and early in the breeding season, yielded superior protection for heifers exposed to bulls harboring *C. fetus* and *T. foetus*.

Keywords: *Tritrichomonas foetus*; *Campylobacter fetus*; Bovine; Vaccines

1. Introduction

Bovine trichomonosis and campylobacteriosis are two sexually transmitted diseases caused, respectively, by *Tritrichomonas foetus* (*T. foetus*) [1] and *Campylobacter fetus* subspecies *venerealis* (*C. fetus venerealis*), subspecies *venerealis* biotype *intermedius* (*C. fetus venerealis intermedius*) or *C. fetus* subspecies *fetus* (*C. fetus fetus*) [2]. In bulls, *T. foetus* and *C. fetus* are confined to the epithelial surface of the gland penis, prepuce, and urethra, where they establish chronic infection without causing observable signs [1,2]. In cows, the micro-organisms persist in the genital secretions and cause early embryonic death, transient infertility, uterine discharge, occasional pyometra, and sporadic abortions [1,2]. In both diseases, initial conception rates are normal but decrease by 70–120 days of gestation [1,2]. Infection by *C. fetus* is unknown in many regions, including parts of North America and the United Kingdom [3]. In addition, little is known about the prevalence of dual infection, although ranch bulls in Argentina had a dual infection rate as high as 11.6% [4]. Thus, areas with extensive cattle raising and natural breeding, such as Latin-America, Africa, Asia, western North America, and some countries of Europe, still have a high prevalence of trichomonosis and campylobacteriosis [4–6].

Because infected animals show no overt signs, identification, and separation of infected animals is difficult. Moreover, legally imposed herd treatments are impractical for *C. fetus*, and without efficacy for *T. foetus*; and artificial insemination, commonly recommended to avoid venereal disease, is impractical under most range conditions. Consequently, since both diseases are similar in their pathogenesis, prevalence, and economic effect, the development of effective dual prophylactic methods has considerable appeal. Vaccines against *C. fetus* conferred at least partial protection against experimental genital infection [7–9]. Regarding *T. foetus*, whole cell vaccines reduced the number of infected females, reproductive losses, and the duration of the genital infection under both experimental and natural challenge conditions [10–13]. On the other hand, some commercial vaccines against *C. fetus* under field conditions were without efficacy [14,15], and others against *T. foetus* were ineffective either in cows [16] or in bulls older than 5 years [17]. At the present time, there is only one vaccine for *T. foetus* and *C. fetus* which is commercially available; although its efficacy in the face of experimental challenge with both pathogens is not known, and the efficacy of dual vaccines under natural simultaneous infection with both micro-organisms was never determined. Finally, that *T. foetus* and *C. fetus* are extracellular and non-invasive encourages the use of mucosal immunization [10,18], which experimentally elicited genital IgA [18–20]; although its performance under natural conditions remains unknown.

The objective of the present study was to investigate, under natural and controlled conditions, reproductive performance, infection status, and serological immune response

in heifers vaccinated with dual vaccines (containing *T. foetus* and *C. fetus* antigens), one experimental and the other commercial. In addition, a new vaccination protocol with different routes of immunization near and during the breeding period was tested.

2. Materials and methods

2.1. Animals

Sixty-two post-pubertal Aberdeen Angus, Hereford, and crossbreed heifers, 18–24 months old, were purchased and identified with ear tags. Selection of females insured that there had been no exposure to *T. foetus* or *C. fetus* and they had cyclic ovarian activity. A total of 21 heifers were vaccinated with the commercial immunogen (group 1) with antigens of *T. foetus*, *C. fetus venerealis*, and *Leptospira canicola*, *grippotyphosa*, *icterohaemorrhagiae* and *pomona* (Trichguard V5L, Fort Dodge, USA). Another 20 heifers were immunized with an experimental vaccine (group 2) containing *T. foetus* and *C. fetus* subspecies antigens according to previous publications [10,21]. In brief, *T. foetus* was originally isolated from a cow with pyometra, and a clone obtained by picking colonies from agar plates [22] was grown in liver infusion medium containing 10% of fetal calf serum [10]. Strains of *C. fetus fetus*, *C. fetus venerealis intermedius*, and *C. fetus venerealis* were obtained from aborted heifers, grown onto Skirrow agar plus 100 mg/mL of Cycloheximide, incubated at 37 °C in a micro-aerobic atmosphere, and typed by biochemical tests [21,23,24]. The cell suspensions of both organisms were killed by the addition of formaldehyde at 0.5% (v/v), and the vaccine was prepared by emulsifying 30:70 volumes of the cell suspension and an oleo adjuvant consisting of Marcol 52, Arlacel C, and Tween 80 [10]. The remaining 21 heifers served as unvaccinated control (group 3) and received adjuvant alone. Heifers from groups 1 and 3 received subcutaneous (sc) treatment with the respective preparation at 60 and 30 days before the 90-day breeding period began (−60 dsb and −30 dsb (days from start of breeding period)). Heifers from group 2 were immunized by two routes: sc, at −30 dsb and +11 dsb, and intravaginal (i.v.), injected at lateral wall of vagina into submucosa, at −9 dsb. The sc doses contained 1.62×10^8 of *T. foetus*, 16 mg of *C. fetus fetus*, 16 mg of *C. fetus venerealis*, and 8 mg of *C. fetus venerealis intermedius*, in a total volume of 8 mL. The i.v. doses contained 4.05×10^6 of *T. foetus*, 0.4 mg of *C. fetus fetus*, 0.4 mg of *C. fetus venerealis*, and 0.2 mg of *C. venerealis intermedius*, in a total volume of 0.2 mL.

2.2. Challenge

All heifers were exposed to infection with *T. foetus* and *C. fetus venerealis* by mating them with two naturally infected bulls over a 90-day breeding period (from 0 dsb to +90 dsb). The bulls were Aberdeen Angus, 5-year-old and deemed to have satisfactory breeding status on the basis of physical soundness, scrotal circumference, and semen examination. Each bull had a *T. foetus* and *C. fetus* venerealis-positive culture for 5 weeks prior to the breeding period, and genital-negative culture for *Haemophilus somnus* and negative serology test for brucellosis. Complementarily, at day +39 dsb, 10 heifers from group

1 (group 1a), 10 from group 2 (group 2a), and 11 from group 3 (group 3a) were supplementally exposed to both infectious agents. Each heifer of additionally challenged subgroups: (a) received an inoculum in vaginal lumen of 1×10^8 live *C. fetus venerealis* cells and 4×10^6 live *T. foetus* cells. The rest of the animals, 11 from group 1 (group 1b), 11 from group 2 (group 2b), and 10 from group 3 (group 3b), were challenged only by natural service to the infected bulls.

2.3. Genital sampling for culture and pregnancy test

Vaginal mucus samples, before and during the breeding period and until 96 days after its end (day +186 dsb) as well as preputial smegma samples of the bulls throughout the 90-day breeding season, were periodically collected by aspiration and cultured to detect *T. foetus* and *C. fetus*. For *T. foetus*, genital samples were cultured in liver infusion medium with antibiotics at 37 °C and examined under a microscope (200–400×) daily for 1 week after collection [10]. For *C. fetus*, samples were transported onto Cary Blair medium [21,23], inoculated onto modified brucellosis broth with antibiotics (Bacitracin 15 IU/mL, Novobiocin 5 μg/mL, Polymyxin B 1 IU/mL, and Cycloheximide 50 μg/mL) and incubated for 2 days at 37 °C in a micro-aerobic atmosphere [21,23]. Superficial growth was transferred onto Skirrow agar plus 100 mg/mL of Cycloheximide and incubated in the same atmosphere for 7 days [21,23]. Culture from preputial samples was processed for *C. fetus* as explained above, but the step of previous enrichment was not done. Morphology and biochemical characterization of *C. fetus* was performed [24]. To determine pregnancy status and fetal loss, a transrectal examination was done once monthly for 330 days, starting 30 days after the onset of breeding.

2.4. Serum antibody response

Sera from all animals were periodically sampled to determine systemic *T. foetus*-IgG by ELISA, as previously described [25] with minor modifications [10]. In brief, 96-well micro-titre plates (Immunoblot, Dynatech, VA, USA) were pre-coated with whole *T. foetus* cells (50 μL at 10^6/mL in PBS (phosphate-buffered saline solution)) and blocked with 3% gelatin (Difco Laboratories, Detroit, MI, USA) in PBS. An aliquot of serum (100 μL per well) was diluted at 1:1,000 in 0.1 M PBS with 0.05% (v/v) Tween 20 and 2 mg/mL of gelatin (PBS Tw-g) before a 30-min incubation at 37 °C. The plates were emptied and washed (3×) in PBS Tw-g. Peroxidase-labeled rabbit anti-bovine whole IgG (Sigma, St. Louis, MO, USA) at 1/5,000 in PBS Tw-g was added (100 μL per well) and incubated 30 min at 37 °C. Plates were then emptied and washed (3×) in PBS Tw-g. For color development [2.2′-azino-die(3-etilbenzotiazolin sulfonate)] (ABTS) (Sigma, St. Louis, MO, USA) was used. A stock solution of 40 Mm ABTS was diluted 1:4 in 0.05 M citric acid pH 4.5 with 0.006% hydrogen peroxide and added (100 μL per well) [10]. After 4 min, the reaction was stopped (50 μL per well) with 2 M sulfuric acid and the optical density at 205 nm was read on an ELISA plate reader [10].

Sera were also collected to detect systemic *C. fetus*-IgG by ELISA test, as described [26] with minor modifications [15]. Strains of *C. fetus fetus*, *C. fetus venerealis intermedius*, and *C. fetus venerealis*, used as antigen, were grown as it was described above and

colonies transferred to 0.5% normal saline for 1 h, centrifuged, and washed (2×) with PBS [26]. Then, it was diluted in 0.5 M potassium chloride with 0.1% cysteine hydrochloride, sonicated on ice (Sonifier 250 Branson Sonic Power, USA) for 10 min at 60% power, and centrifuged (3×) at 20,000 *g* for 15 min [15]. The antigen pellet was re-suspended in carbonate buffer (0.05 M; pH 9.6), and protein concentration determined (Micro BCA Protein Assay Reagent Kit, Pierce, USA). The optimal antigen concentrations per well, determined by checkerboard titration against positive and negative control serum, were found to be 87. 4, 175, and 116.6 μg of protein of *C. fetus fetus*, *C. fetus venerealis intermedius*, and *C. fetus venerealis* antigen, respectively. An aliquot of serum (100 μL per well) at 1:500 in PBS Tw-g was incubated for 2 h at 37 °C and then the plates were washed (3×) in PBS Tw-g. Peroxidase-labeled rabbit anti-bovine IgG immunoglobulins (100 μL per well) at 1:5,000 in PBS Tw-g was added and incubated for 2 h before the plates were washed (3×) in PBS Tw-g. Color was developed with (ABTS) (Sigma, St. Louis, MO, USA) and the reaction at 4 min was read on an ELISA plate reader as described for *T. foetus*.

In both ELISA, all the samples were done in duplicate, and positive and negative control samples were included in each plate to calculate the ELISA value (EV) and to correct variation among plates [EV= ((mean sample OD − mean OD of negative control on the same plate)/(mean OD of positive control on the same plate − mean OD of negative control on the same/plate)) × 100] [10,26].

As positive controls, serum from five heifers subcutaneously immunized weekly for 14 weeks with whole formalized *T. foetus* cells [10], and another five heifers immunized in the same way with the mentioned three formalized *C. fetus* subspecies were used [15]. As negative controls for *T. foetus* and *C. fetus*, serum samples were collected from non-infected and non-exposed virgin heifers [10].

2.5. Statistical analysis

Differences were accepted as statistically significant at the 95% confidence limit ($P < 0.05$). Data regarding the clearance of *T. foetus* or *C. fetus* from the vagina of immunized and control heifers and pregnancy performance were analyzed by Chi-square or Fisher's exact probability test for differences among groups and within the indicated parameters [10]. For ELISA, corrected absorbencies were compared for contrasts among groups by mixed procedure of SAS for dependent samples [10,15].

3. Results

3.1. Culture and reproductive performance

The majority (30/31) of supplementary *T. foetus* challenged heifers (groups 1a, 2a, and 3a) became infected (Table 1). However, vaccinated heifers (groups 1a and 2a) had a shorter duration of infection and a lower number of positive cultures than unvaccinated heifers (group 3a) from day +51 dsb to day +149, with differences at days +86 dsb and +123 dsb (Table 1). No significant difference in the number of positive cultures was found

Table 1
Results of *T. foetus* and *C. fetus venerealis* cultures of vaginal mucus and reproductive performance in heifers supplementally exposed to both micro-organisms by intravaginal inoculation at day +39 (treatment (a))

Number	Days from start of breeding period (+dsb)														
	23		39		51		72		86		123		149		PS
	Tf	*Cfv*	*Tf*	*Cfv*	*Tf*	*Cfv*	*Tf*	*Cfv*	*Tf*	*Cfv*	*Tf*	*Cfv*	*Tf*	*Cfv*	
1a # 1	−	−	−	−	+	−	+	−	+	−	−	−	−	−	NA
1a # 2	−	−	−	−	+	+	+	−	+	−	−	−	−	−	np
1a # 3	−	−	−	−	+	−	+	−	+	−	−	−	−	−	p
1a # 4	−	−	−	+	+	+	−	−	−	−	−	−	−	−	p
1a # 5	−	−	−	−	+	−	+	−	−	−	−	−	−	−	np
1a # 6	−	−	−	−	+	−	+	−	+	−	−	−	−	−	p
1a # 7	−	−	−	−	+	−	+	−	−	−	−	−	−	−	p
1a # 8	−	−	−	−	+	−	+	−	−	−	−	−	−	−	p
1a # 9	−	−	−	−	+	−	+	−	+	−	+	−	−	−	np
1a # 10	−	−	−	−	+	+	+	+	−	−	−	−	−	−	np
	0/10	0/10	0/10	1/10	10/10	3/10	9/10	1/10	5/10	0/10	1/10	0/10	0/10	0/10	5/9
2a # 1	−	−	−	−	+	+	+	−	−	−	−	−	−	−	p
2a # 2	−	−	−	−	+	−	+	−	+	−	−	−	−	−	p
2a # 3	−	−	−	−	+	−	−	−	−	−	−	−	−	−	p
2a # 4	−	−	−	−	+	−	+	−	+	+	−	−	−	−	p
2a # 5	−	−	−	+	+	−	+	−	+	−	−	−	−	−	p
2a # 6	−	−	−	+	+	−	+	−	+	−	−	−	−	−	np
2a # 7	−	−	−	−	−	−	−	−	−	−	−	−	−	−	p
2a # 8	−	−	−	−	+	+	+	−	−	−	−	−	−	−	p
2a # 9	−	−	−	−	+	+	+	−	+	−	−	−	−	−	p
2a # 10	−	−	−	−	+	+	+	−	+	−	−	−	−	−	np
	0/10	0/10	0/10	2/10	9/10	4/10	8/10	0/10	6/10	1/10	0/10	0/10	0/10	0/10	8/10
3a # 1	−	−	−	−	+	−	+	−	+	−	−	−	−	−	p
3a # 2	−	−	−	−	+	+	+	+	+	−	+	−	−	−	np
3a # 3	−	−	−	−	+	−	+	−	+	−	−	−	−	−	p
3a # 4	−	−	−	+	+	+	−	+	+	+	+	+	+	−	np
3a # 5	−	−	−	−	+	+	+	−	+	−	−	−	−	−	p
3a # 6	−	−	−	+	+	+	+	−	+	+	+	+	−	−	np
3a # 7	−	−	−	+	+	+	+	+	+	+	−	−	−	−	p
3a # 8	−	−	−	−	+	+	−	−	−	+	−	−	−	−	p
3a # 9	−	−	−	−	+	+	+	−	+	+	+	−	+	−	np
3a # 10	−	−	−	−	+	+	+	+	+	+	+	+	+	−	np
3a # 11	−	−	−	−	+	+	+	−	+	−	−	−	−	−	p
	0/11	0/11	0/11	3/11	11/11	9/11	9/11	4/11	10/11	6/11	5/11	3/11	3/11	0/11	6/11

Heifers 1a were vaccinated with a commercial vaccine; 2a with an experimental vaccine; and 3a were unvaccinated controls. Heifers were serviced by *T. foetus* and *C. fetus venerealis*-infected bulls from day 0 dsb to day 90 dsb. *Tf*: Tritrichomonas foetus; *Cfv*: *Campylobacter fetus venerealis*; PS: Pregnancy status p: pregnancy np: no pregnancy NA: not available; Breeding period: from day 0 dsb to day + 90, critical risk time at day + 123 dsb.

between vaccinated heifers (groups 1a and 2a) (Table 1). Only naturally challenged heifers from group 3b had a higher proportion of infected heifers than vaccinated heifers (groups 1b and 2b) from days +51 dsb to +149 dsb, but without statistical difference (Table 2). The number of only naturally challenged heifers which remained uninfected throughout the study was high in vaccinated groups (1b and 2b), though without statistical difference with those unvaccinated heifers (3b) (Table 3). In addition, amongst uninfected heifers, 7/8 of group 2b, 2/7 of group 1b, and 2/4 of group 3b (2/4) became pregnant but without significant differences. At day +123 dsb, more *T. foetus*-positive heifers were found in group 3 (two from 3a and two from 3b) followed by group 1 (one from 1a and two from 1b) and group 2 (one from 2b), all these heifers resulting non-pregnant (Tables 1 and 2). With respect to heifers supplementally infected with *C. fetus venerealis*, unvaccinated heifers (group 3a) had higher infection rates than vaccinates (groups 1a and 2a) from days +51 dsb to +149 dsb (Table 1) with statistical differences from days +51 dsb to +86 dsb. No statistical difference was found between vaccinated groups 1a and 2a in the proportion of supplementally *C. fetus*-infected heifers. The number of additionally challenged heifers that remained uninfected for *C. fetus* throughout the study was high in group 1a, followed by group 2a, and group 3a; almost all of these uninfected females (9/12) became pregnant (Tables 1 and 3). In general, heifers challenged by only natural service had a lower rate of infection than those additionally inoculated. Regarding only naturally challenged females, group 3b had a higher proportion of infected animals than vaccinated groups 1b and 2b from days +23 dsb to +149 dsb, with statistical differences at days +23 dsb, +72 dsb, and +86 dsb (Table 2). Vaccinated groups (1b and 2b) were similar in the number of infected heifers (Table 2). Nevertheless, the number of uninfected heifers in those only naturally challenged was higher in group 2b than 1b ($P < 0.052$), and five of six of group 2b became pregnant versus only one of four of group 1b ($P < 0.10$) (Table 3). With respect to simultaneous *C. fetus venerealis* and *T. foetus* infection amongst additionally inoculated heifers, samples with dual infection were 20/77 in group 3a, versus 4/70 in group 1a and 4/70 in group 2a (Table 1). Amongst only naturally serviced heifers, samples with dual infection were 12/70 in group 3b, versus 3/77 in group 1b, and 2/70 in group 2b (Table 2). The total proportion of positive samples was statistically higher in group 3 than in the vaccinated groups 1 and 2, without statistical differences between the vaccinated groups (Tables 1 and 2). The number of samples that were never culture-positive for dual infection was statistically higher in heifers only naturally challenged than supplementally inoculated, and many of these uninfected heifers remained pregnant (Table 3). Regarding only naturally challenged heifers, those from group 2b had a higher proportion of never infected animals than group 1b (P 0.52) and group 3b (P 0.17) (Table 3). The occurrence of dual positive cultures at critical risk time (day +123 dsb) was only observed in heifers from group 3 (three from group 3a and one from group 3b), and it was associated with a negative pregnancy result because all these heifers were not pregnant (Tables 1 and 2).

The general rate of pregnant heifers was highest in group 2, followed by groups 1 and 3 (Tables 1 and 2). However, these values were not significantly different among groups (2a versus 1a: $P < 0.51$; and 2b versus 1b: $P < 0.50$; 2a versus 3a: $P < 0.44$; and 2b versus 3b: $P < 0.10$; and finally, 1a versus 3a: $P < 0.68$; and 1b versus 3b: $P < 0.61$). No abortion or fetal loss was detected in any group.

Table 2
Results of *T. foetus* and *C. fetus* venerealis cultures of vaginal mucus and reproductive performance in heifers challenged only by natural service to *T. foetus* and *C. fetus venerealis*-infected bulls from day 0 dsb to day 90 dsb (treatment (b))

	Days from start breeding period (+dsb)														
	23		39		51		72		86		123		149		PS
	Tf	*Cfv*	*Tf*	*Cfv*	*Tf*	*Cfv*	*Tf*	*Cfv*	*Tf*	*Cfv*	*Tf*	*Cfv*	*Tf*	*Cfv*	
1b # 1	–	–	–	–	–	+	–	–	+	+	+	–	+	–	np
1b # 2	–	+	–	+	–	+	+	+	–	–	–	–	–	–	p
1b # 3	–	–	–	–	–	+	–	–	–	–	–	–	–	–	NA
1b # 4	–	–	–	–	–	+	–	–	–	–	–	–	–	–	np
1b # 5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	np
1b # 6	+	–	+	+	–	+	–	–	+	–	+	–	–	–	np
1b # 7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	np
1b # 8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	NA
1b # 9	–	+	–	+	–	+	+	–	+	–	–	–	–	–	p
1b # 10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	p
1b # 11	–	–	–	–	–	+	–	–	–	–	–	–	–	–	p
	1/11	2/11	1/11	3/11	0/11	7/11	2/11	1/11	3/11	1/11	2/11	0/11	1/11	0/11	4/9
2b # 1	–	–	–	–	–	+	–	–	+	–	+	–	–	–	np
2b # 2	–	–	–	+	–	+	+	+	+	+	–	–	–	–	np
2b # 3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	p
2b # 4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	np
2b # 5	–	–	–	–	–	+	–	–	–	–	–	–	–	–	p
2b # 6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	p
2b # 7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	p
2b # 8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	p
2b # 9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	p
2b # 10	–	–	–	+	–	–	–	–	–	–	–	–	–	–	p
	0/10	0/10	0/10	2/10	0/10	3/10	1/10	1/10	2/10	1/10	1/10	0/10	0/10	0/10	7/10
3b # 1	–	+	–	+	+	+	+	–	+	+	+	–	+	–	np
3b # 2	–	–	–	+	+	+	+	+	+	+	+	–	–	–	np
3b # 3	–	+	–	+	–	–	–	+	+	+	–	–	–	–	np
3b # 4	–	+	–	–	–	+	+	+	+	+	–	–	–	–	np
3b # 5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	p
3b # 6	–	–	–	–	–	+	–	+	+	+	+	+	+	+	np
3b # 7	–	+	–	+	–	+	–	+	–	+	–	–	–	–	np
3b # 8	–	–	–	–	–	+	–	–	–	–	–	–	–	–	NA
3b # 9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	p
3b # 10	–	–	–	–	–	+	+	+	–	–	–	–	–	–	np
	0/10	4/10	0/10	4/10	2/10	7/10	4/10	6/10	5/10	6/10	3/10	1/10	2/10	1/10	2/9

Heifers 1b were vaccinated with a commercial vaccine, those 2b with an experimental vaccine and those 3b unvaccinated controls. *Tf*: *Tritrichomonas foetus*; *Cfv*: *Campylobacter fetus venerealis*; PS: pregnancy status; p: pregnancy; np: no pregnancy; NA: not available; breeding period: from day 0 to day +90, critical risk time at day + 123 dsb.

3.2. Systemic antibody responses

Essentially identical serological patterns were found between heifers which were only naturally serviced and additionally inoculated with *T. foetus* and *C. fetus* antigens (Figs. 1–4). In the case of *T. foetus*, heifers of group 2 had higher EV values than groups 1 and 3 from days −9 to +123, reaching the highest values at day +23 dsb (Fig. 1). These IgG responses of group 2 were statistically higher than group 3 from days −9 dsb to +123 dsb, and than group 1 at day −9 dsb and from days +23 dsb to +86 dsb (Fig. 1). Statistical differences between groups 1 and 3 were present at days −30 dsb, +11 dsb, +23 dsb, and +123 dsb. With regard to *C. fetus fetus*, IgG levels of group 2 were statistically higher compared to groups 1 and 3 beginning about 2 weeks after the first dose (day −9 dsb) until the day +186 dsb, with peak at day +86 dsb. Between groups 1 and 3, the antibody level was statistically different only at days −9 and +11 dsb (Fig. 2). For *C. fetus venerealis intermedius*, group 2 showed statistically higher IgG values than groups 1 and 3 from the day +23 dsb until the day +186 dsb with peak at day +23 dsb (Fig. 3). Groups 1 and 3 had no significant difference in the antibody responses, except on day −9 dsb (Fig. 3). In the case of *C. fetus venerealis*, group 2 presented a lower systemic response than those observed for the other *C. fetus* antigens with maximum values from the day −9 dsb to +72 dsb without a clear

Table 3
Number of heifers remaining uninfected throughout entire study period. Heifers from treatment (a) were supplementally exposed to both micro-organisms by intravaginal inoculation at day +39 and those from treatment (b) were challenged only by natural service to *T. foetus* and *C. fetus venerealis*-infected bulls from day 0 dsb to day 90 dsb

	Treatment	Group	Total uninfected	*P*	np	NA
C. fetus venerealis	a	1	7/10	4	2	1
		2	3/10	3	0	0
		3	2/11	2	0	0
	b	1	4/11	1	2	1
		2	6/10	5	1	0
		3	2/10	2	0	0
T. foetus	a	1	0/10	0	0	0
		2	1/10	1	0	0
		3	0/11	0	0	0
	b	1	7/11	2	3	2
		2	8/10	7	1	0
		3	4/10	2	1	1
Dual infection	a	1	0/10	0	0	0
		2	1/10	1	0	0
		3	0/11	0	0	0
	b	1	4/11	2	1	1
		2	6/10	5	1	0
		3	2/10	2	0	0

Heifers from group 1 were vaccinated with a commercial vaccine, those from group 2 with an experimental vaccine and those from group 3 unvaccinated controls. *p*: pregnancy np: no pregnancy; and NA: not available.

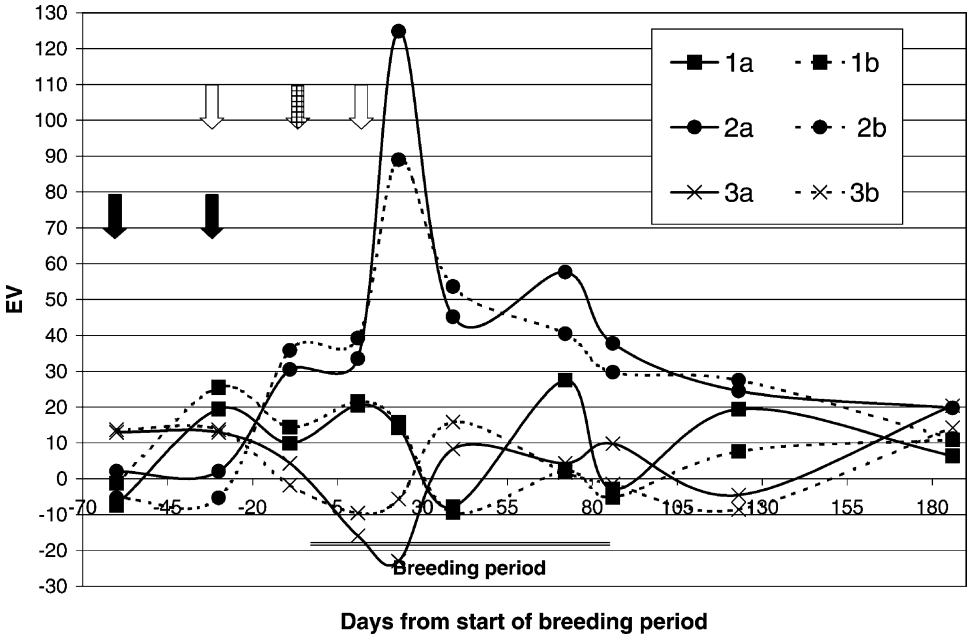

Fig. 1. Serum levels of *T. foetus* specific IgG from vaccinated (groups 1 and 2) and unvaccinated (group 3) heifers. Heifers from treatment: (a) were supplementally exposed to both micro-organisms by intravaginal inoculum at day +39 and those from treatment; (b) were challenged only by natural service to *T. foetus* and *C. fetus venerealis*-infected bulls from day 0 dsb to day 90 dsb. Dark arrows represent subcutaneous vaccinal doses in groups 1 and 3. White arrows represent subcutaneous vaccinal doses in group 2 and quadrille arrow represents vaginal vaccinal doses in group 2.

peak (Fig. 4). However, the IgG response in group 2 was significantly elevated over that of heifers in groups 1 and 3 (Fig. 4). There was no significant difference between groups 1 and 3, except on day −9 dsb.

4. Discussion

In the present study, two vaccines containing *T. foetus* and *C. fetus* antigens were evaluated in vivo in heifers simultaneously challenged with *T. foetus* and *C. fetus*. Heifers vaccinated with both immunogens, commercial and experimental, resisted or quickly cleared infection of both organisms from the genital tract, demonstrated by the statistically lower number of positive cultures from the middle of the breeding season to 2 months after cessation of breeding. Moreover, vaccinated heifers had a higher pregnancy rate and systemic immune response during and after the breeding season.

In a trial with limited numbers of cattle, the experimental dual vaccine, which was given a total of three times (including one dose into the vaginal wall), protected as well as or better than the existing commercial vaccine, which was given SQ on two occasions. Heifers in the experimental group had the highest systemic IgG response for *T. foetus* and the three

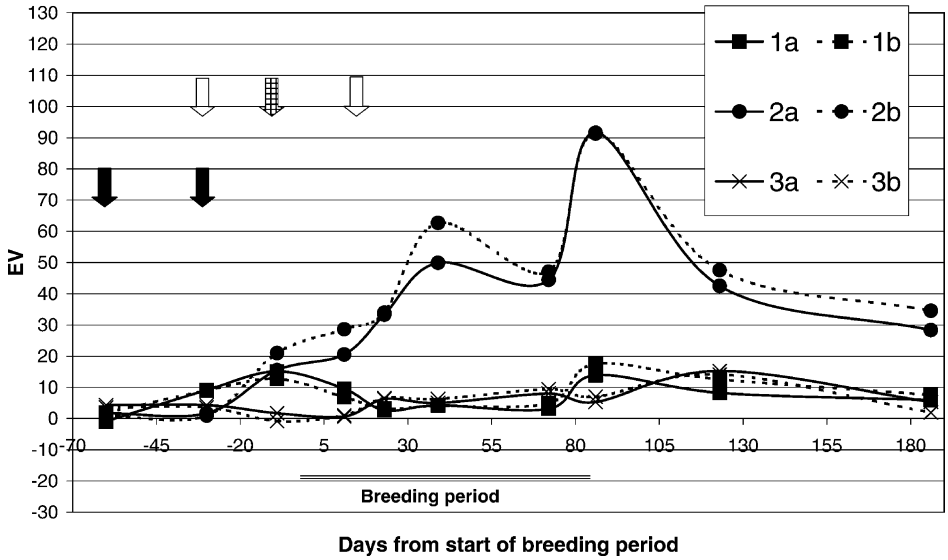

Fig. 2. Serum levels of *C. fetus fetus* specific IgG from vaccinated (groups 1 and 2) and unvaccinated (group 3) heifers. Heifers from treatment: (a) were supplementally exposed to both micro-organisms by intravaginal inoculum at day +39 and those from treatment; (b) were challenged only by natural service to *T. foetus* and *C. fetus venerealis*-infected bulls from day 0 dsb to day 90 dsb. Dark arrows represent subcutaneous vaccinal doses in groups 1 and 3. White arrows represent subcutaneous vaccinal doses in group 2 and quadrille arrow represent vaginal vaccinal doses in group 2.

subspecies of *C. fetus* from before the breeding season to at least 1 month post-breeding (they often had significantly higher titres than heifers that received the commercial vaccine). Heifers vaccinated with the experimental vaccine also had the best pregnancy rates. Since both vaccines contained similar types of antigens (killed cells of *T. foetus* and *C. fetus*), the vaccination protocol used for the experimental immunogen, different from the classic protocol for bovine venereal diseases, could be responsible for the differences between protocols. First of all, doses of the new vaccine plan were applied near and within the breeding period (SQ doses at days −30 dsb and +11 dsb, and intravaginal at day −9 dsb), while the traditional plan consists of two SQ doses at a 3-week interval prior to the breeding period. This revised schedule was based on the knowledge that, for these venereal diseases, the critical period appears to be 63–90 days of gestation [2,27,28], and under field conditions, the breeding period is usually 60–90 days. Hence, protection is necessary for a considerable interval after the onset of breeding, since heifers could theoretically receive an infective service from the first to the last day of the breeding period. However, considering the fact that in natural condition, a high proportion of cycling heifers are serviced during the first month of the 90-day-period, the critical risk time was established to be the last part of the breeding period and 1 month thereafter [10]. According to this assumption, the new vaccination plan succeeded in inducing lasting immune response from the last part of the breeding season until 1 month post-breeding. This extended response was essential to prevent infection, or if infection did occur, it elicited a competent antibody

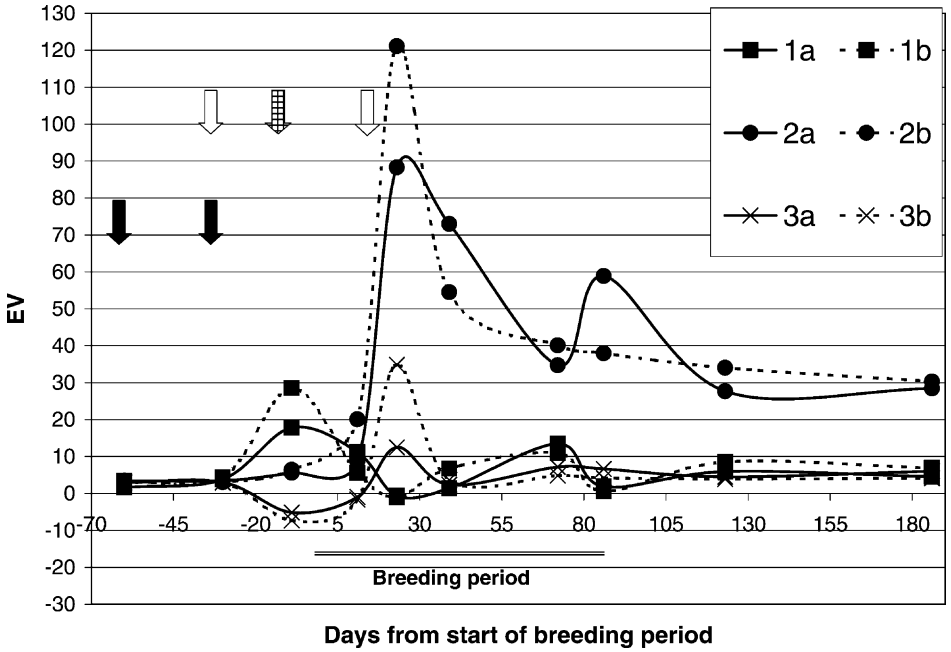

Fig. 3. Serum levels of *C. fetus* venerealis*intermedius* specific IgG from vaccinated (groups 1 and 2) and unvaccinated (group 3) heifers. Heifers from treatment: (a) were supplementally exposed to both micro-organisms by intravaginal inoculation at day +39 and those from treatment; (b) were challenged only by natural service to *T. foetus* and *C. fetus venerealis*-infected bulls from day 0 to day 90 dsb. Dark arrows represent subcutaneous vaccinal doses in groups 1 and 3. White arrows represent subcutaneous vaccinal doses in group 2 and quadrille arrow represents vaginal vaccinal doses in group 2.

response to clear the reproductive tract in time to sustain the pregnancy, as proposed for *T. foetus* [10]. In addition, at the critical time (day +123 dsb), vaccinated heifers had mostly negative cultures for both organisms, mainly those vaccinated with the experimental immunogen that had only one positive culture (2b# 1 for *T. foetus*), and so better pregnancy rates. Also, for *C. fetus*, the vaccination appeared to be more effective when given close to the time of breeding [29].

We concur with previous vaccine studies that the prevention of vaginal colonization by *C. fetus* [7,9,30] or *T. foetus* [10–13] was rare. However, we found a close association, 1 month after the breeding season, between infection (for *T. foetus*, *C. fetus*, or both) and non-pregnancy as well as no infection and pregnancy. What was previously proposed for *T. foetus* alone is confirmed under natural conditions for both micro-organisms [27,28] i.e., that if animals can be quickly cleared (before approximately 7 week of gestation/infection), the endometritis and fetal loss diminish, and pregnancies are not adversely affected. Based on our results, enhancing reproductive tract clearance of either or both organisms can ‘‘rescue’’ a threatened pregnancy. In other words, the success of a vaccine against these venereal diseases depends at least as much on its capacity to reduce the duration of infection as on its ability to actually prevent infection altogether. Contrarily, the traditional

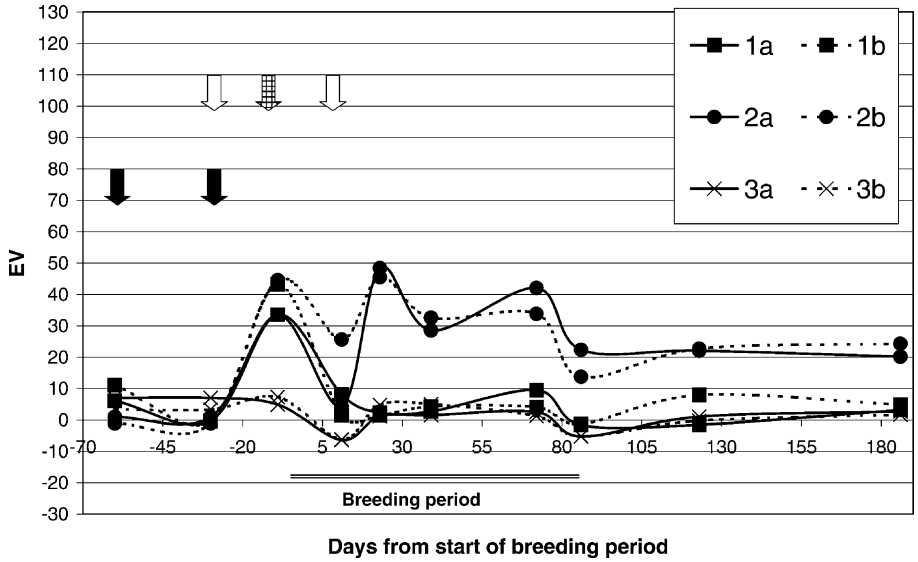

Fig. 4. Serum levels of *C. fetus venerealis* specific IgG from vaccinated (groups 1 and 2) and unvaccinated (group 3) heifers. Heifers from treatment: (a) were supplementally exposed to both micro-organisms by intravaginal inoculum at day +39 and those from treatment; (b) were challenged only by natural service to *T. foetus* and *C. fetus venerealis*-infected bulls from day 0 to day 90 dsb. Dark arrows represent subcutaneous vaccinal doses in groups 1 and 3. White arrows represent subcutaneous vaccinal doses in group 2 and quadrille arrow represent vaginal vaccinal doses in group 2.

vaccine plan would offer an immune response only for the first week of the breeding season since humoral response to a booster immunogen peaks and declines by approximately 21 days, and then the chance of heifers becoming infected increases and reproductive efficiency decreases [29].

Secondly, the employed combination of SQ and intravaginal vaccination routes for the experimental vaccine, different from the SQ route traditionally applied for venereal diseases [7,13,29], would be another reason to explain the better performance. Vaginal immunization, previously examined against *T. foetus* and *Ureaplasma diversum* either injected into the wall or inoculated the whole organism in the lumen, was included by us because it was the unique route that elicited genital IgA [18–20]. Genital IgA to venereal pathogens is of longer duration than IgG1, lasting at least 10 weeks after vaginal vaccination [18–20] and prevents re-colonization of the uterus in carriers [29,30]. However, SQ doses were included because IgG, mainly stimulated by systemic immunizations and apparently transported from serum into the genital secretions, is also involved in the protection of cows against venereal diseases [19,28,30]. Opsonizing properties of IgG, and mainly IgG1, helps to clear infection in concert with IgA of *C. fetus* [29,30] and *T. foetus* [18,19,28]. So, although genital immunoglobulins were not analyzed, but following the presented arguments and, as it was mentioned for *U. diversum* and *T. foetus* alone [18–20], for venereal diseases under field conditions, we used systemic vaccination followed by a booster genital vaccination.

The significantly higher IgG antibody response for *C. fetus fetus* and *C. fetus venerealis intermedius* in heifers vaccinated with the experimental immunogen was consistent with the lack of these *C. fetus* subspecies in the commercial vaccine (it contains *C. fetus venerealis*). The inclusion of the three *C. fetus* subspecies, and not only *C. fetus venerealis*, is important since biotypes *intermedius* and *fetus* were also associated with infertility in cattle and persistence in the genital tract of heifers [2,31]. The *C. fetus venerealis* strain included in the experimental vaccine had minor antigenicity; the concentration antigen was similar to the other *C. fetus* subspecies but elicited a lower antibody response. Further vaccine studies adding more dry weight or improved antigenic quality of *C. fetus venerealis* subspecies is needed to improve the present immunogen.

The supplemental vaginal challenge with *T. foetus* and *C. fetus venerealis* in our experiment was utilized in another *T. foetus*-only immunization trial [13]; we used an inoculation concentration coincident with *C. fetus* [32] and *T. foetus* [18] artificial infections. This allowed us to ensure infectivity (high incidence of dual infection in all groups) and to put the vaccines to an excessive challenge, where the experimental demonstrated a better performance, that was less evident in natural breeding only. In the natural breeding season, copulation temporarily reduces the number of organisms from the surface of the shaft of the penis, and thus the bull's ability to transmit *C. fetus* or *T. foetus* [3,10], masking the real effectiveness of the vaccines. The similar systemic antibody response between supplementally and naturally only challenged groups supported previous evidence for a lack of a relevant systemic response in *C. fetus* or *T. foetus* genital infections [2,10].

In conclusion, systemic priming and vaginal boosting with an experimental dual vaccine applied prior to natural or natural and supplemental challenge resulted in faster clearance of *T. foetus* and *C. fetus* infection before what is considered to be a critical point (70 days after conception and infection). In addition, heifers immunized with this experimental vaccine had a faster rise in systemic antibodies during and after the 90-day breeding period, as well as better pregnancy rates.

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