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Research paper

Evaluation of *Tritrichomonas foetus* infection clearance in heifers immunized with a single intravaginal dose of formaldehyde fixed strain B1 cells

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ABSTRACT

Vaccines against *Tritrichomonas foetus* have been shown to reduce the time of infection after natural or experimental exposure. The object of this study was to assess the protection against *T. foetus* infection conferred by a single vaginal instillation of formaldehyde fixed *T. foetus* cells. Aberdeen Angus virgin heifers were randomly allocated to 3 groups of 12 individuals to receive placebo or formaldehyde fixed *T. foetus* cells prepared following one of two procedures (formalin or freshly prepared solution) and six weeks later they were challenged with 10^6 *T. foetus* trophozoites. The median time for clearance among control heifers was 93.75 days while in animals immunized with formaldehyde fixed *T. foetus* it was 45 days. A single vaginal dose of cells fixed with fresh formaldehyde solution gave a rate of decay of infection per unit of time of 2.54 (CI 95% = 1.07;6.01).

1. Introduction

Trichomonosis is a sexually transmitted disease caused by the flagellate protozoan *Tritrichomonas foetus* in cattle. Infection is asymptomatic in bulls and linked to the development of a chronic condition, without affecting the quality of semen or the libido (Parsonson et al., 1974). The presence of *T. foetus* in cows may manifest through early fetal death, vaginitis, cervicitis, endometritis, salpingitis or postcoital pyometra. It is usually detected by extended breeding periods and lower pregnancy rates. The damage predicted in a herd with 20% prevalence of *T. foetus* is 14% reduction in calf annual crop (Rae, 1989).

T. foetus has a worldwide distribution and the incidence of the disease has significantly decreased in regions where artificial insemination is widely practiced (Bernasconi et al., 2014). In countries with extensive farming systems the systematic approach of trichomonosis control relies upon identification of infected bulls followed by their removal from the herd (Rae and Crews, 2006). The infection is recognized through microscopic test of cultures inoculated with preputial samples but molecular techniques are also available (OIE, 2018; Felleisen et al., 1998; McMillen and Lew, 2006; Oyhenart et al., 2013). Testing and culling is effective in improving reproductive efficiency in a herd (Ondrak, 2016). However to control the presence of *T. foetus* without substantial changes in management appears unlikely.

Vaccine development against *T. foetus* has been pursued since 1983 and progressed slowly because of the cost of maintenance and manipulation of cows, the absence of small animal models and the poor understanding of the pathogenesis (Baltzell et al., 2013 and references therein; Edmondson et al., 2017; Fuchs et al., 2017). *T. foetus* vaccines were mostly based on formalin fixed cells that were mixed with an adjuvant to be systemically delivered through 2–3 subcutaneous (or intramuscular) injections. Commercially available vaccines (eg. TrichGuard®, Boehringer Ingelheim Vetmedica, Inc., Tricovac®, Laboratorio Biológico Tandil, Argentina) are based on similar procedures and used in a similar way.

Vaccine efficacy experiments have been mainly designed to measure a raise in the rate of clearance of infection. Virgin heifers of European breeds can be experimentally infected with high certainty and *T. foetus* will be detectable in cervical vaginal mucus (CVM) samples for a period of 8–14 weeks (Baltzell et al., 2013 and references therein; Edmondson et al., 2017; Fuchs et al., 2017). The main symptom of *T. foetus* infection is low fertility, with an increase in the number of services per conception and the mean intercalving time (Bartlett, 1947; Clark et al., 1983a, 1986). Experiments designed to demonstrate that vaccination against *T. foetus* increases the calving rate were not succesful (Kvasnicka et al., 1992; Cobo et al., 2004; Edmondson et al., 2017). Experimental and commercial vaccines based on killed *T. foetus* trophozoites have been repeatedly shown to reduce the number of infected cows by shortening the period of genital infection of naturally or experimentally infected heifers (Baltzell et al., 2013; Fuchs et al., 2017). There is no vaccine capable of preventing the genital tract colonization by *T. foetus*.

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Whole cell based vaccines have been the most common form used in *T. foetus* immunization. Antigen concentration in the form of cell membranes or purified proteins (or glycolipids), have been shown to be effective but no more than whole cells (BonDurant et al., 1993; Voyich et al., 2001; Cobo et al., 2002). *T. foetus* cells in culture have been used without inactivation (alive) or have been killed with formalin (Clark et al., 1983b; Herr et al., 1991; Corbeil et al., 1998a; Cobo et al., 2002). There are no published studies ascertaining whether live or fixed *T. foetus* are better but the latter should be preferred from a safety and stability point of view. Formaldehyde fixation is the most used method in *T. foetus* vaccine manufacture. However there is great variation among fixation protocols and there is no information addressing the impact of formalin treatment on the quality of a vaccine (Kiernan, 2000).

Most of the currently available vaccines in a cattle vaccination scheme are applied via the parenteral route. A vaccine against *T. foetus* that will be applied via subcutaneous or intramuscular injection can be quickly incorporated in a general vaccination scheme. The commercial vaccines against *T. foetus* are offered in combination with *Leptospira* spp and *Campylobacter fetus* bacterins. The parenteral delivery of *T. foetus* antigens is associated with an increase of serum IgG antibodies against the pathogen but there is no change in IgA or IgG concentration in the CVM (BonDurant et al., 1993; Gault et al., 1995; Corbeil et al., 2001). Some vaccination schemes have included an epithelial delivery to reinforce immune response (Corbeil et al., 1998b; Voyich et al., 2001; Cobo et al., 2002, 2004; Fuchs et al., 2017). In these works there is no evidence that direct application of *T. foetus* antigens onto the cervical epithelium can stimulate the immune system and promote the defense from infection.

The aim of this study was to test if cervical priming with fixed *T. foetus* can prevent *T. foetus* infection. A single instillation of fixed *T. foetus* cells was given to heifers and, after experimental infection, the clearance kinetics was determined.

2. Materials and methods

2.1. T. foetus isolates and in vitro culture

T. foetus B1 strain used in this study is a clone obtained from an isolate from a cow with pyometra (Cobo et al., 2002). *T. foetus* was cultured for 24–48 h at 37 °C in liver infusion broth containing 1 g/L streptomycin, 100,0000 IU/L ampicillin and 10% v/v heat inactivated horse serum. A batch for immunization was obtained from a 250 mL of culture incubated at 37 °C until mid-log phase (24–36 h). The cell suspension was centrifuged at 1400 × *g* for 15 min and the pellet was washed twice with phosphate buffered saline buffer (PBS, 137 Mm NaCl, 10 mM phosphate, pH 7.4). Cells were fixed at a final concentration of 2×10^8 cells/ml in 0.5% formaldehyde for 15 min and immediately washed twice in PBS. Formaldehyde containing fixing solutions were prepared by dilution of 37% formalin (FA) to 4% in PBS or by dissolving paraformaldehyde (PFA) powder to the same concentration, in freshly prepared PBS heated at 60 °C while vigorously stirring. Both solutions were immediately used. Washed cells were diluted to a final concentration of 1×10^8/mL in PBS and 1 m L was used for instillation in the cranial vagina using a sterile Cassou pipette.

2.2. Cattle and experimental design

The experimental animals were 45 Aberdeen Angus virgin heifers (with no sexual exposure to bulls since weaning) 18–24 months old and weighting 330–380 kg. Animals were kept under extensive grazing conditions, in a herd free of brucellosis, campylobacteriosis and trichomonosis for more than ten years. The absence of *T. foetus* in the experimental animals was confirmed through culture of two CVM samples taken before the trial and 30 days after immunization. The experimental plan is depicted in Fig. 1. Heifers were randomly assigned to receive PBS (Control, n = 12), *T. foetus* B1 cells fixed with formalin solution (group FA, n = 12) or *T. foetus* B1 cells fixed with freshly prepared formaldehyde (group PFAn = 12). A group of 9 animals was used as infection control (Ci) that were neither immunized, nor infected. Randomization was performed with the "randbetween(1;4)" function in LibreOffice Calc (The Document Foundation) that returned a value for each ear tag identification number.

One month after immunization estrus cycle was synchronized in control, FA and PFA groups. For estrus synchronization heifers were given vaginal progesterone (500 mg) releasing devices (Zoovet, Santa Fe, Argentina) and 2 mg estradiol benzoate intramuscularly. Seven days later, progesterone releasing devices were extracted and 500 µg D-cloprostenol was injected. The following day every animal received 1 mg estradiol benzoate and 24 h later they were infected. Infection consisted of instilation of 1 mL of PBS containing 10^6 *T. foetus* B1 (> 99% viability) in the cranial vagina by using a Cassou pipette. Heifers were evaluated for clinical signs and inflammatory response every second week until the end of the trial at day 120 post infection (pi). The protocol and procedures used in this study were approved by the Animal Ethics Committee of INTA. Heifers were handled by trained personnel according to standards of good practices and conditions.

2.3. Sample collections

CVM samples were obtained fortnightly for a total of 4 months using a sterile Cassou pipette. Approximately 0,5 mL of CVM were immediately suspended in 3 mL of liver infusion broth media. Fractions of CVM samples were distributed in 2 mL sterile vials and, as soon as possible, frozen at −20 °C until PCR tests and ELISA quantification of anti-*T. foetus* IgA antibodies.

2.4. Quantification of anti-T. foetus IgA antibodies

The concentration of anti-*T. foetus* immunoglobulins was assessed by indirect ELISA assay as previously indicated (Fuchs et al., 2017). Briefly, 96-well plates 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 ethanol, each plate was incubated and stored at −20 °C until use. Each well was blocked with 100 µL of PBS containing 0.05% (v/v) Tween 20 and 1% (w/v) porcine skin gelatin (Sigma, St. Louis, MO) 183 (PBS-TG) for 2 h at 37 °C in agitation.

Samples of CVM were sonicated in an ice bath with a team Sonics Vibra Cell (Newtown, USA) at 80% power for 10 s and then diluted 1:100 and 1:1000 in PBS-TG. Mucus from animals hyperimmunized against *T. foetus* were used as positive controls and samples from heifers without contact with the parasite were used as negative controls (Fuchs et al., 2017).

Samples were tested in duplicates, and positive and negative controls were included in each plate. The presence of IgA was revealed through the binding of a rabbit anti-bovine IgA conjugated to horseradish peroxidase (Bethyl Lab., Texas, USA) diluted 1:4000 in PBS-TG. For color development, TMB (3,3′,5,5′-Tetramethylbenzidine, Sigma, St. Louis, MO) was used. After 10 min, the reaction was stopped with 50 µL of 2 M sulfuric acid and optical densities (OD) were read on an ELISA microplate reader (Multiskan EX, Labsystems, Helsinki, Finland) at 405 nm (Fuchs et al., 2017). The relative absorbance values were obtained as follows: Abs corrected = (Abs test serum − Abs negative control)/(Abs Positive 197 Control − Abs negative control).

2.5. Polymerase chain reaction

The primer pairs TFR1-TFR2 were used for amplification of DNA from the trichomonad group and TFR3-TFR4 for specific amplification of *T. foetus* DNA (Oyhenart et al., 2013). PCR reactions were performed in 25 µL containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2.0 mM

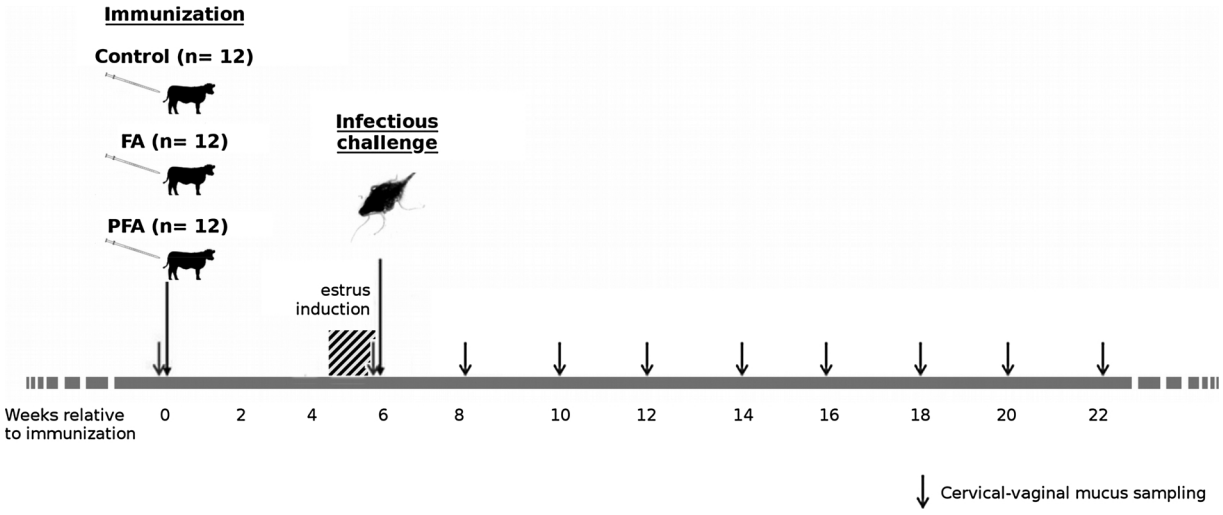

Fig. 1. Experimental scheme. Three groups of twelve cows were immunized by vaginal instillation of *Tritrichomonas foetus* killed with formalin (FA), *T. foetus* cells killed with fresh formaldehyde (PFA) or PBS only (Control). One month later, estrus was synchronized and all the cows were artificially infected with alive *T. foetus*. Cervical-vaginal samples were collected every two weeks for infection test and immunoglobulin quantification.

$MgCl_2$, 0.5 µM each primer (TFR1 and TFR2, or TFR3 and TFR4), 140 µM each dNTP and 2 U of Taq DNA polymerase (PB-L, Bernal, Argentina). Two to five µL of crude sonicated mucus sample were used as templates. Amplification comprised 36 cycles of denaturation at 207 94 °C for 30 s, annealing at 67 °C for 30 s and extension at 72 °C for 90 s, with a final extension step of 15 min at 72 °C. At the end of amplification 6 µL of loading buffer (50% glycerol, 11 mM EDTA, 0.017% SDS, 0.015% bromophenol blue in 3.3 mM Tris–HCl pH 8.0) were added and 10 µL of the sample were resolved in 2% agarose gels at 10 V/cm for 30 211 min. Gels were stained with 0.5 µg/ml ethidium bromide (Oyhenart et al., 2013).

2.6. Protein analysis

T. foetus cells were subcultured for 24 h, centrifuged, washed 3 times in PBS, counted and lysed in 1X SDS Laemmly buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue and 62.5 mM Tris HCl, pH 6.8). Replicates were fixed before lysis by adding formalin or freshly prepared formaldehyde solution at a final concentration of 4% and incubated for 0–24 h at room temperature. Treated cells were then washed 3 times in PBS, counted and suspended in 1X Laemmly buffer. A total of 5×10^3 cells were loaded in each lane and electrophoresed in 10% discontinuous SDS-PAGE. Gels were stained or transferred onto Hybond-P membranes (Amersham). For silver staining, gels were submerged in fixing solution (10% ethanol, 0.5% acetic acid) and were subsequently stained with 35 mM $AgNO_3$. After brief washing in H_2O, gels were incubated in developing solution (3% NaOH, 0.25% formaldehyde) and the reaction was stopped by submerging the gel in fixing solution (45% methanol/10% acetic acid). Blotted membranes were washed twice with distilled water, stained with 0.1% (w/v) Ponceau S in 5% acetic acid to verify uniform transfer and blocked 1 h with 5% nonfat dry milk in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.4. Bovine anti *T. foetus* or pre-immune sera were diluted at 1:1000. The secondary antibody was rabbit anti-bovine IgG (Fc specific) coupled to alkaline phosphatase (Sigma Aldrich) and it was diluted at 1:10,000. Three TBS-T washes were performed between steps. Reactive bands were detected after incubation in 5-bromo-4233 chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT Pomega, USA).

2.7. Statistical analysis

Statistical analysis was performed with the R software package (cran.r-project.org). Cumulative times to reach remissions were calculated by the Kaplan-Meier method. A sample size of 12 cows per group was determined to be sufficient to detect, with 83% power, a 60% reduction in *T. foetus* infection by week 7 after experimental infection (Chinn, 2000). The statistical significance of the obtained differences was tested with the log rank test. Hazard ratios were calculated by following Cox proportional model. Differences in immunoglobulin levels among the treatment groups were evaluated with Kruskal-Wallis test with post-hoc analysis of significant differences with Tukey-Kramer (Nemenyi) test. Correlation between remission times and immunoglobulin concentrations were analyzed 246 using Spearman rank coefficient.

3. Results

3.1. Effect of formaldehyde fixatives on T. foetus proteins

The effect of formaldehyde on *T. foetus* cells was studied in protein profiles. Proteins from untreated cells distributed in a typical pattern after migration through 10% acrylamide gels. Proteins from cells incubated for a short time in fixatives showed an increment of high molecular weight bands proportional to the concentration of FA or PFA (Fig. 2A). A long term exposure to the fixative was more evident when a stacking gel was stained altogether with the resolving gel section. While proteins in untreated extracts went throughout the stacking gels and evenly distributed in the resolving gel according to size, fixed proteins accumulated in the stacking gel as well as on top of resolving gels. The amount of high molecular weight adducts was higher in FA than in PFA treated cell extracts (Fig. 2B). Western blots probed with bovine anti-*T. foetus* serum also showed differences between control and fixed antigenic proteins. Non fixed showed most of the signal in a unique band of ~250 kDa and a faint signal at ~220 kDa. Among proteins treated with formaldehyde solutions, reactive proteins mostly accumulated at ~220 kDa and in a higher size band in the stacking gel that was absent in untreated cell extracts. The signal intensity of the most represented bands in formaldehyde treated extracts was consistently higher in FA than in PFA treated extracts. FA protein profiles showed also more adducts of high and low molecular weight (Fig. 2B).

3.2. T. foetus clearance

Two weeks after infection 12/12 cultures in the control group, 11/12 in the FA group and 10/12 in the PFA group were positive for *T.*

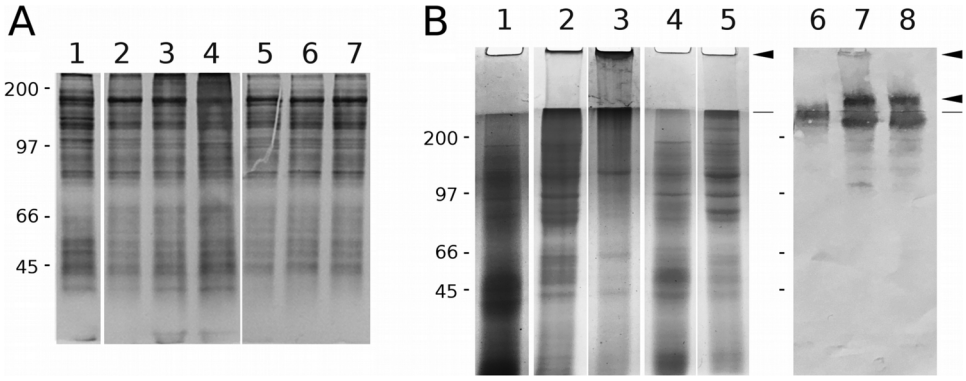

Fig. 2. Effect of formaldehyde solutions on *Tritrichomonas foetus* proteins. (A) Total *T. foetus* protein extracts either untreated (lane 1) or treated in 0,1%, 0,2% or 0,5% formalin in PBS (lanes 2–4) or in 0,1%, 0,2% or 0,5% fresh formaldehyde solution in PBS (lanes 5–7) before separation through 10% acrylamide gels. (B) Silver staining (left) and western blot (right) hybridized with bovine anti-*T. foetus* serum of *T. foetus* proteins directly suspended in Laemmly buffer (lanes 1 and 6), previously treated with 0,1% formalin for 15 min (lane 2) or 12 h (lanes 3 and 7); 0,1% fresh formaldehyde solution for 15 min (lane 4) or 12 h (lanes 5 and 8). A horizontal lane at right shows the frontier between stacking (top) and resolving gel (bottom). Arrowheads point to high molecular products that accumulate in the stacking gel.

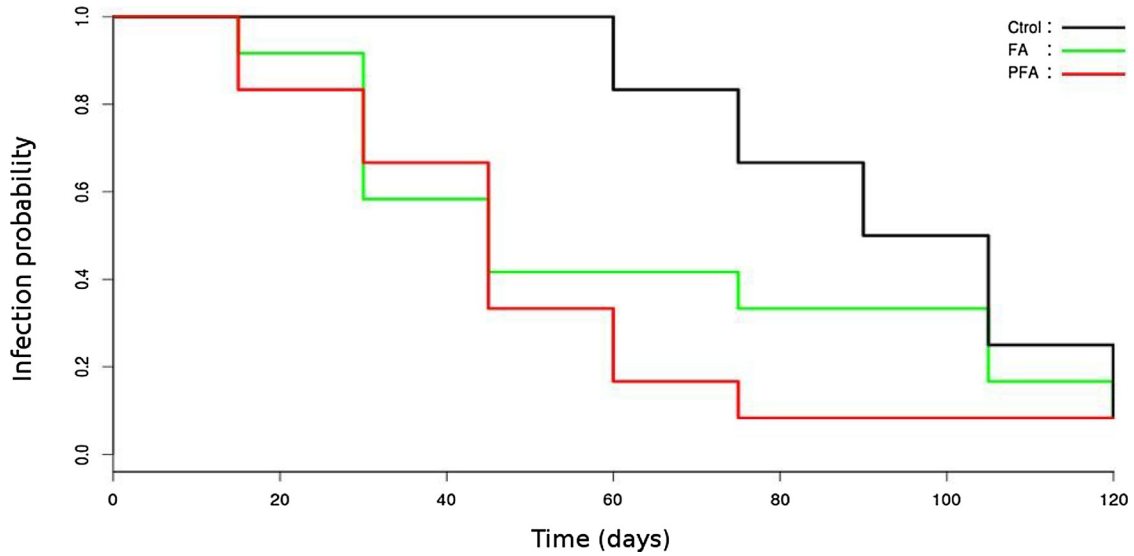

Fig. 3. Kaplan-Meier infection probabilities. K–M curves for*Tritrichomonas foetus* infection in groups of animals immunized by instillation with a single dosis of PBS (≡ Ctrol), formalin fixed cells (≡ FA), or cells fixed with fresh formaldehyde solution (≡ PFA) in the lumen of the vagina.

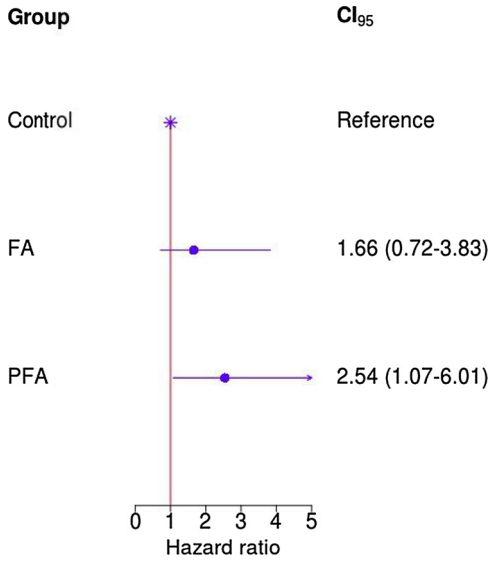

Fig. 4. Association between immunization and the rate of clearance of*Tritrichomonas foetus*. Forest plot for unadjusted Cox model. Circles indicate unadjusted hazard ratios for a given immunization treatment and horizontal lines indicate 95% 552 confidence intervals (CI.95) for the Control group: vehicle only, FA: formalin treated cells, PFA: fresh formaldehyde solution treated cells.

foetus. Direct microscopic observation of negative samples in the FA and the PFA group showed static trichomonad-like structures (5–8 μm long, elongated to round, rarely with external flagella). PCR amplification with TFR3-TFR4 primers gave a typical band of amplification of 347 bp in all three animals. Thus, all three samples were interpreted as positive. The persistence of the infection was variable among the groups. Culture results are shown in supplementary Table 1. All animals in the control group remained infected by day 42 p.i. thereafter showing regularly negative cultures. In every vaccinated group negative cultures were observed in samples 281 from day 27 after infection. By the end of the trial at day 120 p.i. 1 out of 12 animals was still infected in every group. Kaplan-Meier time-to-cure curves depict the rate of infection clearance (Fig. 3). The mean time for clearance among non vaccinated heiffers was 93.75 days while immunized animals showed mean values of 62.5 days in FA group and 48.7 days in PFA group. The median time for clearance among control heifers was 97.5 days while immunized animals in either FA or PFA group showed median values of 45.0 days. Generalized Wilcoxon test showed that, at least, one experimental procedure was significantly different from the control ($p = 0.0102$). Therefore vaccinated groups were 289 tested individually against control group. The difference between the FA treatment and the control group did not reach a significant difference. However the difference between the 291 PFA group and the control group can statistically significant ($p = 0.00652$).

Hazard ratios were used to measure the rate of clearance of infection by unit of time in every treated group as well as in the control

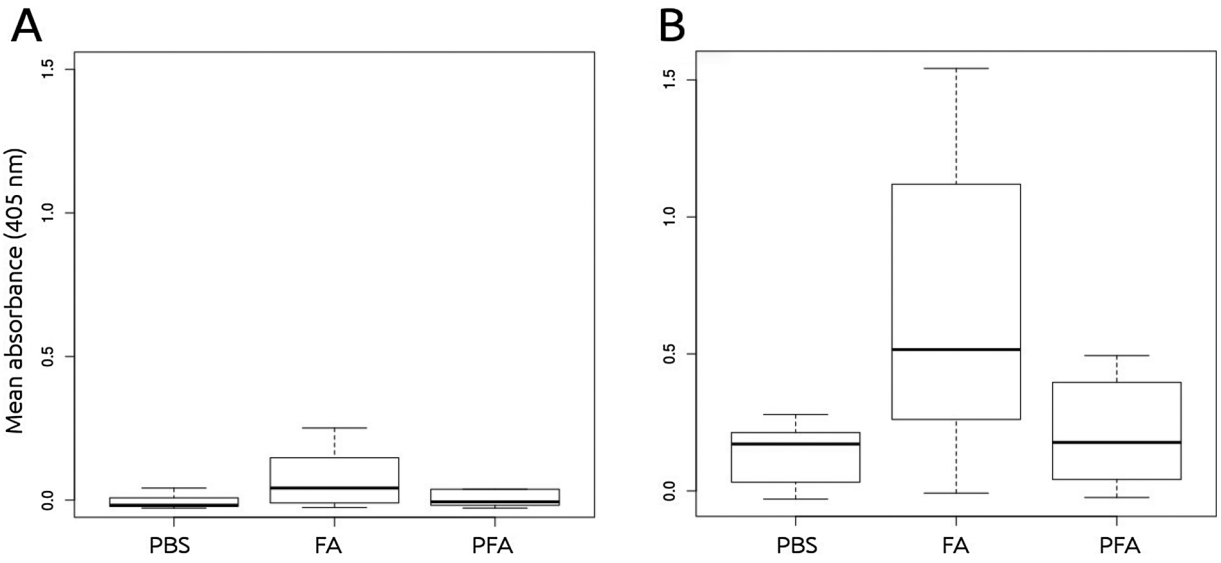

Fig. 5. Immunoglobulin A levels in cervical vaginal mucus. Box and wisker plot of IgA concentrations in cervical vaginal mucus from cows receiving a single vaginal dose of either placebo (PBS), *T. foetus* cells fixed with formalin solution (FA) or *T. foetus* cells fixed with fresh formaldehide solution (PFA). Measures were taken just before infection (day 42 after immunization) (A) and 45 days after infection (B).

group. As seen in Fig. 4 significant values for the rate of decay of infection per unit of time were found in the PFA group immunized with a single vaginal dose of cells fixed for a short time with freshly prepared formaldehyde solution. Hazard ratio in the FA group was 1.66 but the low limit of confidence interval was still close to the reference value (CI 95% = 0.72;3.83, p = 0.2393). A significant difference (p = 0.0338) was observed in the PFA group clearance, with a rate of 2.54 (CI 95% = 1.07;6.01) times the clearance observed in the control group. Evidence of non- proportional hazards was noted (p = 0.03) but the difference is supported by the mean 302 clearance time (48.7 days with PFA against 93.75 days in vehicle-only).

3.3. IgA concentration after intravaginal immunization and experimental infection

Basal concentrations of IgA were observed in animals without contact with the parasite. Similar values were observed in every animal before immunization. Samples obtained just before infection (42 days after immunization) showed IgA levels slightly higher in FA treated animals (Fig. 5A). However no significant increase in IgA was observed in the FA or the PFA group ($p = 0.3915$). Otherwise, an increase in IgA concentration was evident after experimental infection (Fig. 5B). Kruskal-Wallis test on day 45 p.i. showed significant differences between groups ($p = 0.043$) and post analysis suggested that FA immunization induced a significant raise in IgA concentration when compared to vehicle only treatment (p 314 = 0.034).

IgA level in CVM did not seem to be directly related to vaginal immunization with formaldehyde fixed cells. Spearman correlation analysis between mean IgA values after vaccination (infection time) and mean time for clearance in every group showed no significant value supporting this hypothesis. After infection, an inverse relationship between IgA levels and the rate of clearance of *T. foetus* can be predicted but Spearman rank correlation 321 analysis was still non significant ($\rho_s = -0.625$; $p = 0285$).

4. Discussion

After natural or experimental infection, the presence of *T. foetus* in the female genital tract can be easily demonstrated through standard culture technique. Depending on the inoculum, the *T. foetus* strain, the host breed, the health, nutritional and hormonal status, the infection period can extend from 70 days to more than a year (Herr et al., 1991; BonDurant et al., 1993). Experimental infection of Aberdeen Angus, Hereford and crossbred heifers with 10^6–10^7 *T. foetus* B1 cells has given mean infection times of approximately 70–105 days (Cobo et al., 2002, 2004; Fuchs et al., 2017). Immunization of heifers with fixed *T. foetus* cells have been shown to be 333 effective in reducing the time of pathogen residence in the host cervix (Kvasnicka et al., 1992; Herr et al., 1991; BonDurant et al., 1993; Corbeil et al., 1998a; Cobo et al., 2002, 2004; Fuchs et al., 2017). Two doses of fixed cells applied systemically can help to reduce clearance time of infection to half of the time observed in non immunized animals (Kvasnicka et al., 1992; Herr et al., 1991; Cobo et al., 2002, 2004). A third dose, intended to shorten this period of infection, has been used in several trials, while applied systemically, intravaginally or via nasal instillation (Corbeil et al., 1998b; Voyich et al., 2001; Cobo et al., 2002, 2004; Riccio et al., 2008; Fuchs et al., 2017). These works have not concluded that mucosal application of *T. foetus* antigens can promote the defense from infection. A great variation in test procedures and the low number of animals tested can explain this difficulty. Here, animals exposed to a single dose of fixed cells delivered in the cranial vagina cleared the infection in less than half the time needed by control animals. The median time of 45 days attained in two treatment procedures against 97 days in control animals is clearly indicative that the vaginal route is a valuable way to ameliorate *T. foetus* vaccination.

Fixatives have desirable as well as undesirable effects in vaccine preparation (Nencioni et al., 1991). Whole cells have to be inactivated while preserving its antigenic properties. Formalin inactivation is cheap and easy to carry out but it is not advisable as the active compound is not always present at the indicated concentration and excipients added to stabilize the solution can change the fixative properties (Kiernan, 2000; Metz et al., 2004). Freshly prepared solutions made by dissolving paraformaldehyde under heating at slightly basic pH are the only known way to have a certain amount of formaldehyde monomer that can give us an effective and reproducible fixation procedure. Formalin and fresh formaldehyde solutions showed similar results on *T. foetus* proteins as both procedures did change the total protein profiles observed in gel electrophoresis. Both fixatives were also shown to induce changes in the size of antigenic proteins. Moreover, evidence has been provided in the sense that treatment of *T. foetus* with formalin could have different effect on immunogenic properties than freshly prepared formaldehyde. Immunization with formalin treated cells induced an

increase of IgA secretion that was not observed in cows treated with vehicle or with fresh formaldehyde treated cells. The IgA response was variable and it was not directly related to the rate of clearance of *T. foetus*.

After infection with *T. foetus*, cows respond with a significant IgA (and IgG) antibody 366 response in vaginal secretions (BonDurant et al., 1993; Ikeda et al., 1995; Corbeil et al., 1998a; Fuchs et al., 2017). It is therefore thought that a specific IgA increase after infection contributes to clearing the infection and reestablishing fertility after a few weeks. However systemic, intranasal or intravaginal immunization with formalin inactivated *T. foetus* was never shown capable to increase vaginal IgA concentration (Voyich et al., 2001; BonDurant et al., 1993; Fuchs et al., 2017). There is still a need for a different *T. foetus* antigen preparation procedure.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2018.03.019.

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