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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

Gracia Martinez Florencia[a], Fuchs Lumila[b], Ramirez Rosana[c], Breccia Javier[a], Oyhenart Jorge[a,*]

[a] INCITAP, CONICET, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa, Santa Rosa, La Pampa, Argentina
[b] Instituto Nacional de Tecnología Agropecuaria (INTA), Anguil, La Pampa, Argentina
[c] CONICET, Universidad Nacional del Litoral, Paraje el Pozo, Santa Fe, Argentina

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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*.

* Corresponding author.
E-mail address: jorgeoyhenart@exactas.unlpam.edu.ar (O. Jorge).

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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