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Short communication

The route of *Besnoitia besnoiti* tachyzoites inoculation does not influence the clinical outcome of the infection in calves

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ABSTRACT

In a previous attempt, an experimental model of bovine besnoitiosis was established in calves that were intravenously inoculated with different doses of *Besnoitia besnoiti* tachyzoites. Despite the fact that all infected calves developed the acute stage of disease, only microscopic findings characteristic of chronic besnoitiosis were reported. In the present study, calves were inoculated by subcutaneous and intradermal routes with *B. besnoiti* tachyzoites with the aim of developing clinical signs and macroscopic lesions characteristic of chronic besnoitiosis.

Nine 3-month-old male calves were randomly distributed into three groups of three animals each. Next, 10^6 tachyzoites were inoculated by either the subcutaneous (G1) or intradermal route (G2). The negative control group (G3) was inoculated with PBS. Daily clinical monitoring and regular blood collection were performed. At 70 days post-infection (pi), animals were euthanized, and tissues were collected to investigate lesions and parasites.

Infected animals developed mild-moderate acute besnoitiosis characterized by lymphadenopathy from four days to 47 days pi, and sporadic fever peaks were only observed in one calf from G2. However, other clinical signs and macroscopic lesions characteristic of chronic besnoitiosis were not detected. Only nine tissue samples were *B. besnoiti*-DNA-positive, eight of which belonged to reproductive and respiratory tracts tissues from G1. Finally, the kinetics of the immune responses were similar in both infected groups. However, delayed and lower cellular and humoral immune responses were observed in G1 followed by G2 and were compared with intravenously inoculated calves. The differences observed among the three inoculation routes could be due to different effector mechanisms of the host early innate immune response against *B. besnoiti*.

Accordingly, the inoculation route of *B. besnoiti* tachyzoites does not significantly influence the clinical outcome of the infection in calves. Thus, a further refinement of this experimental model of bovine besnoitiosis is needed to reproduce macroscopic lesions characteristic of chronic stage disease.

1. Introduction

Besnoitia besnoiti is an apicomplexan protozoan responsible for bovine besnoitiosis. This chronic and debilitating disease is characterized by both systemic and local manifestations. In particular, low body score, skin lesions and reproductive failure are the major consequences of the infection. Indeed, males may develop infertility or even sterility and dams may occasionally abort, giving rise to substantial economic losses in the infected farms (Álvarez-García et al., 2014; Gutiérrez-Expósito et al., 2017). Unfortunately, there are neither effective drugs nor a licensed vaccine in Europe. Thus, the disease spreads rapidly in the absence of control tools, and it is considered to be re-emerging in Europe (European Food Safety Authority, 2010). Proof-of-concept studies performed in *in vitro* systems have shown that new generation drugs and commercially available drugs such as decoquinate and diclazuril are effective against parasite invasion and proliferation (Jiménez-Meléndez et al., 2017, 2018). Nevertheless, these promising therapeutic tools need to be tested in an *in vivo* bovine model able to

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reproduce characteristics clinical signs from both the acute and the chronic stages of the disease.

In the 1960s, considerable efforts were invested to develop an experimental model of bovine besnoitiosis with inconclusive results due to the diversity of the inocula employed and experimental designs in the absence of serological and molecular tests. Both asexual infective parasite stages described in cattle thus far, tachyzoites (responsible for the acute stage) and bradyzoites (responsible for the chronic stage), were inoculated with unfruitful results in most cases. However, tachyzoites seem to be the most convenient inoculum since they are routinely maintained in *in vitro* systems and well-characterized isolates can be obtained (Frey et al., 2016). In a recent experimental infection in calves and bulls intravenously inoculated with *B. besnoiti*-Spain 3 tachyzoites, infected animals developed clinical signs compatible with acute stage disease and microscopic lesions characteristic of the chronic stage of the disease (Diezma-Díaz et al., 2018) regardless of the parasite dose and host age. However, taking into account the crucial role that the direct contact and hematophagous vectors play in parasite transmission (Álvarez-García et al., 2014), other inoculation routes that may emulate natural transmission should be considered to refine the previously developed experimental model. The calf model is quite convenient since it exhibits several advantages over adult animals (cost, space, infrastructure and management measures).

Accordingly, herein, the influence of subcutaneous and intradermal inoculation of *B. besnoiti* tachyzoites was investigated with the aim of developing macroscopic clinical signs and lesions characteristic of chronic besnoitiosis in an experimental calf model.

2. Materials and methods

All experimental procedures were approved by the Animal Welfare Committee of the Community of Madrid, Spain (PROEX 92/14, Law 32/2007, and R.D. 53/2013) and Council Directive 2010/63/EU. Animals were housed in clinical facilities belonging to the Faculty of Veterinary Sciences, Complutense University of Madrid (Number of register: ES280790000101).

The nine three-month-old calves used in this study came from a Holstein Friesian dairy herd located in Madrid province and were free from bovine besnoitiosis and the main contagious production diseases, including tuberculosis, brucellosis, bovine respiratory syndrome (IBR) and bovine viral diarrhoea (BVD), among others. We conducted the experimental design, clinical monitoring and samplings as previously described by Diezma-Díaz et al. (2018) for 70 days. Briefly, animals were selected after they were assessed for the absence of specific antibodies against *B. besnoiti* and the closely related coccidian parasites *N. caninum*, *T. gondii* and *Sarcocystis* spp. Prior to inoculation, a vaccination protocol and a quarantine-adaptation period was established, and animals were randomly allocated into three different groups, G1, G2 and G3, which were composed of three animals each. G1 calves were subcutaneously inoculated in the left pre-scapular area. G2 calves were inoculated by the intradermal route in the thigh area, with the dosage subdivided into ten inoculation points of five on each side of the animal, using an intradermal syringe Dermojet HR (Akra DermoJet®, Pau, France). A non-infected control group was intravenously inoculated with PBS (G3). The inocula consisted on 10^6 *B. besnoiti* tachyzoites from the Bb-Spain 3 isolate (Diezma-Díaz et al., 2017) freshly purified from infected cell cultures under sterile conditions, free of BVD and *Mycoplasma* spp. Inoculation doses were prepared as previously described in Diezma-Díaz et al. (2018).

Rectal temperatures and other clinical signs characteristic of either acute (lymphadenopathy, oedema, orchitis, respiratory signs, lameness and anorexia) and chronic phase disease (conjunctival cysts and skin lesions) were monitored daily in the calves. Next, a clinical scoring system was established (Table 1) (Diezma-Díaz et al., 2018). Blood samples were collected seven days before the inoculation, twice a week for the first month post-infection (pi) and once a week until the end of assay. Innate interferon-gamma (IFN-γ) and humoral immune responses were determined in sera samples. IFN-γ levels were measured with the Bovine IFN-γ ELISA Development Kit (Mabtech AB, Stockholm, Sweden) according to the manufacturer's instructions (Sánchez-Sánchez et al., 2018). *Besnoitia besnoiti*-specific IgGs were determined by ELISA-SALUVET 2.0 (García-Lunar et al., 2017), and the IgG1/IgG2 ELISA was essentially conducted as described by Diezma-Díaz et al. (2018). IFN-γ adaptive immune responses were measured in heparinized blood samples. A peripheral blood stimulation assay was conducted, and IFN-γ production was evaluated as previously described by Sánchez-Sánchez et al. (2018).

At the end of the experiment, animals were sedated and immediately euthanized following approved procedures and a previous experimental infection (Diezma-Díaz et al., 2018). Next, necropsies were carried out and tissue samples from the reproductive (testis, epididymis head, body and tail, vasa deferential, bulbourethral gland, prostatic gland, seminal vesicles and penis), respiratory tracts (nostrils, nasal turbinates, larynx, pharynx, epiglottis, trachea, bronchi and lungs), lymphatic system (submandibular, subscapular, inguinal and tracheobronchial lymph node, thymus, tonsils and spleen), skin of different locations (neck, upper eyelid, carpus, tarsus, perineum, pinna, thigh and scrotum) and other organs (tongue, ventricle, atrium, ocular conjunctiva, sclera, distal fascia-tendon from the rear leg and hoof corium) were collected. These tissue samples from both infected and non-infected calves were maintained in 10% neutral buffered formalin and stored at −80 °C for histopathological and PCR analyses, respectively. After seven days of fixation with 10% neutral buffered formalin, tissue samples were dehydrated using a graded series of alcohols and were embedded in paraffin using an automatic tissue processor (TP1020, Leica Mycrosystems). Tissue sections of 4–7 µm in thickness were cut from each sample with a motorized rotary microtome (RM2255, Leica Mycrosystems) and stained with haematoxylin and eosin (H/E) using a linear staining system (4040, Leica Mycrosystems) for the histopathological evaluation. Samples were observed with an optical microscope (BX50, Olympus).

Genomic DNA was extracted from frozen tissue samples, and ITS-1 rDNA PCR was carried out. The forward primer ITS1F (50-TGACATTT AATAACAATCAACCCTT-30) and the reverse primer ITS1R (50-GGTT TGTATTAACCAATCCGTGA-30) were added at a concentration of 10 µM, and the rest of reagents were incorporated in the mixture, as indicated by Frey et al. (2013). The amplified products with the expected size of 231 base pairs were visualized after electrophoresis on a 1.5% agarose gel containing 0.1 µl/ml GelRed™ Nucleic Acid Gel Stain (Biotium, USA). DNA extraction and PCR were performed in separate laboratories under biosafety level II conditions (BIO II A Cabinet, TELSTAR, Spain) to avoid cross contamination. The positive control was DNA extracted from *in vitro* cultured tachyzoites of *B. besnoiti*, and PCR grade water was used as the negative control.

A repeated measures two-way ANOVA test and a Tukey post-test were performed with parametric data (rectal temperatures, IFN-γ levels and IgG/IgG1-IgG2). Differences among clinical scores were assessed by a non-parametric Tukey test followed by a Dunn's multiple range test for all pairwise comparisons (GraphPad Prism 6.01 software).

3. Results

Regarding clinical inspection, only one calf inoculated subcutaneously (G1T3) showed sporadic fever peaks (> 39.5 °C) during the first 14 days pi. In contrast, all infected animals (G1 and G2) developed enlarged pre-crural lymph nodes until 47 days pi, and this was more evident for the first four days pi. In addition, respiratory signs, such as nasal discharge and cough, were observed in two of the three G1 animals during the first week pi. The outcome of the acute infection was classified as a 'mild-moderate infection' according to the clinical score shown in Table 1. Animals did not develop either other signs characteristic of the acute stage (oedema, orchitis or lameness) or any

Table 1
Clinical score. **G1**: Calves inoculated by the subcutaneous route; **G2:** Calves inoculated by the intradermal route; **G3:** Control group.

	Week pi	1		2		3		4		5		6		7		8- 10
	Days pi	1-4	5-7	8-11	12-14	15-18	19-21	22-25	26-28	29-32	33-35	36-39	40-42	43-46	47-49	50-70
G1	C1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0
	C2	1	1	0	0	1	1	0	1	1	1	1	1	1	0	0
	C3	2	1	1	2	1	1	1	1	1	1	1	1	1	1	0
G2	C1	1	1	0	1	1	1	0	1	2	1	1	1	1	0	0
	C2	1	1	0	0	1	1	1	1	1	1	1	1	1	0	0
	C3	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0
G3	C1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

pi: Post-infection.
G: Group.
C: Calf.
Clinical score: 0, None (Absence of infection); **1**, Mild (Local lymphadenopathy); **2**, Moderate (Fever, systemic lymphadenopathy, cough/nasal secretion and/or congestive conjunctiva) (Diezma-Díaz et al., 2018).

clinical signs or lesions characteristic of the chronic stage, such as macroscopic tissue cysts in the conjunctiva and skin lesions.

In general, the G1 animals showed earlier and higher IFN-γ innate and adaptive responses compared with the G2 animals (Fig. 1). Serum IFN-γ levels were higher compared with the control group from four days to 28 days pi in animals inoculated by subcutaneous routes (G1) (P values: from 0.014 to 0.049) and from seven days to 21 days pi in the intradermally inoculated group (G2) (P values: from 0.032 to 0.05). G1 showed higher IFN-γ levels than G2 between four and 11 days pi (P values: from 0.012 to 0.026) and between 21 and 28 days pi (P values: from 0.023 to 0.042). IFN-γ levels peaked at 7 days pi in G1 *vs.* 14 days pi in G2 (Fig. 1A). On the other hand, an increase in IFN-γ levels in the cell culture supernatants was observed at days 11 pi in G1 and 14 pi in G2 with respect to the control group ($P < 0.001$) (Fig. 1B). Differences were maintained until the end of the trial in G1 (P values: to 0.001 to 0.047) and until 21 days pi in G2 compared with G3 (P values: from 0.001 to 0.019) (Fig. 1B). IFN-γ levels reached maximum values at day 14 pi for G1 ($P < 0.001$) and on 18 days pi for G2 ($P < 0.001$). G1 displayed higher levels than G2 from 11 days to 26 days pi (P values: from 0.001 to 0.024) and at 67 days pi ($P = 0.018$) (Fig. 1B).

As shown in Fig. 2A, seroconversion was detected in G1 and G2 at 21 and 26 days pi, respectively, and the antibody levels were higher in infected calves than in control group onwards (P values: from 0.001 to 0.048). G1 showed higher antibody levels compared with G2 starting at 26 days ($P = 0.025$) and remained higher from 40 days pi until the end of the trial (P values: from 0.001 to 0.048) (Fig. 2A). Regarding IgG1 and IgG2 kinetics, a similar pattern was observed from 26 days pi onwards. IgG1 and IgG2 levels were higher in the infected groups compared with uninfected animals ($P = 0.015$ and $P = 0.004$, respectively) and IgG1 levels were maintained higher than IgG2 levels in both infected groups. Differences between infected groups were only observed for IgG2 levels in G1 compared with G2 at 47 days pi ($P = 0.008$) and from 61 days to 70 days pi (P values: from 0.001 to 0.034) (Fig. 2B and C).

Parasite DNA was detected by PCR in nine tissue samples from infected calves. Seven samples (left epididymis body, proximal vas deferens, right testicular parenchyma, left nostrils, nasal turbinates, tonsils and brain) belonged to one calf inoculated by the subcutaneous route (G1C1). The upper- eyelid from another animal of the same group (G1C3) and epiglottis from one calf inoculated by the intradermal route (G2C2) were also positive. When samples, both PCR- positive and negative, were analysed by histopathological techniques, neither tissue cysts nor lesions compatible with the infection were evidenced. All samples from control group were negative by both techniques.

4. Discussion

The route of inoculation is a host-dependent variable that has an impact on microbial infection outcome (Benávides et al., 2014). Thus, we have evaluated two different routes of *B. besnoiti* administration (subcutaneous and intradermal route), and, remarkably, this is the first study where tachyzoites were intradermally inoculated. Herein, similar results were observed compared with a previous intravenous inoculation of the same dose of Bb-Spain 3 tachyzoites (Diezma-Díaz et al., 2018) except for a few remarkable differences.

Both inoculation routes together with the intravenous route have

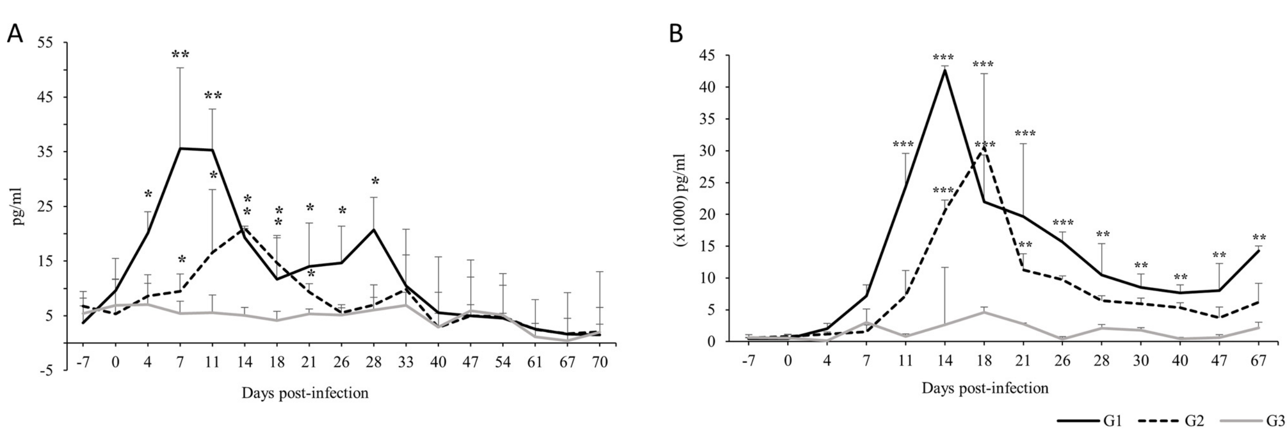

Fig. 1. Cellular immune response: **A**: Innate IFN-γ responses measured in serum samples; **B**: Adaptive IFN-γ responses measured in cell culture supernatants.

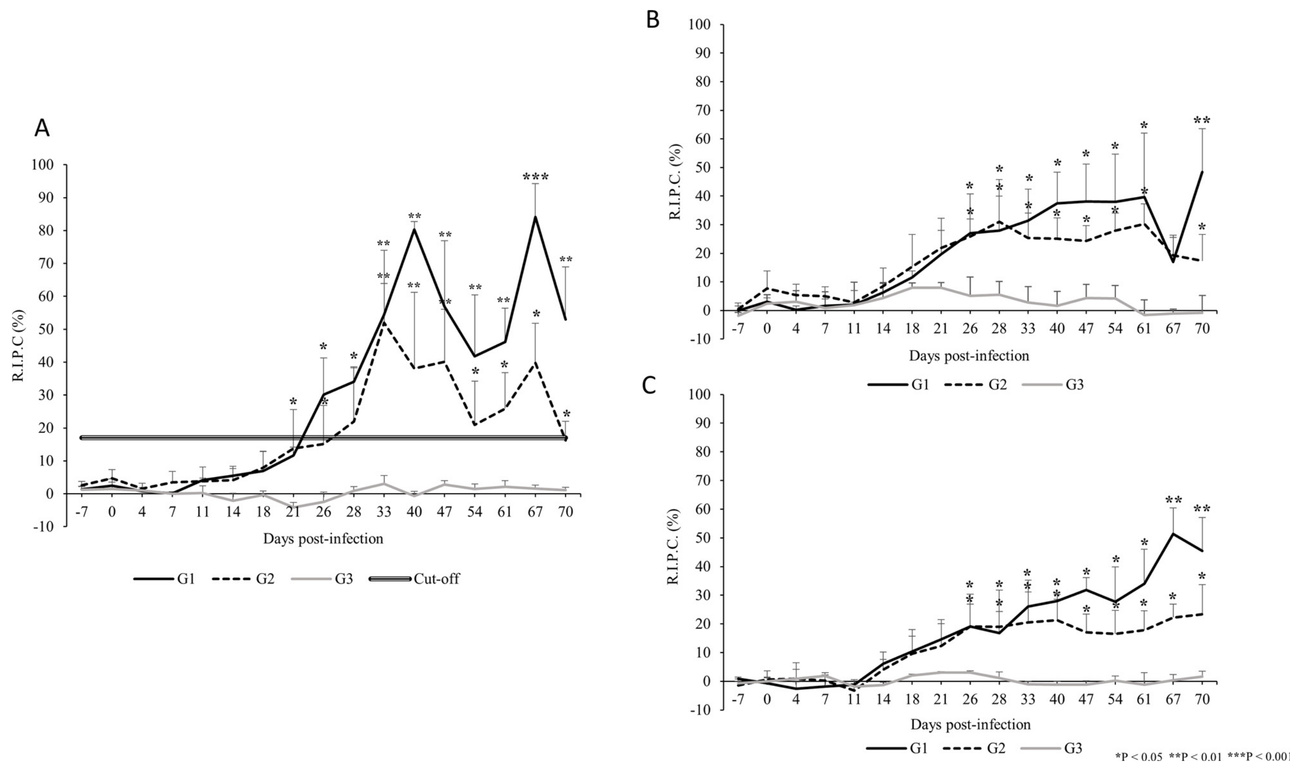

Fig. 2. Humoral immune response: **A**: *Besnoitia besnoiti*–specific IgG responses; **B**: IgG1 antibody levels; **C**: IgG2 antibody levels. Mean Relative Index (+Standard Deviation). (R.I.P.C: Relative index per cent).

been studied in the same experimental calf model of *B. besnoiti* infection. The three routes mimic parasite transmission in nature since parasites can be transmitted by blood suckling arthropods and direct contact has also been suggested based on epidemiological evidence (Álvarez-García et al., 2014).

Infected calves developed "mild-moderate" acute stage bovine besnoitiosis characterized by lymphadenopathy around the first month pi and mild respiratory signs. Fever was not detected in the intradermally infected animals, and a lower febrile response was developed by subcutaneously infected animals compared with animals infected by the intravenous route. The later showed fever earlier from one day pi until seven days pi. Fever might not be a necessary requisite for a successful infection since the majority of naturally infected animals apparently do not develop fever, or at least it goes undetected, and these animals remain as parasite carriers. However, fever is likely associated with the first replication cycles of the parasite (Benávides et al., 2014) and might be indicative of disease severity since severely affected animals during the acute stage of the infection develop and maintain a high fever (Álvarez-García et al., 2014).

Although previous experimental trials are not comparable, it is worthwhile mentioning that the subcutaneous route was employed in two previous trials. Bigalke et al. (1974) inoculated 2×10^6 or 10^6 tachyzoites from the blue wildebeest strain by the subcutaneous route in combination with the intravenous route, and fever and lymphadenopathy were observed in all inoculated animals. However, the incidence of scleral cysts, the only clinical signs characteristic of the chronic stage monitored, was very low (from 0 to 1.79%) over the period of observation. Later, Diesing et al. (1988) inoculated 3.7×10^8 tachyzoites in 3- to 6-month-old calves (one of them immunosuppressed with cortisone), and the infected animals only developed fever from three days pi until 27 days pi. Other routes of inoculation, such as oral, nasal or conjunctival routes, were also explored in cattle, and scarce fruitful results were obtained (Bigalke, 1968). Fever and tissue cysts were reported in the upper eyelid of one animal that was inoculated orally and in the jugular, facial and peripheral veins of two animals that were inoculated in the nostrils. These routes should not be ruled out, since these authors inoculated adult cattle with unknown health statuses and serological tests were not employed, which is crucial as immunity to re-infections has been reported (Álvarez-García et al., 2014).

In our work, regardless of the absence of macroscopic and microscopic lesions characteristic of the chronic stage of the infection, parasite DNA was detected in a few target tissues, mostly from reproductive tract as in agreement with previous reports (Frey et al., 2013; Diezma-Díaz et al., 2018). In contrast, intravenous inoculation of tachyzoites gave rise to scattered vasculitis, and a tissue cyst was observed in the carpus region. The absence of tissue cyst detection is explained by the low parasitic load. Curiously, *B. besnoiti* DNA was detected in the brain from one subcutaneously infected calf. There were a few reports of PCR-positive brain samples corroborating that *B. besnoiti* is able to cross the blood brain barrier, although its ability to form cysts remains elusive (Basso et al., 2011; Diezma-Díaz et al., 2017). Taking into account the low parasite intra-organic distribution observed previously (Diezma-Díaz et al., 2018) and herein (nine positive tissue samples), a likely efficient cellular immune response elicited by infected animals is expected to have cleared out most parasites.

In the present study, the kinetics of immune responses were similar in both infected groups. However, delayed and lower cellular and humoral immune responses were observed in both groups compared with intravenously inoculated calves (Diezma-Díaz et al., 2018). First, an innate IFN-γ response was detected for the first and second weeks pi (7–14 days pi) followed by an adaptive IFN response at the second week pi (14–18 days pi), and the animals developed humoral immune responses from the third week pi onwards (21–26 days). In contrast, with the intravenous route, in which an IFN- γ response was detected at 4 days pi and seroconversion was detected at approximately 17 days pi. The differences observed among the three inoculation routes could be due to different effector mechanisms of the host early innate immune response against *B. besnoiti* in the different tissues and parasite dissemination related to tissue vascularization (Muñoz-Caro et al., 2014). The dermis and subcutaneous tissue, where the parasite is inoculated by intradermal and subcutaneous injections, respectively, may elicit distinct local innate immune responses based on the different innate cells populations present. In the dermis, mast cells, macrophages, T cells and mainly dendritic cells play an important role in the immune response (Hunsaker and Perino, 2001). In subcutaneous tissue, macrophages are the predominant immune cells. Moreover, a higher blood vessel calibre in the subcutaneous tissue is responsible for a more rapid absorption compared with the intradermal route. Consequently, in the dermis, dermal dendritic cells need to take up the antigen, migrate to draining lymph nodes, and present processed antigen to T-cells (Hunsaker and Perino, 2001). However, in subcutaneous tissue, the antigen bypasses the skin's immune cells, and the migration into lymph nodes is more efficient leading to a more rapid immune response (Escobar-Chávez, 2010). In contrast, after intravenous inoculation (Diezma-Díaz et al., 2018), the pathogens in the bloodstream signify a breach in barrier and are met with a full-blown systemic response and are rapidly distributed to different tissues that might explain the earliest and highest cellular and humoral immune responses compared with the subcutaneous or intradermal route (Iwasaki and Medzhitov, 2015). Whether the inoculation route is a crucial variable when inoculating other parasite stages remains to be investigated.

According to our results, the inoculation route of *B. besnoiti* tachyzoites does not significantly influence the clinical outcome of infection in calves. Thus, a further refinement of this experimental model of bovine besnoitiosis is needed to reproduce both the acute and the chronic stages of bovine besnoitiosis with macroscopic lesions. Since host dependant factors, such as age and inoculation route, and parasite-dependant factors, such as tachyzoite dose, have been already tested, further trials should evaluate the other parasite stage described in bovines, the bradyzoites.

Conflict of interest

The authors have no conflict of interest.

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