UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

TESIS DOCTORAL

Desarrollo de un modelo bovino de infección experimental por *Besnoitia besnoiti*

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Carlos Diezma Díaz

Directores

Gema Álvarez García
Ignacio Ferre Pérez
Francisco Javier Blanco Murcia

Madrid

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

Departamento de Sanidad Animal

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Autor: Carlos Diezma Díaz

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Francisco Javier Blanco Murcia

Madrid, 2019

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DESARROLLO DE UN MODELO BOVINO DE INFECCIÓN EXPERIMENTAL POR *Besnoitia besnoiti*

y dirigida por: Gema Álvarez García, Ignacio Ferre Pérez y Francisco Javier Blanco Murcia

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Animal Health Department

DEVELOPMENT OF AN EXPERIMENTAL MODEL OF *Besnoitia besnoiti* INFECTION IN CATTLE

DOCTORAL THESIS

Author: Carlos Diezma Díaz

Supervisors: Gema Álvarez García

Ignacio Ferre Pérez

Francisco Javier Blanco Murcia

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«Olvida lo que has dado para recordar todo lo que has recibido»

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LISTADO DE ABREVIATURAS

%	Porcentaje	Percentage
°C	Grados centígrados	Grades centigrade
µl	Micro litro	Micro liter
µm	Micrómetro	Micrometer
µg	Microgramo	Microgram
µM	Micro molar	Micro molar
AC	Número medio de quistes	Average number of tissue cysts
AD	Diámetro medio de quistes	Average diameter of tissue cysts
ADN/DNA	Ácido desoxirribonucleico	Desoxyribonucleic acid
ARN/RNA	Ácido ribonucleico	Ribonucleic acid
AI	Índice de avidez	Avidity index
ANOVA	Análisis de la varianza	Analysis of variance
Bb	*Besnoitia besnoiti* (prefijo)	*Besnoitia besnoiti* (prefix)
BHK	Fibroblastos de riñón de criceto	Baby hamster kidney fibroblasts
BKI	Inhibidores de la proteína quinasa	Bumped kinase inhibitors
BUVEC	Células endoteliales de vena umbilical bovina	Bovine umbilical vein endothelial cells
BVD	Diarrea vírica bovina	Bovine viral diarrhoea
BVDV	Virus de la diarrea vírica bovina	Bovine viral diarrhea virus
Bz	Bradizoítos	Bradyzoites
C	Ternero	Calf
C-	Testigo negativo	Negative control
C+	Testigo positivo	Positive control
CO2	Dióxido de carbono	Carbon dioxide
CRFK	Células de riñón de gato	Feline renal cell line
DEET	N-dietil-3-metil-toluamida	N-diethyl-3-m-toluamide
EDTA	Ácido etilendiaminotetraacético	Ethylenediaminetetraacetic acid
EFSA	Autoridad Europea de Seguridad Alimentaria	European Food Safety Authority
ELISA	Ensayo inmunoenzimático	Enzyme-linked immunosorbent assay
Esp/Sp	Especificidad	Specificity
EU	Unión Europea	European Union
FITC	Isotiocianato de fluoresceína	Fluorescein isothiocyanate
G	Grupo	Group
g	Gramo	Gram
H/E	Hematoxilina y Eosina	Haematoxylin and Eosin
HeLa	Células epiteliales de tumor de cérvix humano	Human cervical cancer cells
HFF	Fibroblastos de prepucio humano	Human foreskin fibroblasts
HP	Histopatología	Histopathology
hpi	Horas post-infección	Hours post-infection
IBR	Síndrome respiratorio bovino	Bovine respiratory syndrome
IC50	Concentración inhibitoria 50	Inhibitory Concentration 50
IFI/IFAT	Inmunofluorescencia indirecta	Indirect immunofluorescence assay
IFN-γ	Interferón gamma	Gamma Interferon
IgG	Inmunoglobulinas isotipo G	Immunoglobulin isotype G
IgG1	Inmunoglobulina isotipo G 1	Immunoglobulin isotype G 1
IgG2	Inmunoglobulina isotipo G 2	Immunoglobulin isotype G 2
IgM	Inmunoglobulina isotipo M	Immunoglobulin isotype M
IHQ/IHC	Inmunohistoquímica	Immunohistochemistry
im	Intramuscular	Intramuscular

in	Intranasal	Intranasal
ip	Intraperitoneal	Intraperitoneal
IR	Tasa de invasión	Invasion rate
IRPC/RIPC	Índice relativo por ciento	Relative index per cent
ITS-1	Espacio transcrito interno-1	Internal transcribed space-1
iv	Intravenosa	Intravenous
kDa	Kilodalton	Kilodalton
KH-R	Células embrionarias de corazón de ternero	Embryonic calf heart cells
l	Litros	Litres
Ln	Linfonódulos	Lymph nodes
LSTH-RA/ RML	Células embrionarias de garrapata	Embryonic tick cells
M	Molar	Molar
m	Metro	Metro
m^2	Metro cuadrado	Square metre
MARC	Células epiteliales de riñón de mono Rhesus	Rhesus monkey fetal kidney cells
MDBK	Células epiteliales de riñón bovino (Madin-Darby)	Madin-Darby bovine Kidney epithelial cells
min	Minutos	Minutes
ml	Mililitros	Millilitres
mm	Milímetros	Millimetres
mM	Milimolar	Milli molar
MS	Microsatélites	Microsatellite
n	Número de muestra	Sample number
NA42	Neuroblastos de ratón	Mouse neuroblastoma cells
NA	No aplicable	Not appicable
ND	No determinado	Not determined
ng	Nano gramo	Nano gram
nM	Nano molar	Nano molar
nm	Nanómetro	Nanometer
NRC	Consejo Nacional de Investigación	National Reserch Council
OD	Densidad óptica	Optical density
Ooq	Ooquistes	Oocysts
P	Picaduras	Bites
p.ej./e.g.	Por ejemplo	For instance
PAS	Ácido Peryódico de Schiff	Periodic Acid Schiff
pb/bp	Par de base	Base pair
PBMC	Células mononucleares de sangre periférica	Peripheral blood mononuclear cells
PBS	Tampón fosfato salino	Phosphate Buffer Saline
PCR	Reacción en cadena de la polimerasa	Polymerase chain reaction
pi	Post-infección	Post-infection
PMN	Neutrófilos polimorfo-nucleares	Polimorfo nuclear neuthropiles
PV	Vacuola parasitófora	Parasitophorous vacuoles
qPCR	PCR cuantitativa	Quantitative PCR
R.D.	Real Decreto	
S	Sacrificado	Slaughtered
sc	Subcutánea	Subcutaneous
SD	Desviación estándar	Standar deviation
Se	Sensibilidad	Sensitivity
SNC/CNS	Sistema nervioso central	Central nervous system
sp./ spp.	Especie/ Especies	Species
T-cells	Linfocito T	T-lymphocyte

Td	Tiempo de duplicación	Doubling time
TY	Producción de taquizoítos	Tachyzoite yields
Tz	Taquizoítos	Tachyzoites
EE.UU/USA	Estados Unidos de América	United States of America
Vero	Células epiteliales de riñón de mono verde africano	African green monkey epithelial kidney cells
vs.	Versus	Versus
WB	Western blot	Western blot

CAPÍTULO I

RESUMEN/ABSTRACT

La besnoitiosis bovina, causada por el protozoo apicomplejo *Besnoitia besnoiti*, es una enfermedad crónica y debilitante del ganado vacuno caracterizada por signos clínicos locales y sistémicos. La European Food Safety Authority (EFSA) consideró a la besnoitiosis bovina como una enfermedad re-emergente en Europa debido a un notable aumento en el número de casos. Actualmente, el control de la enfermedad solo es posible mediante medidas de manejo asociadas a un diagnóstico temprano, ya que fármacos y/o vacunas no están disponibles en Europa.

Se han testado *in vitro* diversos fármacos efectivos contra el estadio de taquizoíto responsable de la fase aguda de la enfermedad. Sin embargo, estos candidatos terapéuticos no han sido probados en un modelo animal de infección experimental ya que, por el momento, no está disponible. Se han infectado diversas especies de roedores de laboratorio con resultados inconclusos. Únicamente, los conejos han mostrado cierta susceptibilidad a la infección, manifestando signos clínicos de la fase aguda y de la fase crónica. Sin embargo, el establecimiento y la adaptación de la enfermedad a un hospedador heterólogo son difíciles de conseguir. Por lo tanto, el desarrollo de un modelo de infección experimental en el bovino (la especie de destino) sería el abordaje más adecuado. Entre la década de los 60 y de los 80 del siglo pasado, se realizaron algunas infecciones experimentales en bovino bajo diferentes condiciones experimentales y con inóculos de procedencia muy diversa. Esta heterogeneidad en los ensayos no permitió desarrollar un modelo de infección reproducible en bovino. Además, la mayoría de los resultados se basaron únicamente en la inspección clínica e histología, ya que los métodos serológicos y moleculares no estaban disponibles.

Por todo ello, en esta Tesis Doctoral, se ha desarrollado un modelo de infección experimental en el ganado vacuno para la infección por *B. besnoiti*. Se han inoculado en bovinos, bajo el mismo diseño experimental, los dos estadios infectivos conocidos hasta el momento del parásito (taquizoíto y bradizoíto). Además, todos los ensayos se complementaron con estudios histológicos y moleculares en los órganos diana y con el estudio de la respuesta inmune. Se aislaron, por primera vez, taquizoítos de *B. besnoiti* procedentes de una ternera con infección crónica (Sub-objetivo 1.1). Tras su aislamiento, se determinó la identidad de *Besnoitia* sp. por genotipado. El nuevo aislado (Bb-Spain 3) mostró *in vitro* una baja invasión y proliferación, con un ciclo lítico similar al de otros aislados españoles y europeos. Además, se describió el primer caso clínico de besnoitiosis crónica en una ternera menor de seis meses, la cual evidenció una distribución intraorgánica del parásito y unas lesiones muy similares a las observadas previamente en animales adultos.

Posteriormente, se inocularon por vía intravenosa tres dosis diferentes (10^8, 10^7 y 10^6) de taquizoítos (procedentes del aislado Bb-Spain 3) en terneros y, a continuación, se administraron 10^6 taquizoítos en novillos por la misma vía. El objetivo era determinar el efecto de la dosis y el de la edad en el desarrollo de la infección (Sub-objetivo 1.2). La duración de las infecciones experimentales fue 70 y 115 días en terneros y novillos, respectivamente, e incluyeron una monitorización clínica

diaria de los animales inoculados, así como una extracción semanal de sangre y de biopsia de piel. Se determinó la respuesta inmune celular y humoral en suero y tras la eutanasia de los animales, se recogieron tejidos de piel, ojos, tracto reproductivo y respiratorio, entre otros órganos, para estudiar la presencia del parásito. Se reprodujo la fase aguda de la enfermedad en todos los animales inoculados con una gravedad leve-moderada, sin detección de signos clínicos macroscópicos de la fase crónica. Únicamente, se observo un quiste tisular en piel y una escasa detección de ADN del parásito en un reducido número de tejidos diana. En una segunda infección experimental, se testaron la ruta de inoculación subcutánea e intradérmica en terneros inoculados con la misma dosis de 10^6 taquizoítos (Sub-objetivo 1.3). El diseño experimental fue el mismo y la infección se extendió durante 70 días. De nuevo, la detección de la besnoitiosis aguda de la enfermedad y la escasa distribución intraorgánica del parásito fueron los hallazgos más relevantes. Por lo tanto, ni la dosis parasitaria ni la ruta de inoculación fueron factores determinantes del parásito y del hospedador en el desarrollo de la fase crónica de la besnoitiosis bovina cuando se inocularon taquizoítos.

En un nuevo ensayo, se determinó el efecto de la inoculación del otro estadio del parásito (los bradizoítos) por diferentes vías de inoculación en el mismo modelo experimental establecido previamente en terneros. Los bradizoítos, empleados como inóculo, fueron obtenidos de un toro infectado crónicamente. Estos se inocularon en terneros (10^6 bradizoítos por animal) por vía intravenosa, subcutánea e inradérmica, respectivamente (Sub-objetivo 2.1). Clínicamente, la infección aguda se clasifico como leve-moderada y la fase crónica, el resultado más relevante, como moderada-grave. Se observaron quistes oculares patognomónicos de la enfermedad en todos los animales infectados a partir de los 43 días post-infección (pi) siendo más abundantes en el grupo de terneros inoculados por vía intradérmica. Además, uno de los terneros de este grupo desarrolló también lesiones en piel. Los quistes tisulares microscópicos y el ADN del parásito se detectaron en muestras de la piel (incluida la piel de escroto), seguido del sistema reproductivo y del tracto respiratorio, con más carga en el grupo intradérmico (Sub-objetivo 2.2). A los 90 días pi, los quistes tisulares estaban completamente desarrollados, con un diámetro medio de 181,2 µm. Sin embargo, estos no alcanzaron el tamaño máximo observado en otros ensayos previos y las lesiones detectadas fueron moderadas, asociado posiblemente a un buen control de la infección por la respuesta inmune del hospedador. Se observaron en los terneros lesiones compatibles tanto con la fase aguda (trombos, edema e inflamación) como con la fase crónica de la enfermedad (lesiones en piel, hiperqueratosis y dilatación de glándulas sudoríparas) que no estuvieron relacionadas, en muchos casos, con la presencia de quistes. En el aparato reproductor, el daño vascular y la inflamación fueron las lesiones más frecuentes. Se observaron principalmente en piel de escroto seguido de parénquima testicular y epidídimo. Sin embargo, estas lesiones podrían no haber afectado a la función reproductiva de los terneros en su vida adulta ya que el epitelio seminífero de los testículos y las células de la línea germinal no estaban dañadas. Estudios futuros deberían esclarecer la cronobiología y la relevancia de

los diferentes mecanismos patogénicos en la esterilidad desarrollada por los sementales infectados. Por lo tanto, nuestros resultados indican que el estadio parasitario (bradizoíto) y la ruta de inoculación (intradérmica) son variables determinantes en el establecimiento de la infección crónica.

Además, las infecciones experimentales nos proporcionaron un amplio panel de sueros bien caracterizados donde se estudió por primera vez la cinética de las inmunoglobulinas del isotipo M (IgM) en la infección por *B. besnoiti*. El objetivo fue mejorar el diagnostico temprano *in vivo* de la besnoitiosis aguda debido a las limitaciones de las técnicas serológicas empleadas habitualmente y basadas en la detección del isotipo G (IgG) (Objetivo 3). Para ello, se desarrolló un ELISA para la detección de anticuerpos específicos IgM, los cuales se determinaron también en sueros procedentes de infección natural, con animales en fase aguda y crónica de la enfermedad. A continuación, se monitorizó la avidez de los anticuerpos IgG en todos los sueros. Se detectó una rápida respuesta de IgM previa a la seroconversión de IgG (7-19 vs. 17-26 días pi) que estuvo asociada con el inicio de la fiebre en los terneros infectados experimentalmente. En la infección natural, los animales en la fase aguda de la enfermedad mostraron resultados positivos de IgM y negativos de IgG, seguido de seroconversión 2-3 semanas después. En la fase crónica, se observaron resultados positivos tanto para IgM como IgG. En resumen, la detección pareada y simultánea de IgM/IgG sería de utilidad en el diagnóstico temprano de la besnoitiosis aguda junto a la presencia de signos clínicos. Con respecto a la avidez de la IgG, se detectó un incremento progresivo a medida que avanzó la infección en los terneros infectados experimentalmente, aunque los valores de baja avidez coexistieron con quistes visibles en la conjuntiva ocular. Se detectó una baja avidez, al tiempo de la seroconversión, en los animales infectados naturalmente con signos clínicos de fase aguda, en contraste con la alta avidez detectada en los de la fase crónica. Sin embargo, el tiempo necesario para alcanzar una alta avidez tras la infección sigue sin esclarecerse. Deberían realizarse nuevos estudios para monitorizar la cinética de avidez de los anticuerpos IgG, tanto individualmente (estudios longitudinales) como en rebaño (brotes vs. zonas endémicas de besnoitiosis) y determinar su uso en el diseño de programas de control.

En conclusión, se han desarrollado dos modelos de infección experimental en terneros. Uno de ellos representativo de la besnoitiosis subclínica (con la inoculación de taquizoítos) y otro representativo de la besnoitiosis crónica con detección de abundantes quistes tisulares macroscópicos (bradizoítos inoculados por vía intradérmica). Además, se ha mejorado el diagnóstico *in vivo* de la besnoitiosis aguda mediante el empleo de un ELISA de IgM y de avidez.

Bovine besnoitiosis, caused by the cyst forming apicomplexan parasite *Besnoitia besnoiti*, is a chronic and a debilitating cattle disease characterized by both local and systemic manifestations. It is considered re-emerging by the European Food Safety Authority due to an increase number of cases in Western and Central European countries. Currently, the control of this disease is only possible through management measures coupled to diagnosis since chemotherapeutics are not available and no vaccines are licensed in Europe.

Several drugs have been tested in *in vitro* systems for their efficacy against *B. besnoiti* tachyzoites responsible for the acute stage. As a next step, these drugs should be tested in experimental animal models that are not available yet. Laboratory rodent species have been infected with *B. besnoiti* showing inconclusive results. Rabbits showed susceptibility to experimental infection by developing clinical signs compatible with acute and chronic phases of the disease. However, the establishment of *B. besnoiti* and its adaptation to a heterologous host is difficult. Thus, an experimental bovine model (the target species) is the ideal approach. A few attempts were performed between the 1960s and 1980s. Unfortunately, all the inoculations were carried out under different experimental conditions and the inocula were obtained from different sources. This heterogeneity did not allow the development of a reproducible experimental model of besnoitiosis in cattle. Additionally, most results were based on clinical inspection and histopathology, since molecular tests were not still available and serological assays were rarely employed.

Thus, in the present Doctoral Thesis, a bovine model for *B. besnoiti* infection was developed. The two known infective parasite stages described in the intermediate host (tachyzoite and bradyzoite) were inoculated in cattle under the same experimental conditions and design. Moreover, these trials were accompanied by histological and molecular analyses performed in target tissues and the study of immune responses. Initially, *B. besnoiti* tachyzoites were isolated from a chronically infected calf for the first time (Sub-objective 1.1). The identity of the *Besnoitia* species was determined after parasite isolation and molecular genotyping. According to the *in vitro* results, the new isolate, named Bb-Spain 3, was categorized as a low invader and low prolific isolate with a lytic cycle similar to other Spanish and European isolates. Moreover, the first case report of chronic besnoitiosis in a young animal was reported in a female calf less than 6 months-old with parasite intraorganic distribution and lesions as previously observed in adults.

Later, different tachyzoite doses (10^8, 10^7 and 10^6) from this isolate (Bb-Spain 3) were intravenously inoculated in calves and subsequently, 10^6 tachyzoites were administrated in bulls in order to study parasite- (dose) and host- (age and inoculation route) dependent factors that may also determine the outcome of the infection (Sub-objective 1.2). The trial lasted for 70 and 115 days post-infection (pi) respectively, and included daily clinical monitoring as well as weekly skin biopsies and blood sampling. Sera were obtained to analyse both cellular and humoral responses. Once the calves

were euthanized, tissues from the skin, eyes, respiratory and reproductive tracts, among others, were collected to investigate the presence of parasite. Infected animals developed mild-moderate clinical signs compatible with acute besnoitiosis without macroscopic lesions of chronic besnoitiosis. One tissue cyst in skin sample and parasite DNA in a reduced number of target tissues were detected. In a second experimental infection, subcutaneous and intradermal inoculation routes were also tested in experimentally infected calves (10^6 tachyzoites) with the aim of developing macroscopic lesions characteristic of chronic besnoitiosis (Sub-objective 1.3). The experimental design was similar and the trial lasted for 70 days pi. Acute besnoitiosis and intraorganic distribution of the parasite were the most relevant findings and similar to the previous trial. Accordingly, the dose and the inoculation route as well as the animal age did not seem to be key parasite and host dependant factors in the clinical outcome of the infection when *B. besnoiti* tachyzoites were inoculated.

Using the previously stablished calf model, bradyzoite stage and inoculation routes were studied simultaneously. Bradyzoites obtained from a chronically infected bull were inoculated via intravenous, subcutaneous and intradermal routes in calves (10^6 bradyzoites per animal) (Sub-objective 2.1). Clinically, the infection was classified as mild to moderate for the acute stage and the most relevant results were achieved during the chronic stage that was classified as moderate to severe. Pathognomonic conjunctival cysts were observed in all infected calves from 43 days pi onwards and were more abundant in intradermally inoculated calves. Moreover, one calf from this group developed skin lesions at 49 days pi. The microscopic tissue cysts and *Besnoitia*-DNA were detected primarily in skin, reproductive tract and respiratory tissue samples and parasite load was higher in calves inoculated by intradermal route. Tissue cysts were more abundant and lesions were more frequent in calves inoculted via intradermal route that previously showed more visible tissue cysts (Sub-objective 2.2). The most parasitized tissues harbouring tissue cysts were skin, including scrotum (40.8% of positive samples), nostril and nasal turbinate. Tissue cysts were fully developed at sampling time, with an average tissue cyst diameter of 181.2 µm. However, tissue cysts did not reach the maximum size reported, and lesions were mild, possibly due to the host immune response control. Microscopic lesions were detected mainly in skin samples, followed by reproductive and upper respiratory tracts, and were not necessarily associated with the presence of tissue cysts. Lesions compatible with both acute (thrombus, oedema and inflammation) and chronic besnoitiosis (skin lesions, hyperkeratosis and dilated sweat glands) coexisted. Vascular damage and inflammation were more frequently observed in skin (including scrotum), followed by testicular parenchyma, epididymus and pampiniform plexus. Herein, histological findings were not expected to impair fertility during adult life since the seminiferous epithelium of the testes did not show alterations. Future studies should clarify the chronobiology and relevance of the different pathogenic events in the sterility of naturally infected breeding bulls. Based on the results obtained, the parasite stage (bradyzoite) and the

inoculation route (intradermal) were key factors that influence the clinical outcome of chronic infection.

In addition, the experimental infections provided well-coded panel sera in which the kinetics of immunoglobulin isotype M (IgM) were analysed for the first time in *B. besnoiti* infection. The aim was to improve an early *in vivo* diagnosis of bovine besnoitiosis due to the limitations of the serological tools routinely employed based on IgG detection (Objective 3). Therefore, a novel ELISA to detect specific IgM antibodies was developed and IgM levels were also determined in naturally infected cattle with either acute or chronic infection. Finally, IgG avidity index was monitored in both experimentally and naturally infected animals. A prompt specific IgM response was detected prior to specific IgGs (7-19 vs. 17-26 days pi) and was associated with an onset of fever in experimentally infected calves. Naturally and acutely infected animals showed IgM positive and IgG negative results, followed by IgG seroconversion 2-3 weeks later. Chronically infected cattle showed IgM and IgG positive results. Thus, IgM/IgG pair-wise serology comparison would be useful for the early diagnosis of bovine besnoitiosis coincided with clinical signs compatible with acute phase. A progressive increase of avidity index was observed in all experimentally infected calves during the course of the experimental trials, although low avidity values coexisted with visible tissue cysts. In naturally and acutely infected cattle, low avidity was detected when animals seroconverted, in contrast to high avidity values detected in chronically infected cattle. However, the time needed to detect high avidity values remains to be clarified. Further studies to monitor IgG avidity kinetics at individual (longitudinal studies) and at herd level (outbreaks vs. herds with endemic besnoitiosis) should be performed in order to determine IgG avidity usefulness in the design of control programmes.

In conclusion, two experimental models of *B. besnoiti* infection has been developed in calves representative of subclinical besnoitiosis (with inoculated tachyzoites) and chronic besnoitoisis with macroscopic tissue cysts (with inoculated bradyzoites by intradermal route). Moreover, we have improved the diagnosis of acute besnoitiosis with IgM and avidity ELISAs.

CAPÍTULO II

ANTECEDENTES

1. La besnoitiosis bovina

La besnoitiosis bovina, causada por el protozoo apicomplejo *Besnoitia besnoiti*, es una enfermedad crónica y debilitante del ganado vacuno que se caracteriza por la presencia de lesiones en la piel y alteraciones sistémicas en los animales infectados. Desde sus primeras descripciones, la enfermedad recibió diversas denominaciones como globidiosis, sarcosporidiosis cutánea bovina o elefantiasis, denominándose entonces el agente causal *Globidium besnoiti* o *Sarcocystis besnoiti* (Cuillé et al., 1936; Pols, 1954). Posteriormente, el agente etiológico se encuadró dentro de un nuevo género, *Besnoitia*, denominándose definitivamente *B. besnoiti* (Jellison, 1956). Las primeras descripciones clínicas de la enfermedad en Europa datan de finales del siglo XIX y principios del siglo XX en la zona de los Pirineos (Francia) y el Alentejo (Portugal) (Cadéac, 1884; Besnoit y Robin, 1912; Franco y Borges, 1915). Sin embargo, se piensa que la enfermedad tuvo su origen en el continente africano, ya que en Portugal se detectaron animales infectados en ganado procedente de Angola, antigua colonia portuguesa (Leitao, 1949).

En África, la enfermedad se describió por primera vez en Sudáfrica en 1945 (Hofmeyr, 1945). Bigalke (1968) propuso que la introducción de animales infectados procedentes de las tribus nómadas bantúes y hotentotes a partir del siglo XV habría propagado la enfermedad. Desde los años 50 hasta los 80 del siglo pasado, la besnoitiosis bovina cobró especial importancia en este país. De hecho, fue en Sudáfrica donde se obtuvieron los primeros aislados de *B. besnoiti* que procedían de ungulados infectados naturalmente, tanto domésticos (bovino) como silvestres (impala y ñu). Esto permitió realizar las primeras infecciones experimentales en bovinos, roedores y lagomorfos; y permitió además dilucidar algunos aspectos relacionados con la transmisión de la enfermedad (Pols, 1960; Bigalke et al., 1967; Basson et al., 1970). Basson et al. (1965, 1970) y McCully et al. (1966) llevaron a cabo los primeros estudios sobre la patogénesis y distribución intraorgánica del parásito. Estos trabajos sentaron las bases para el posterior desarrollo de una vacuna viva basada en un aislado de baja virulencia procedente de un ñu y que evitaba el desarrollo de signos clínicos en los animales vacunados (Bigalke et al., 1974). Hay que destacar también en esta etapa el trabajo realizado por Kumi-Diaka et al. (1981), relacionado con las lesiones asociadas a la presencia del parásito en el aparato genital de toros con una infección crónica y a la infertilidad asociada. También en estos años se llevaron a cabo los primeros estudios morfológicos del parásito, así como los primeros ensayos *in vitro* e *in vivo* para evaluar posibles tratamientos frente a la infección (Shkap et al., 1985; Shkap et al., 1988). Los estudios más relevantes en este aspecto incluyeron la valoración de una vacuna basada en un aislado avirulento de origen bovino en diferentes especies animales (Shkap, 1986; Shkap et al., 1987). La seguridad de esta vacuna aún se desconoce. Sin embargo, se sigue empleando actualmente de forma habitual en Israel para evitar el desarrollo de signos clínicos en los sementales importados.

Desde las primeras descripciones de la besnoitioisis bovina en Europa (Francia y Portugal) (Cadéac, 1884; Franco y Borges, 1915), la enfermedad pasó desapercibida en este continente hasta

los años noventa del siglo XX. El número creciente de nuevos casos en zonas donde la enfermedad es endémica (Juste et al., 1990; Cortes et al., 2004; 2005; 2006a; 2006b; 2006c; Alzieu, 2007; Fernández-García et al., 2009b), así como la aparicición de nuevos brotes en países cercanos (Alemania, Croacia, Hungría, Italia y Suiza) hizo que fuera declarada como una enfermedad re-emergente por la European Food Safety Authority en 2010 (European Food Safety Authority, 2010). Desde entonces, se han realizado grandes avances en el estudio de esta enfermedad. Como ejemplo, destaca el desarrollo de nuevas técnicas diagnósticas (Schares et al., 2013; García-Lunar et al., 2017), diversos estudios de prevalencia e incidencia de la infección (Esteban-Gil et al., 2017; Gazzonis et al., 2017; Gutiérrez-Expósito et al., 2017a) descripción de nuevos casos (Nieto-Rodríguez et al., 2016; Ryan et al., 2016) y el avance en el conocimiento de la cronobiología de la infección durante las fases aguda y crónica de la enfermedad en condiciones naturales (Frey et al., 2013; Langenmayer et al., 2015b; 2015c). Sin embargo, aún quedan por esclarecer otros aspectos importantes relacionados tanto con el parásito (ciclo biológico completo) como con la patogenia de la enfermedad (respuesta inmune y bases moleculares de la interacción parásito-hospedador). Además, en la actualidad no existen fármacos o vacunas comercializadas en Europa y no se ha desarrollado hasta el momento un modelo animal de infección adecuado, que sería necesario para evaluar la seguridad y eficacia de nuevos candidatos terapéuticos (Jiménez-Meléndez et al., 2017; 2018; Cervantes-Valencia et al., 2018; Müller et al., 2019).

1.1. Etiología

El agente etiológico responsable de la besnoitiosis bovina es *Besnoitia besnoiti*. Esta especie se encuadra taxonómicamente en el Phylum Alveolata, Subphylum Apicomplexa, Clase Coccidia, Familia Sarcocystidae, Subfamilia Toxoplasmatinae y Género *Besnoitia*. Hasta el momento se han descrito diez especies dentro del Género *Besnoitia* que pueden dividirse en dos grupos según parasiten a pequeños *(B. akodoni, B. jellisoni, B. darlingi, B. neotomofelis, B. oryctofelisi, B. wallacei*) (Jellison, 1956; Wallace y Frenkel, 1975; Nganga, 1994; Dubey et al., 2002; Dubey y Lindsay, 2003; Dubey et al., 2003b; 2003c) o a grandes mamíferos (*B. besnoiti, B. bennetti, B. caprae, B. tarandi*) (Besnoit y Robin, 1912; Dubey et al., 2003a; 2004; 2005; Oryan et al., 2014) (Tabla 1).

Besnoitia besnoiti, B. bennetti, B. caprae y *B. tarandi* producen signos clínicos similares en sus respectivos hospedadores intermediarios, bóvidos, équidos, cabras y cérvidos respectivamente (Olias et al., 2011). De hecho, diversos estudios han cuestionado su diferenciación específica. Esto se debe, principalmente, al desconocimiento de la identidad del hospedador definitivo, la ausencia de diferencias ultraestructurales (Dubey et al., 2003a) y a las reacciones cruzadas serológicas observadas entre *B. besnoiti* y *B. tarandi* (Gutiérrez-Expósito et al., 2012) y entre *B. besnoiti* y *B. bennetti* (Ness et al., 2012). Además, diversos estudios moleculares han demostrado que estas especies están muy relacionadas entre sí y que poseen idénticas regiones ITS-1, 18S y 5,8S del ARN ribosómico (Ellis et al., 2000; Schares et al., 2009; Olias et al., 2011). Sin embargo, el estudio de microsatélites ha

permitido diferenciar estas cuatro especies e incluso establecer diferencias entre distintos aislados de *B. besnoiti* (Madubata et al., 2012; Gutiérrez-Expósito et al., 2016).

En los años sesenta y ochenta del siglo pasado se obtuvieron en Sudáfrica los primeros aislados de *B. besnoiti* procedentes de ungulados infectados naturalmente (Bigalke, 1967; 1968; Basson et al., 1970), aunque ninguno de éstos se ha conservado hasta la actualidad.

Sin embargo, durante los últimos años se han obtenido numerosos aislados de origen bovino a partir de biopsias de animales infectados crónicamente (Bb-Israel, Bb1-Evora 03, Bb2-Evora 03, Bb-Spain 1, Bb-Spain 2, Bb-GER 1, Bb-Italy 1, Bb-IPZ-1-CH, Bb-IPZ-2-CH, Bb-IPZ-3-CH, Bb-French) (Dubey et al., 2003a; Cortes et al., 2006b; Schares et al., 2009; Fernández-García et al., 2009b; Gentile et al., 2012; Basso et al., 2013; Frey et al., 2016). Muchos de estos aislados presentan una identidad genética idéntica en las secuencias ribosomales 18S e ITS-1 (Schares et al., 2009), con escasas variaciones en alguno de sus microsátelites (Gutiérrez-Expósito et al., 2016; Nieto-Rodríguez et al., 2016). Sin embargo, se desconoce si estas diferencias moleculares conllevan asociadas diferencias respecto a la virulencia de los distintos aislados.

Tabla 1. Especies del género *Besnoitia*.

Especie	Hospedador intermediario	Hospedador definitivo	Localización geográfica	Referencias
B. besnoiti	Bovinae / antilopinae	Desconocido	África, Asia, Europa, Venezuela	Besnoit y Robin, 1912 Dubey et al., 2003a
B. bennetti	Equidae	Desconocido	África, Francia, EE.UU.	Dubey et al., 2005
B. caprae	Caprinae	Desconocido	Kenia, Irán, Nigeria	Oryan et al., 2014
B. tarandi	Cervidae	Desconocido	EE.UU., Canadá, Finlandia, Rusia, Suecia	Dubey et al., 2004
B. akodoni	Cricetinae[1]	Desconocido	Brasil	Dubey et al., 2003c
B. jellisoni	Neotominae[2]	Desconocido	EE.UU.	Jellison, 1956
B. darlingi	Didelphinae[3]	Gato y lince rojo	EE.UU., Panamá	Dubey et al., 2002
B. neotomofelis	Cricetinae[4]	Gato	EE.UU.	Dubey y Yabsley , 2010
B. oryctofelisi	Leporidae[5]	Gato	Argentina	Dubey y Lindsay, 2003 Dubey et al., 2003b
B. wallacei	Murinae[6]	Gato	EE.UU., Japón, Kenia	Wallace y Frenkel, 1975 Ngabga, 1994

1: *Akodon montensis*; **2:** *Dipodomys* spp., *Peromyscus maniculatus*; **3:** *Didelphis marsupialis*; **4:** *Neotoma micropus*; **5:** *Oryctolagus cuniculus*; **6:** *Rattus rattus.*

1.2. Ciclo biológico y transmisión

El ciclo biológico de *B. besnoiti* no se conoce con exactitud. Se considera heteroxeno, donde bóvidos (Pols, 1960) o antílopes (Basson et al., 1965) actuarían como hospedadores intermediarios. En ellos se desarrollarían los dos estadios conocidos del parásito: el taquizoíto y el bradizoíto responsables, respectivamente, de la fase aguda y crónica de la enfermedad. Los taquizoítos se multiplican rápidamente en las células endoteliales de los vasos sanguíneos. Los bradizoítos se agrupan y multiplican lentamente acantonándose en el interior de quistes tisulares, algunos de ellos macroscópicos, que se localizan principalmente en el tejido conjuntivo subcutáneo (fibroblastos, miofibroblastos e histiocitos). Ambos estadios son infectantes para los hospedadores intermediarios, como se ha demostrado en diversas infecciones experimentales (Pols, 1960; Bigalke, 1967; 1968; Diesing et al., 1988).

Recientemente se ha estudiado el papel de los rumiantes silvestres que conviven con el ganado bovino en el ciclo biológico del parásito. Un corzo procedente de los Pirineos (área donde la enfermedad es endémica) manifestó signos clínicos compatibles con la besnoitiosis crónica y se pudo identificar al parásito en el interior de quistes tisulares. Por lo que estos animales pueden actuar, excepcionalmente, como hospedadores intermediarios de la enfermedad (Arnal et al., 2016). Además, se detectó la presencia de anticuerpos en ciervos procedentes también de zonas donde la enfermedad es endémica, lo cual indicaría una cierta circulación del parásito en estos animales (Arnal et al., 2016; Gutiérrez-Expósito et al., 2016). La implicación de los pequeños rumiantes (ovejas y cabras) en el ciclo biológico del parásito parece descartada debido a la ausencia de anticuerpos séricos frente a *Besnoitia* spp. en estos animales (Gutiérrez-Expósito et al., 2017b).

El hospedador definitivo aún no se conoce con exactitud. Por similitud con otras especies de *Besnoitia* (*B. darlingi*, *B. wallacei*, *B. oryctofelisi* y *B. neotomofelis*) (Tabla 1) se ha considerado al gato doméstico (*Felis silvestris catus*) como potencial hospedador definitivo de *B. besnoiti*. Sin embargo, de las pocas infecciones experimentales realizadas hasta el momento (Diesing et al., 1988; Basso et al., 2011), únicamente Peteshev et al. (1974) detectaron ooquistes en las heces de tres gatos alimentados con tejido de ganado bovino con besnoitiosis crónica. Los ooquistes, eliminados a partir de los 13-16 días pi durante un corto periodo de tiempo (3-5 días), fueron, al parecer, infectantes para marmotas, ratones, cabras, ovejas y terneros, los cuales desarrollaron posteriormente la enfermedad. La ausencia de datos sobre el manejo, método de cría y alimentación de los animales, así como la ausencia de métodos serológicos o moleculares que confirmaran los hallazgos hacen que estos resultados sean difícilmente explicables (Basso et al., 2011). De hecho, estudios similares realizados posteriormente no pudieron confirmarlos. Diesing et al. (1988) alimentaron varias especies de reptiles, aves y mamíferos, incluidos cuatro gatos de edad desconocida, con tejidos que contenían quistes de *B. besnoiti*. Ninguna de estas especies, incluidos los gatos, eliminaron en sus heces formas compatibles con ooquistes. Solo un leopardo (*Panthera pardus*) excretó en sus heces unos pocos

ooquistes de tipo *Cystoisospora* spp., no pudiéndose identificar posteriormente a la especie implicada. Basso et al. (2011) realizaron una infección experimental similar en cinco gatos adultos infectados con taquizoítos de *B. besnoiti* de la cepa Bb-GER 1 (dos gatos) y con bradizoítos contenidos en quistes de tejido de piel de vacas infectadas naturalmente (tres gatos). Aunque no se observaron ooquistes de *B. besnoiti* en las heces, dos de los tres gatos alimentados con bradizoítos desarrollaron anticuerpos específicos frente a *B. besnoiti* detectados mediante inmunofluorescencia indirecta (IFI) desde la semana cinco después de la infección y que, posteriormente, fueron confirmados por Western blot (WB).

Por lo tanto, hasta el momento, se han identificado en el hospedador intermediario dos estadios diferentes del parásito: los taquizoítos (endozoítos) y los bradizoítos (cistozoítos). Un hipotético hospedador definitivo eliminaría ooquistes que, aunque no han sido observados en *B. besnoiti*, se piensa que serían similares a los descritos en otras especies de *Besnoitia*. Los ooquistes se eliminarían sin esporular con las heces del hospedador definitivo, esporulando en el ambiente cuando las condiciones de temperatura, humedad relativa y oxigenación fueran adecuadas. Los ooquistes esporulados tendrían un tamaño aproximado de entre 10 y 14 µm con dos esporoquistes albergando cuatro esporozoítos cada uno, según lo observado en los de *B. darlingi* (Dubey et al., 2002) y *B. neotomofelis* (Dubey y Yabsley, 2010).

El taquizoíto y el bradizoíto son morfológicamente muy similares entre sí (Figura 1A y 1B). Estos zoítos presentan forma ovoide o de media luna, con un extremo anterior acabado en punta y un extremo posterior redondeado. Su ultraestructura se parece a la ya descrita para otros protozoos apicomplejos (Dubey, 1998; Speer et al., 1999). Están rodeados por una película formada por tres capas: la membrana externa (plasmalema) que rodea la superficie y una doble capa interna que forma el complejo de membrana interno (Langenmayer et al., 2015b). Presentan organelas típicas de la célula eucariota (núcleo, retículo endoplasmático, aparato de Golgi y mitocondria), así como otras organelas (apicoplasto, micronemas, roptrias y gránulos densos) y estructuras (anillos polares anterior y posterior, conoide, plasmalema y complejo interno de membrana) propias de los Apicomplexa (Figura 1C).

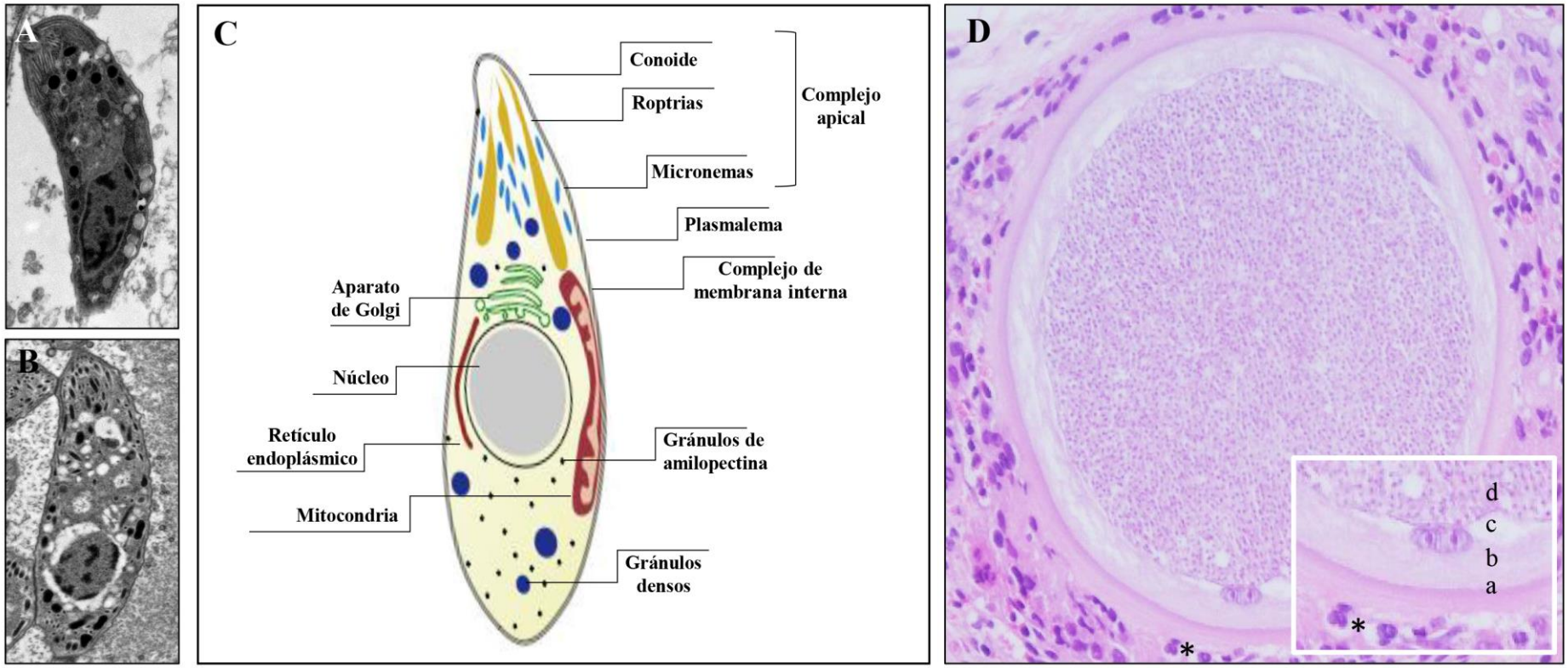
Los taquizoítos, formas proliferativas del parásito, se multiplican en el interior de las células endoteliales de los vasos sanguíneos, aunque también pueden invadir otras células como histiocitos, neutrófilos, fibroblastos y hepatocitos (Pols, 1954; Basson et al., 1970; Schares et al., 2009; Muñoz-Caro et al., 2014; Langenmayer et al., 2015c). Se piensa que los taquizoítos invaden la célula hospedadora de forma similar a como lo hacen otros Toxoplasmatinae, con una secuencia de procesos que forman parte de lo que se denomina ciclo lítico y que incluyen la adhesión e invasión de los taquizoítos en la célula, su multiplicación intracelular y, finalmente, su liberación, tras la lisis celular, en un proceso llamado egresión (Black y Boothroyd, 2000).

En cambio, los bradizoítos se acantonan en el interior de quistes tisulares localizados en el tejido conjuntivo del hospedador intermediario, principalmente en la piel y en las mucosas y, en menor

medida, en otras localizaciones como tendones, tejido vascular, esclerótica, tracto respiratorio superior, testículo y epidídimo en los machos y vestíbulo vaginal en las hembras (Kumi-Diaka et al., 1981; Basso et al., 2013; Frey et al., 2013; Langenmayer et al., 2015c). El tamaño del quiste varía según el tiempo de infección transcurrido (Bigalke, 1968). El crecimiento de los quistes parece asincrónico, por lo que sería posible detectarlos simultáneamente con signos clínicos compatibles con la fase aguda de la enfermedad (Basson et al., 1970; Gollnick et al., 2015). A las 4 semanas pi pueden aparecer quistes en la piel con un tamaño de hasta 100 µm de diámetro. Éstos evolucionan hasta alcanzar su máximo desarrollo como quistes maduros (330-390 µm de diámetro) a los 70 días pi (Basson et al., 1970). La pared de un quiste desarrollado está formada por dos capas: una capa externa que contiene múltiples fibras de colágeno entrelazadas y una capa interna formada por proteoglicanos y extensiones filamentosas que se proyectan hacia la membrana externa (Langenmayer et al., 2015b). El citoplasma de la célula hospedadora aparece deformado y contiene el núcleo y las diferentes organelas celulares. La vacuola parasitófora está inmediatamente por debajo del citoplasma celular y limitada por una membrana de estructura granular. En su interior se encuentran numerosos bradizoítos en diferente estado evolutivo (Langenmayer et al., 2015b) (Figura 1D). Los quistes tisulares maduros pueden contener hasta 200.000 bradizoítos (Bigalke, 1968).

Se han observado también quistes degenerados, que son aquellos que han perdido su morfología esférica y presentan la pared externa rota, con un infiltrado de células inflamatorias y los bradizoítos en su interior son difíciles de identificar correctamente (Frey et al., 2013). Los quistes tisulares degenerados se pueden comenzar a observar a partir de los 30 días pi (Basson et al., 1970).

Figura 1: Morfología de *B. besnoiti*. Taquizoíto **(A)** y Bradizoíto **(B)** (Microscopía electrónica de transmisión) (Nótese en el bradizoíto la cantidad de gránulos de amilopectina con respecto al taquizoíto); Estructura general de un zoíto apicomplejo **(C)**; Bradizoítos en el interior de un quiste tisular y estructura de la pared **(D)** (Hematoxilina-eosina, x40) (**a:** capa externa; **b:** capa interna; **c:** núcleo desplazado de la célula hospedadora; **d:** bradizoítos).



*Fuente: A, B y C (Doctoral Thesis, García-Lunar, 2015b).

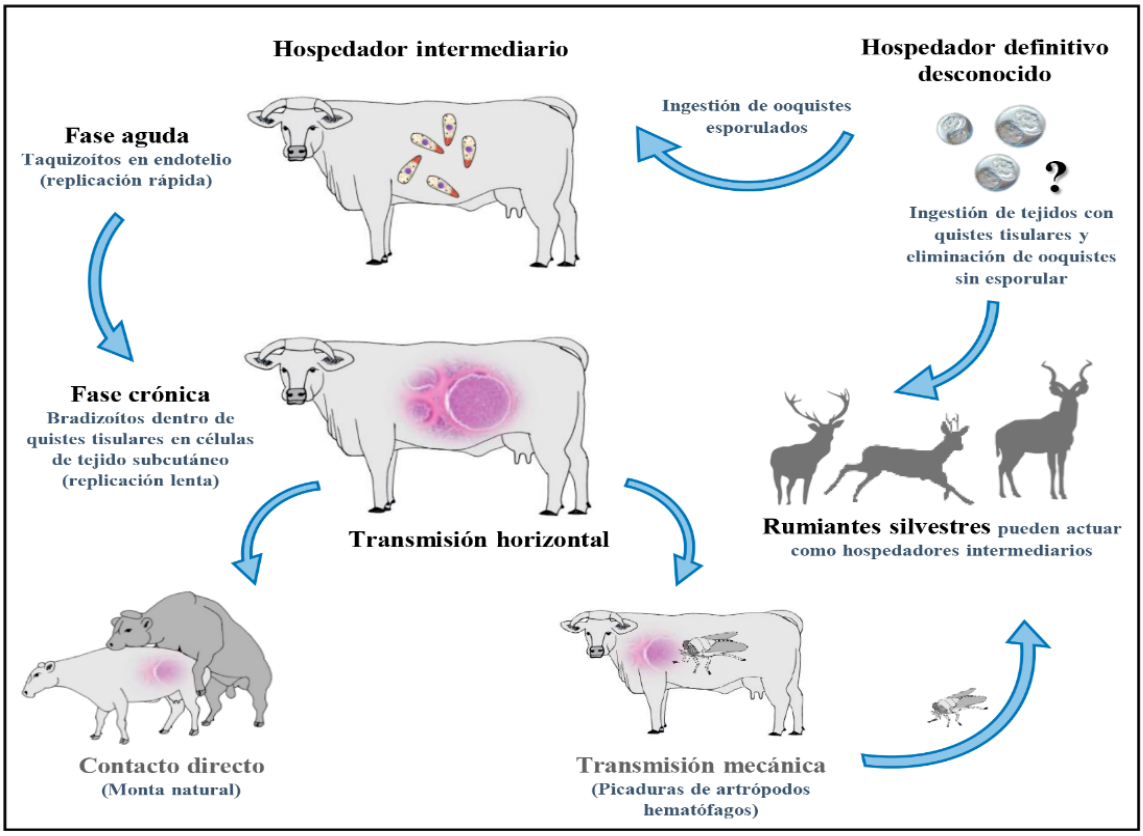
Los datos epidemiológicos disponibles sugieren que el principal modo de transmisión de *B. besnoiti* es el horizontal (Álvarez-García et al., 2013), bien por contacto directo (p.ej. monta natural, laceraciones) o por transmisión mecánica a través de vectores artrópodos de las familias Tabanidae y Muscidae. La transmisión horizontal por ingestión de ooquistes no se ha descrito todavía puesto que no se ha identificado el hospedador definitivo y la transmisión venérea parece improbable (Figura 2).

Con respecto a la transmisión horizontal, se ha visto que el estadio de bradizoíto es capaz de atravesar la barrera mucosa (Bigalke, 1968). Dado que los quistes tisulares se localizan superficialmente en la mucosa del aparato reproductor masculino y femenino (Nobel et al., 1981; Kumi-Diaka et al., 1981), éstos podrían romperse durante la monta natural y los bradizoítos al ser liberados, serían capaces de infectar a un nuevo hospedador. Es por ello que el semental podría suponer un riesgo para las hembras o viceversa, que hembras infectadas supongan un riesgo para el semental durante el periodo de monta. Del mismo modo, la distribución mayoritaria de los quistes en la zona más superficial de la dermis (dermis papilar) (McCully et al., 1966; Dubey et al., 2013; Langenmayer et al., 2015c) facilitaría la exposición de los bradizoítos al medio externo tras la ruptura de éstos por heridas o excoriaciones.

En la transmisión mecánica a través de artrópodos hematófagos, tanto los animales con infección aguda (taquizoítos en sangre circulante) como los animales con infección crónica (quistes tisulares en la piel) podrían ser una fuente de infección. Diferentes especies de tábanos y *Stomoxys* pueden transmitir el parásito de forma mecánica, si bien es cierto que la diseminación del parásito a largas distancias parece bastante improbable ya que se piensa que el tiempo de supervivencia del parásito en los insectos es limitado (Bigalke, 1968). Otros autores también detectaron ADN de *Besnoitia* spp. en *Stomoxys calcitrans* que habían estado en contacto con la piel de un animal infectado crónicamente (Liénard et al., 2012) o que fueron recolectadas durante un estudio de cohabitación con animales infectados por *B. besnoiti* y no infectados (Gollnick et al., 2015). Recientemente, se ha revaluado el papel de *Stomoxys calcitrans* como vector de *B. besnoiti*. *Stomoxys calcitrans* que estuvieron en contacto con vacas infectadas crónicamente fueron capaces de transmitir el parásito a un conejo (especie susceptible a la enfermedad) que desarrolló signos clínicos característicos de besnoitiosis aguda (Sharif et al., 2019). Sin embargo, parece que es necesario un número elevado de picaduras para que se produzca la transmisión. Hornok et al. (2015) sugirieron que además de la transmisión mecánica de *B. besnoiti* por las moscas hematófagas, las moscas secretófagas también deberían evaluarse como vectores potenciales. Este autor observó un alto riesgo de propagación de la enfermedad en la temporada de mayor actividad de estas moscas cuando los terneros permanecieron durante la lactancia con sus madres infectadas.

La transmisión por consumo de ooquistes ha sido estudiada en varias ocasiones a la vez que se intentaba esclarecer el ciclo biológico completo del parásito. Sin embargo, esto no se ha conseguido en las infecciones experimentales mencionadas anteriormente (Diesing et al., 1988; Basso et al., 2011).

Figura 2: Ciclo biológico y transmisión de *B. besnoiti.*



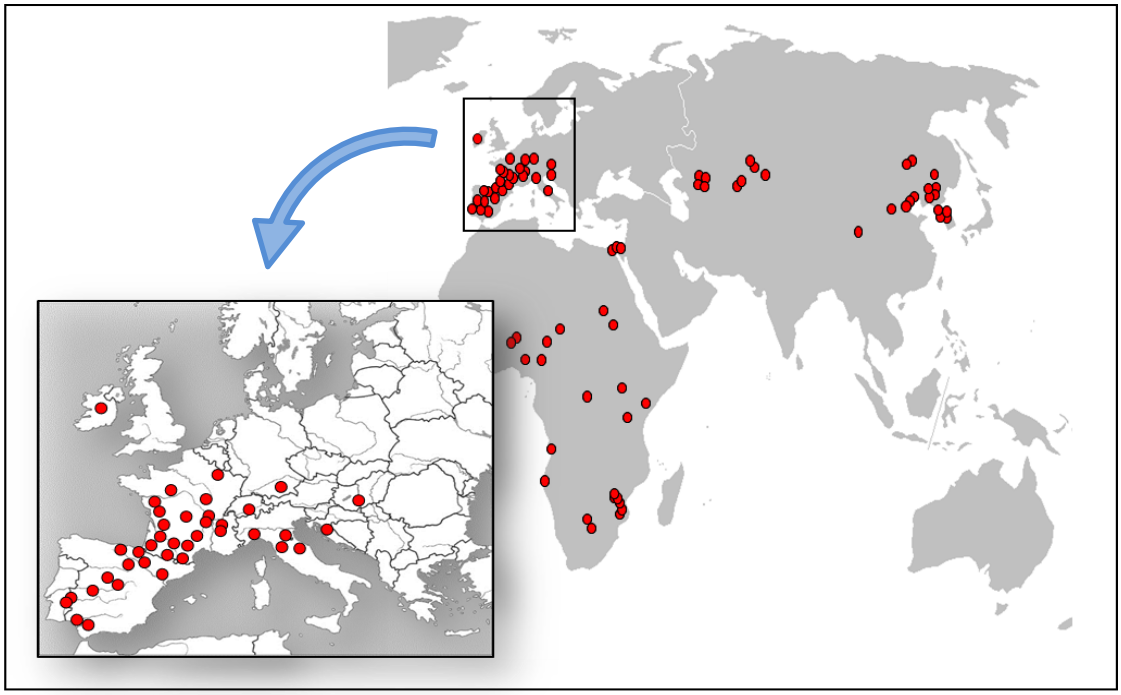
A pesar del tropismo que ha mostrado el parásito por el tracto reproductivo de los machos infectados (Kumi-Diaka et al., 1981), no se ha detectado ADN del parásito en el semen de toros seropositivos con o sin signos clínicos evidentes de besnoitiosis crónica (Esteban-Gil et al., 2014). Por ello, la transmisión de la enfermedad por esta vía parecería descartada. No obstante, la presencia de ADN del parásito en semen tampoco confirmaría la transmisión sexual ya que otros factores como la viabilidad del parásito serían determinantes para causar una infección. La transmisión vertical de madre a hijo por vía transplacentaria o por ingestión de calostro no ha sido confirmada, aunque también parece poco probable. Hornok et al., (2014) descartaron la transmisión vertical al no detectar anticuerpos precalostrales en 15 terneros nacidos de madres seropositivas y la ausencia de ADN de *B. besnoiti* en el calostro. Anteriormente, Shkap et al. (1994) tampoco detectaron anticuerpos precalostrales en terneros cuyas madres estaban infectadas crónicamente, si bien, tras el encalostrado los títulos de anticuerpos medidos por IFI oscilaron entre 1:64 y 1:1024.

1.3. Distribución geográfica.

La besnoitiosis bovina fue descrita por primera vez en Sudáfrica (Hofmeyr, 1945), siendo ampliamente estudiada desde los años 60 hasta los 80 del siglo pasado (Pols, 1960; Bigalke, 1968). Posteriormente, fue reportada en otros países del África Sub-sahariana como Botsuana, Namibia, Zimbabue, Angola, Congo, Kenia, Tanzania, Uganda, antigua República del Sudán, Camerún, Nigeria y, recientemente, Ruanda (Hofmeyr, 1945; Chatikobo et al., 2009; Habarugira et al., 2019). Además, se ha descrito en algunas regiones de Asia (China, Corea, Jordania, Israel, Kazajistán, Rusia y Uzbekistán) (Lee et al., 1970; Olias et al., 2011; Talafha et al., 2015) y en Venezuela (Vogelsang y Gallo, 1941).

En Europa, durante años la besnoitiosis bovina estuvo restringida a los Pirineos y a la zona del Alentejo en Portugal (zonas endémicas de la enfermedad) (Besnoit y Robin, 1912; Leitao, 1949; Bourdeau et al., 2004), dónde paso desapercibida hasta los años 90 del siglo XX. Desde entonces, la enfermedad se ha diseminado desde las zonas endémicas del Alentejo portugués (Cortes et al., 2004; 2005; 2006b) y en la zona centro, este y oeste de Francia (Alzieu, 2007; Jacquiet et al., 2010). En 1994 se detectaron animales infectados en el ganado exportado a Italia desde Francia (Agosti et al., 1994) y, desde 2009, se han descrito números casos de la enfermedad en la zona norte de los Montes Apeninos, donde ya se considera que la besnoitiosis bovina es endémica (Gentile et al., 2012). En los últimos años, se han descrito varios brotes de la enfermedad asociados al movimiento de animales infectados desde zonas donde la enfermedad es endémica. Como en otras regiones de Portugal (Malta y Silva, 1991; Waap et al., 2014), Alemania, Suiza y Croacia (Majzoub et al., 2010; Rostaher et al., 2010; Lesser et al., 2012). Tras la importación de 178 animales aparentemente sanos procedentes de Francia se describieron en Hungría los primeros casos clínicos de besnoitiosis bovina, tanto en el ganado importado como en el ganado autóctono (Hornok et al., 2014). En otros países mediterráneos, como Grecia, se sospecha la presencia de la enfermedad, ya que se han detectado animales seropositivos en explotaciones lecheras que albergaban animales procedentes de Alemania, Francia y los Países Bajos (Papadopoulos et al., 2014). Una reciente descripción de la enfermedad en el ganado vacuno lechero de Irlanda ha demostrado que la enfermedad no se limita únicamente a la Europa continental, sino que también esta presente en las Islas Británicas (Figura 3). Los hallazgos clínicos hicieron sospechar que la enfermedad mostraba en el rebaño una presentación endémica, por lo que otras granjas contiguas podrían estar también infectadas. Esto pone de manifiesto la importancia de la bioseguridad y la necesidad de elaborar planes de control frente a esta enfermedad re-emergente en Europa (Álvarez-García, 2016; Ryan et al., 2016).

Figura 3: Distribución geográfica de la besnoitiosis bovina (actualización de la EFSA, 2010).



En España, los Pirineos, la Sierra de Urbasa y Andía (Navarra), la zona del Maestrazgo (Teruel), Sierra Norte de Guadalajara, Extremadura, Sierra de Madrid, La Rioja y diversas zonas de Castilla-León (Salamanca, Ávila) también se consideran en la actualidad zonas donde la besnoitiosis bovina es endémica. Además, se han descrito brotes en otras zonas del norte, centro y suroeste de España (Fernández-García et al., 2010; Álvarez-García et al., 2013; Nieto-Rodríguez et al., 2016), lo que pone en evidencia su notable expansión dentro de nuestro país.

1.4. Prevalencia e incidencia

Los primeros estudios sobre la prevalencia de la besnoitiosis bovina fueron realizados en Sudáfrica, describiéndose tasas de prevalencia entre 10 y 50% tras detectar quistes en la conjuntiva ocular y la presencia de lesiones en la piel (Bigalke, 1968). Cuando se utilizaron, además, técnicas serológicas, por ejemplo, en Israel y Sudáfrica, se reportaron tasas de prevalencia mayores, que oscilaron entre 64,4 y 66,9% en ganado de carne de Israel (Neuman, 1972; Frank et al., 1977) y del 50% en animales sin signos clínicos en Sudáfrica (Janitschke et al., 1984). En relación a estudios más recientes, se han descrito tasas de seroprevalencia individual del 13,5% en Egipto usando técnicas ELISA (Ashmawy y Abu-Akkada, 2014). En Jordania se han reportado tasas de seroprevalencia individual y de rebaño del 6% y del 28,7%, respectivamente, en ganado vacuno lechero que no mostraba signos clínicos compatibles con besnoitiosis (Talafha et al., 2015).

Respecto a las tasas de prevalencia serológica y de presencia de signos clínicos en zonas de Europa, la besnoitiosis bovina fue detectada en la década de los 90 del siglo XX en una explotación localizada en el noroeste de Italia que albergaba ganado de carne procedente de Francia (Agosti et al.,

1994). Posteriormente, Gentile et al. (2012) realizaron el seguimiento de la enfermedad en Italia y describieron los primeros casos autóctonos, notificando una seroprevalencia de 41,2% con un 23,4% de los animales mostrando signos clínicos. Sin embargo, un estudio posterior, describió una baja prevalencia de la enfermedad (0,3% y 3,9% de seroprevalencia individual e intra-rebaño, respectivamente) en la zona noroeste (Lombardia, Piemonte y Liguria) y en la isla de Cerdeña (Gazzonis et al., 2014). En Francia se ha observado una tasa de seroprevalencia intra-rebaño del 50% en una explotación de leche localizada en una zona libre de la enfermedad (Genest, 2008). En otra explotación localizada en el noroeste de Francia, zona donde también la enfermedad es endémica, se describió una seroprevalencia individual de 33,8% con 10% de los animales mostrando signos clínicos. El seguimiento de la misma explotación durante 14 meses reveló un aumento de la seroprevalencia (89,5%) y un mantenimiento del porcentaje de animales con signos clínicos (12,3%) (Liénard et al., 2011). En la zona sur de Portugal se describió que hasta 42% de los sementales bovinos investigados y aparentemente sanos presentaban quistes de *B. besnoiti* detectables por histopatología (Cortes et al., 2005). En la región del Alentejo (zona en la cual la enfermedad es endémica) se describió una seroprevalencia del 5,1% y una seroprevalencia media intra-rebaño del 33% (Waap et al., 2014). En un estudio reciente en la zona de Baviera y realizado en el primer rebaño de vacuno de carne infectado naturalmente con *B. besnoiti* en Alemania, se detectaron tasas altas tanto de seroprevalencia (89,4-100%) como de prevalencia clínica (23,5-36,6%) (Gollnick et al., 2018). El 42% de los animales de una explotación estaban infectados y el 37% mostraban signos clínicos en un brote detectado en Croacia (Beck et al., 2013). En otra explotación del este de Hungría se observó que 12 animales estaban infectados y que 10 de ellos mostraban signos clínicos de besnoitiosis bovina, aunque en este estudio el resto del rebaño no fue investigado (Hornok et al., 2014). Se ha descrito

también la infección endémica en una granja de vacuno lechero en Irlanda, donde se detectó una seroprevalencia de 68% y la presencia de quistes en hasta 50% de los animales en la conjuntiva ocular y hasta 68% de las hembras en la mucosa vulvar (Ryan et al., 2016). El parásito circula por otros países europeos como Grecia, dónde se reportaron datos de seroprevalencia individual de hasta el 22% sin detectar animales con signos clínicos (Papadopoulos et al., 2014). En este tipo de estudios, sin clínica evidente y seroprevalencias altas, una combinación de técnicas serológicas sería lo más adecuado para obtener datos fiables sobre la presencia real de la enfermedad.

Con respecto a España, diversos estudios han puesto de manifiesto que la enfermedad se encuentra ampliamente distribuida en zonas endémicas de la misma. Tras realizar un estudio transversal en ganado bovino de carne de Navarra se describió una tasa de seroprevalencia individual del 16%. Cabe destacar que los animales seropositivos estaban ubicados en zonas de montaña donde previamente se habían detectado casos clínicos de la enfermedad (Sierra de Urbasa y Andía y los Pirineos) (Álvarez-García et al., 2014a). Tras analizar un total de 3.211 vacas de 63 rebaños y todos los sementales (587) del Pirineo oscense, se estimó una prevalencia del 48,7% en machos y 51,9% en hembras; y una prevalencia de rebaño del 87,3% (Gutiérrez-Expósito et al., 2017a). En un estudio

reciente realizado en el Pirineo catalán, se han descrito prevalencias individuales del 25,1% y de rebaño del 46% en ganado vacuno extensivo (Garrido-Castañé et al., 2019). Fernández-García et al. (2010) analizaron un brote de la enfermedad en la Sierra de Guadalajara, reportando hasta el 90,8% de las hembras y el 71,4% de los machos seropositivos. Además, 43,2% de los animales presentaron algún signo clínico compatible con la besnoitiosis aguda o crónica. En otro brote descrito recientemente en el suroeste de España, la prevalencia serológica fue del 40% (Nieto-Rodríguez et al., 2016).

En relación a la incidencia de la enfermedad, se han realizado dos estudios en zonas donde la enfermedad es endémica con un periodo de observación de cuatro años en ambos casos. En el primer estudio, realizado en España en la Sierra de Urbasa y Andía, se describió una tasa de incidencia serológica del 23,5 por 100 animales-año, junto con una incidencia en la presencia de signos clínicos de entre 12,5 y 16,7 por 100 animales-año (Gutiérrez-Expósito et al., 2017a). Mayores tasas de incidencia serológica (40,5 por 100 animales-año) y tasas muy similares de incidencia clínica (16,7 por 100 animales-año) se detectaron en otro estudio longitudinal en un rebaño con besnoitiosis endémica en la zona Baviera (Alemania) (Gollnick et al., 2018). Ambos estudios han puesto de manifiesto un incremento considerable en la trasmisión de la enfermedad en ausencia de planes de control específicos.

1.5. Factores de riesgo

El poder identificar los factores de riesgo asociados con la enfermedad tiene una gran importancia a la hora de evaluar e implantar medidas de control adecuadas. Esto es especialmente importante en la besnoitiosis bovina, ya que la mayoría de los factores de riesgo asociados a la infección están estrechamente relacionados con la aptitud ganadera y el sistema de manejo; y en menor medida con factores individuales como el sexo y la raza (Álvarez-García et al., 2013).

El movimiento de animales infectados desde zonas donde la enfermedad es endémica a zonas libres de la enfermedad supone el mayor riesgo para su diseminación. Influyen notablemente las prácticas asociadas al manejo de razas de aptitud cárnica, donde se practica la monta natural y en las que los animales comparten pastos, ya que puede existir mayor contacto directo entre ganado infectado y sano, así como con otros posibles reservorios silvestres. Se ha descrito también cierta estacionalidad de la enfermedad, ya que la mayoría de los casos nuevos ocurren durante los meses cálidos cuando el ganado comparte pastos (Alzieu, 2007; Fernández-García et al., 2010; Gutiérrez-Expósito et al., 2017a) y los artrópodos hematófagos, que pueden actuar como vectores del parásito, están en su periodo de máxima actividad (Hornok et al., 2015).

Con respecto a los factores individuales, se ha observado un incremento de la seroprevalencia conforme aumenta la edad de los animales (Bigalke, 1968; Fernández-García et al., 2010). Los animales adultos tienen más posibilidades de infectarse, ya que el tiempo de exposición es mayor que en los jóvenes. Sin embargo, Hornok et al. (2014) detectaron la presencia de anticuerpos frente a *B.*

besnoiti en animales con edades comprendidas entre los seis y los 12 meses. La explicación de este hecho podría ser la detectión de anticuerpos calostrales que, en principio, podrían ser protectores (Shkap et al., 1994). Sin embargo, los signos clínicos de la enfermedad se suelen observar con mayor frecuencia en animales de dos a cuatro años de edad en comparación con animales mayores. De hecho, los animales infectados crónicamente pueden experimentar una aparente disminución de la gravedad de los signos clínicos, pero permanecen infectados de por vida y sus producciones quedan mermadas (Kumi-Diaka et al., 1981; Nobel et al., 1981). Se ha observado que en zonas donde la enfermedad es endémica, un alto porcentaje de animales infectados que presentan anticuerpos específicos frente a *B. besnoiti* no presentan signos clínicos detectables, es decir, son animales portadores del parásito (Gutiérrez-Expósito et al., 2017a).

En cuanto a las razas, todas son susceptibles a la infección por *B. besnoiti*, sin embargo, se ha descrito principalmente en razas de aptitud cárnica, asociada a las prácticas de manejo (Álvarez-García et al., 2014b). De hecho, los trabajos que describen la enfermedad en explotaciones lecheras son escasos (Goldman y Pipano, 1983; Ryan et al., 2016; Garrido-Castañé et al., 2019). Nieto-Rodríguez et al. (2016) han reportado diferencias de susceptibilidad entre razas locales (Avileña) e importadas (Limousin), ya que los únicos animales que manifestaron signos clínicos fueron los que pertenecían a esta última raza. Aunque esto debería ser estudiado con mayor profundidad ya que también se ha descrito la enfermedad en rebaños de raza Avileña (Carvajal-Valilla et al., 2017). El sexo no se ha considerado un factor de riesgo ya que tanto machos como hembras presentan similares tasas de seroprevalencia. Sin embargo, en los sementales los signos clínicos parecen ser más graves o se detectan con mayor facilidad (Gutiérrez-Expósito et al., 2017c). Varias infecciones experimentales realizadas en el pasado con animales inmunosuprimidos sugieren que ciertas situaciones fisiológicas o enfermedades concomitantes que provoquen inmunosupresión (alteración de la respuesta inmunitaria) podrían ser responsables de una mayor gravedad de la enfermedad (Bigalke, 1968; Álvarez-García et al., 2014b).

1.6. Patogenia, signos clínicos y lesiones

Después de la infección y tras un periodo de incubación de 1 a 13 días de duración, la enfermedad se desarrolla en dos fases consecutivas. En primer lugar, una fase aguda producida por la multiplicación rápida del parásito (taquizoítos) en el interior de las células endoteliales de los vasos sanguíneos. En segundo lugar y, a continuación de la anterior, una fase crónica o de escleroderma producida por la multiplicación lenta del parásito (bradizoítos en el interior de quistes tisulares) en células de origen mesenquimatoso del tejido conectivo (fibroblastos y miofibroblastos) o en histiocitos (Pols, 1960; Álvarez-García et al., 2014b). La muerte puede ocurrir tanto en la fase aguda como en la fase crónica de la enfermedad, aunque las tasas de mortalidad son bajas (1%) (Jacquiet et al., 2010).

La fase aguda febril de la enfermedad, que dura aproximadamente 12-13 días, se caracteriza por la aparición de signos clínicos inespecíficos que pueden pasar desapercibidos, como hipertermia (40,8-41,6ºC), taquicardia, taquipnea, linfadenitis, disminución de la ingesta voluntaria de alimento y pérdida de peso (Álvarez-García et al., 2014b). En la hembra gestante, el cuadro febril puede ocasionar aborto. Además, puede aparecer hiperemia conjuntival, lacrimeo, fotofobia y descarga ocular y nasal mucopurulentas (Figura 4A). La proliferación de taquizoítos en las células endoteliales de los vasos sanguíneos puede producir un fallo circulatorio caracterizado por vasculitis, trombosis y necrosis de vénulas y arteriolas. En los casos graves, aparecen edemas alveolares e intersticiales en pulmones con los consiguientes signos respiratorios, llegando a producir la muerte del animal en escasas ocasiones. A esta fase le sigue una fase aguda de anasarca, que puede prolongarse hasta las 4-5 semanas pi y que se caracteriza por la presencia de edemas que se localizan fundamentalmente en la cabeza y el cuello; y se desplazan hacia la parte inferior del animal, como pecho, papada, prepucio, ubre y extremidades (Figura 4B). En los machos es frecuente la aparición de orquitis (Figura 4C).

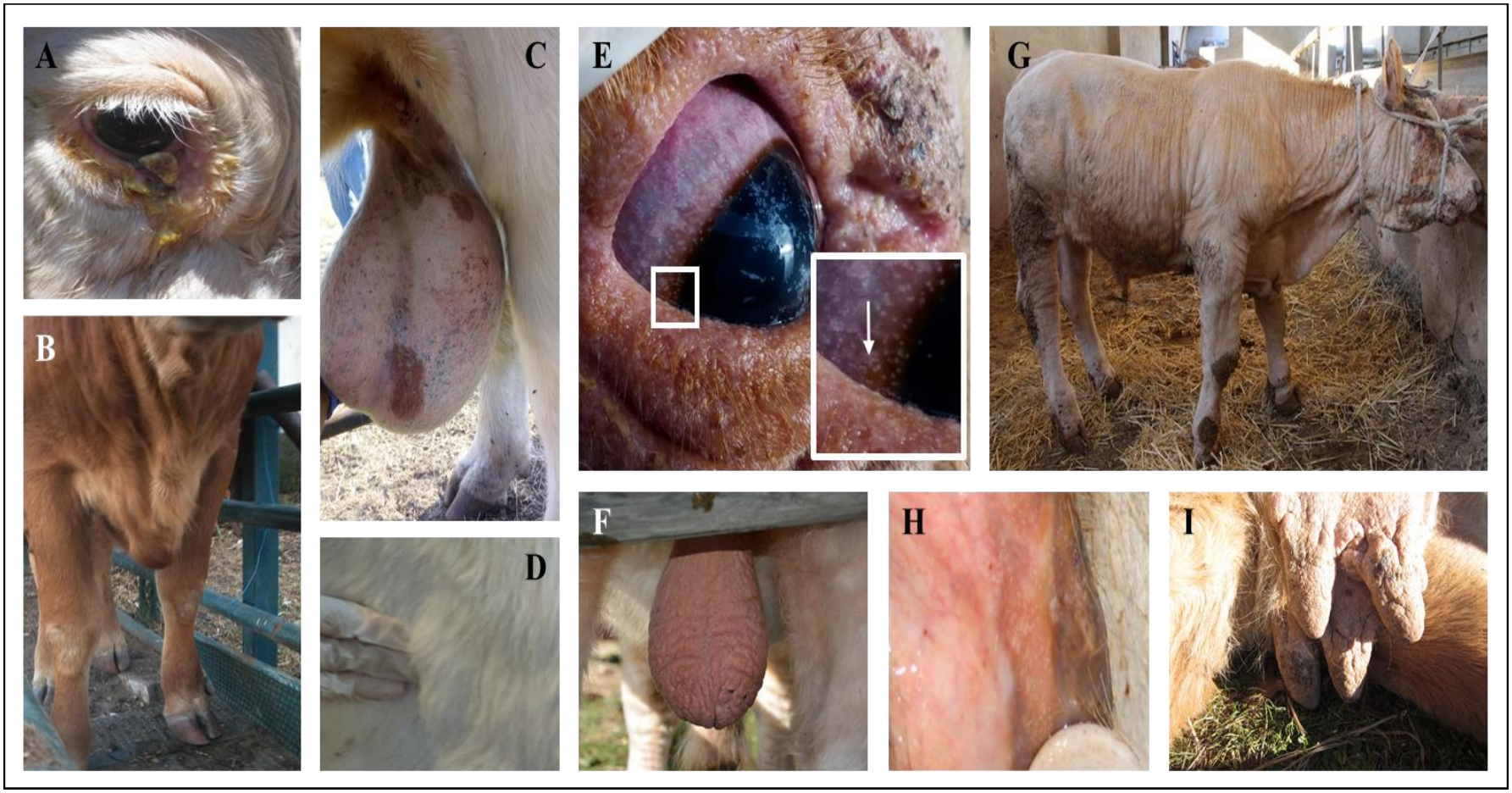
Posteriormente, en la fase crónica, conocida como fase de escleroderma, se puede producir un deterioro gradual de la condición corporal. A partir de las 6-7 semanas pi se pueden observar quistes macroscópicos que aparecen inicialmente en las conjuntivas palpebral y esclerótica y, posteriormente, en el vestíbulo vaginal (Figura 4E y H). Estos quistes también se desarrollan en el endotelio, dermis, fascias, mucosas principalemente del tracto respiratorio superior y aparato genital tanto de machos como de hembras. Como consecuencia del desarrollo de quistes en el tejido subcutáneo, se puede observar en la piel lesiones características de una besnoitiosis crónica como engrosamiento, endurecimiento y formación de pliegues en la piel (Figura 4G). En las zonas de mayor carga parasitaria se puede observar alopecia. Se ha descrito esterilidad en los machos a consecuencia de las siguientes alteraciones: i) los quistes tisulares presentan tropismo por las paredes vasculares del plexo pampíniforme produciendo una disminución del aporte sanguíneo al testículo (Kumi-Diaka et al., 1981); ii) los propios quistes tisulares localizados en los túbulos seminíferos comprimen directamente al tejido produciendo necrosis y atrofia testicular (Kumi-Diaka et al., 1981; Dubey et al., 2013) y iii) el engrosamiento de la piel del escroto por la presencia de quistes dificulta la termoregulación del testículo (Kumi-Diaka et al., 1981) (Figura 4F). En las hembras pueden aparecer nódulos y grietas en las ubres y pezones (Figura 4I) que se pueden complicar por infecciones secundarias oportunistas. Gollnick et al. (2015) describieron laminitis en animales gravemente afectados por besnoitiosis crónica debido a la presencia de quistes en el corion laminar y en los vasos de la dermis de las pezuñas.

No se han descrito alteraciones hematológicas y bioquímicas notables en los animales infectados. Solo en los casos más graves se puede presentar leucopenia y anemia, así como una disminución de la concentración de calcio y de la albúmina sérica. Se ha observado también una disminución de los valores de urea y magnesio, todo ello asociado a la reducción de la ingestión voluntaria de alimento durante los primeros días de infección (Langenmayer et al., 2015a). La destrucción de la integridad

de las fibras musculares puede ser responsable del aumento de la actividad enzimática de la aspartato aminotransferasa y de la creatinín-quinasa. Además, la degeneración muscular producida en los animales gravemente afectados causa un aumento de la creatinina. Las concentraciones altas de globulinas, tanto en la fase aguda como en la crónica de la infección, pueden ser debidas al desarrollo de anticuerpos específicos frente al parásito (Langenmayer et al., 2015a).

El número de animales que muestran signos clínicos de enfermedad aguda o crónica representan solo la punta del iceberg, tanto en zonas donde la enfermedad es endémica (1-10%) como en los brotes epidémicos (15-40%) (Legrand, 2003; Fernández-García et al., 2010; Jacquiet et al., 2010; Liénard et al., 2011). Sin embargo, en un rebaño infectado, el porcentaje más alto del efectivo (hasta un 17% y un 40% en zonas donde la enfermedad es endémica y en brotes, respectivamente) está compuesto por animales infectados con carga parasitaria baja y sin signos clínicos detectables (infección subclínica), que representan un factor de riesgo muy importante para la transmisión del parásito (Fernández-García et al., 2010; Liénard et al., 2011; Basso et al., 2013; Frey et al., 2013).

Figura 4: Signos clínicos de la besnoitiosis aguda (A, B, C, D). A: Secreción ocular; **B:** Edema en zonas declives del cuerpo; **C:** Orquitis; **D:** Linfadenomegalia. **Signos clínicos de la besnoitiosis crónica (E, F, G, H, I). E:** Quistes en la conjuntiva ocular; **F:** Orquitis necrotizante; **G:** Lesiones en la piel; **H:** Quistes en la mucosa vulvar; **I:** Grietas y nódulos en las ubres y pezones.



Fuente: **A** y **H** (Doctoral Thesis, García-Lunar, 2015b). **B**, **E** e **I** cedidas por Jesús Casablanca. **C** cedida por Javier Blanco Murcia. **F** (Gutiérrez-Expósito et al., 2017c). **G** cedida por Javier Balado Albiol.

1.7. Diagnóstico

El protocolo de diagnóstico de la besnoitiosis bovina debe estar basado, fundamentalmente, en la combinación de una adecuada inspección clínica y de un correcto diagnóstico serológico (Figura 5). El diagnóstico epidemiólogico, aunque no es determinante sino solo orientativo, nos puede proporcionar información muy relevante. Es importante conocer si la explotación se encuentra en una zona donde la enfermedad es endémica o libre de la misma. En el primer caso, se deben considerar las prácticas de manejo asociadas al sistema de explotación que favorezcan la trasmisión del parásito, tales como la existencia de monta natural, uso de pastos comunales o el grado de exposición a vectores artrópodos (la aparición de casos clínicos tras la época estival es más frecuente). En zonas libres de la enfermedad es importante prestar atención a la entrada de nuevos animales a la explotación, especialmente si la valoración sanitara previa no incluye el diagnóstico de la besnoitiosis bovina. El diagnóstico clínico de la enfermedad durante la fase aguda es complicado. La presencia de sígnos clínicos inespecíficos como hipertermia, depresión, taquicardia, taquipnea, anorexia y pérdida de peso se pueden confundir con otras enfermedades de gran importancia veterinaria como la lengua azul o las piroplasmosis, o incluso pueden ser leves y pasar desapercibidos. A medida que progresa la enfermedad, la aparición de edemas en zonas declives del cuerpo del animal, así como la presencia de orquitis pueden ser más fáciles de detectar. En la fase crónica, las lesiones en la piel son las más características (hiperqueratosis, formación de pliegues, alopecias). Sin embargo, cualquier enfermedad que curse con alteraciones cutáneas, como sarna, dermatofitosis, fotodermatitis o incluso la deficiencia de algunos oligoelementos y/o alergias, deben contemplarse en el diagnóstico diferencial (Rostaher et al., 2010). En los machos la degeneración testicular es evidente y en las hembras infectadas es posible encontrar nódulos y grietas en los pezones.

La presencia de quistes tisulares en la conjuntiva ocular y en la mucosa vulvar son patognomónicos de la enfermedad, aunque pueden ser difíciles de detectar mediante inspección visual, sobre todo cuando se presentan en un número bajo. El diagnóstico clínico debe combinarse con un diagnóstico serológico, ya que como se ha indicado anteriormente, un gran número de los animales infectados presentan una infección subclínica. Existen tanto técnicas directas para la detección de parásito o de su ADN en tejidos (Tabla 2), como técnicas serológicas (indirectas) para la detección de anticuerpos específicos (Tabla 3) (Gutiérrez-Expósito et al., 2017c).

Con respecto al diagnóstico directo en animales infectados crónicamente, la visualización de quistes en biopsias de piel mediante compresión en placas de cristal confirmaría la presencia del parásito. La biopsia de piel debe tomarse de las zonas afectadas, aunque recientemente se ha recomendado tomar las biopsias de la grupa o zona interior del muslo por su fácil acceso y por el mayor establecimiento del parásito en estas localizaciones (Schares et al., 2016). La histopatología (HP) y la inmunohistoquímica (IHQ) pueden ser otra opción para la detección de quistes en los tejidos.

Aunque son técnicas con elevada especifidad (Esp), la sensibilidad (Se) es baja en los animales con poca carga parasitaria. Por tanto, si no se detectan quistes en una biopsia y/o HP/IHQ no puede descartarse la infección (Tabla 2). Las técnicas de PCR, tanto cualitativas como cuantitivas, son las más empleadas en investigación, aunque pueden ser de utilidad en el diagnóstico laboratorial tanto para la detección del parásito en tejidos como en la sangre (Tabla 2). Por ejemplo, en la fase aguda se pueden emplear para la detección de parasitemia, ya que las técnicas serológicas que se emplean habitualmente no detectan anticuerpos al no haberse producido aún la seroconversión de los animales infectados antes de las 3-4 semanas pi (García-Lunar et al., 2013b). La detección de ADN del parásito en muestras de testículo (p. ej. piel de escroto) puede ser una buena herramienta de diagnóstico post-morten en animales que mueren durante la besnoitiosis aguda. La PCR convencional fue desarrollada por Cortes et al. (2007b) y se basa en la amplificación de un fragmento de 231 pb correspondiente a la región ITS-1 del ADN del parásito (esta es una región muy conservada en el género *Besnoitia* spp.). Posteriormente, Cortes et al. (2007b) y Schares et al. (2011b) desarrollaron una PCR cuantitativa a tiempo real que fue empleada por Frey et al. (2013) y Basso et al. (2013) para estudiar la distribución intraorgánica del parásito en animales con baja carga parasitaria.

Sin embargo, la serología es el método indirecto más empleado en el diagnóstico laboratorial de la besnoitiosis bovina. Entre las técnicas desarrolladas para la detección de anticuerpos específicos frente a *B. besnoiti* se encuentran la técnica IFI, la aglutinación, el WB y el ensayo inmunoenzimático (ELISA), siendo esta última la más empleada, tanto en el diagnóstico como en estudios epidemiológicos (Tabla 3) (Cortes et al., 2006a; Fernández-García et al., 2010; Schares et al., 2010; 2013; Waap et al., 2011; García-Lunar et al., 2013b; 2017). La técnica IFI fue la primera técnica serológica desarrollada (Goldman y Pipano, 1983) y su Esp y Se dependen, especialmente, de la experiencia del observador a la hora de interpretar los resultados. La técnica de aglutinación, escasamente utilizada para el diagnóstico de *B. besnoiti* (Waap et al., 2011), se ha empleado como una adaptación de la técnica de aglutinación desarrollada para la toxoplasmosis (Desmonts et al., 1981). Esta técnica ha sido empleada en animales silvestres y donde no hay anticuerpos secundarios disponibles (Mazuz et al., 2018), aunque su Se es moderada y su interpretación puede ser también un tanto subjetiva. Se han desarrollado diversas pruebas ELISA para la detección de anticuerpos específicos frente a *B. besnoiti*, con diferencias, principalmente, en el antígeno empleado. Se ha empleado el extracto soluble de taquizoítos (Cortes et al., 2006c; Fernández-García et al., 2010), el extracto de taquizoítos enriquecido con proteínas de membrana (Schares et al., 2013), el extracto total de taquizoítos (Schares et al., 2011a) y, por ultimo, taquizoítos liofilizados (García-Lunar et al., 2017). Todos ellos, además de dos pruebas ELISA comerciales (INGEZIM BES 1.2.BES.KI INGENASA, ID SREEN ® BESNOITIA INDIRECT), han mostrado elevados valores de Se y Esp (García-Lunar et al., 2013b; García-Lunar et al., 2017). Sin embargo, las técnicas serológicas ELISA tienen limitaciones que deben tenerse en cuenta. Por un lado, se han descrito reacciones cruzadas entre *B.*

besnoiti y otros protozoos apicomplejos como *N. caninum* y *Sarcocystis* spp. (García-Lunar et al., 2015a). Además, existen fluctuaciones de los valores de anticuerpos séricos en los animales infectados a lo largo del tiempo, por lo que un animal seropositivo podría tener resultados negativos en muestreos seriados. En áreas donde la enfermedad es endémica esto puede constituir un riesgo muy importante si se piensa en el control de la enfermedad (Liénard et al., 2011; Gutiérrez-Expósito et al., 2017a). También existe un porcentaje muy bajo de animales infectados (1-3%) que mostrando signos clínicos evidentes de besnoitiosis crónica tienen resultados negativos en la serología. Otra importante limitación ocurre en la fase aguda de la enfermedad puesto que las técnicas serológicas actuales basadas en la detección de inmunoglobulinas del isotipo G (IgG), no permiten diagnosticar animales infectados antes de la tercera semana pi, momento aproximado en el que se produce la seroconversión (Jacquiet et al., 2010; Schares et al., 2013; García Lunar, 2015b). En estos casos, habría que volver a remuestrear al animal pasadas dos o tres semanas. El WB es la técnica de referencia en *B. besnoiti* (García-Lunar et al., 2013b; Gutiérrez-Expósito et al., 2017c). Para el WB se han desarrollado pruebas basadas en extractos tanto de taquizoíto como de bradizoíto y en condiciones tanto reductoras como no reductoras (Fernández-García et al., 2009a; Schares et al., 2010; García-Lunar et al., 2013; 2015b) mostrando valores de Se y Esp cercanos al 100%. Por tanto, se recomienda su uso en casos dudosos o en animales muy valiosos como los sementales (Gutiérrez-Expósito et al., 2017c). Sin embargo, en la fase aguda de la enfermedad, un resultado negativo de WB no sería concluyente y un remuestreo del animal estaría también indicado. El WB no se emplea habitualmente debido a su laboriosidad y a su elevado coste.

Tabla 2: Técnicas diagnósticas directas, histopatológicas y moleculares empleadas en la besnoitiosis bovina (adaptado de Ferre y Álvarez-García, 2019).

	Biopsia*	**Histopatología/ Inmunohistoquímica***	**PCR***
Tiempo de realización	Rápido	Varios días	1 día
Número de muestras	Pocas	Pocas	Pocas
Tipo de muestra*	Piel	Piel	Piel/ testículos/ sangre
Interpretación de los resultados	Fácil	Fácil	Fácil
Requerimiento de laboratorio	No, en campo	Sí, especializado	Sí, especializado
Coste	Bajo	Elevado	Moderado
Sensibilidad	Baja	Baja	Elevada
Especificidad	Elevada	Elevada	Elevada
Utilidad	Confirmar una infección crónica	Investigación	Confirmar una infección aguda en testículos Detección de parasitemia

* Las muestras de piel se recogerán preferentemente de las zonas con lesión, cara interna del muslo, grupa, escroto, cuello y cara.

Tabla 3: Técnicas diagnósticas serológicas empleadas en la besnoitiosis bovina (adaptado de Ferre y Álvarez-García, 2019).

	ELISA*	**Western blot***	**IFI**	**Aglutinación**
Tiempo de realización	1 día	2-3 días	1 día	2 días
Número de muestras	Elevado	Bajo	Bajo	Bajo
Interpretación de los resultados	Objetiva	Objetiva (experiencia)	Subjetiva (experiencia)	Subjetiva
Requerimiento de laboratorio	Sí	Sí, especializado	Sí	Sí
Coste	Bajo	Elevado	Elevado	Elevado
Sensibilidad	Elevada	Elevada	Elevada	Moderada
Especificidad	Elevada	Elevada	Elevada	Elevada
Utilidad	Diagnóstico laboratorial y en estudios epidemiológicos	Confirmación de casos dudosos	No se recomienda en diagnóstico laboratorial	Empleo en animales silvestres donde no hay anticuerpos secundarios disponibles

* Técnicas empleadas con más frecuencia en el diagnóstico laboratorial.

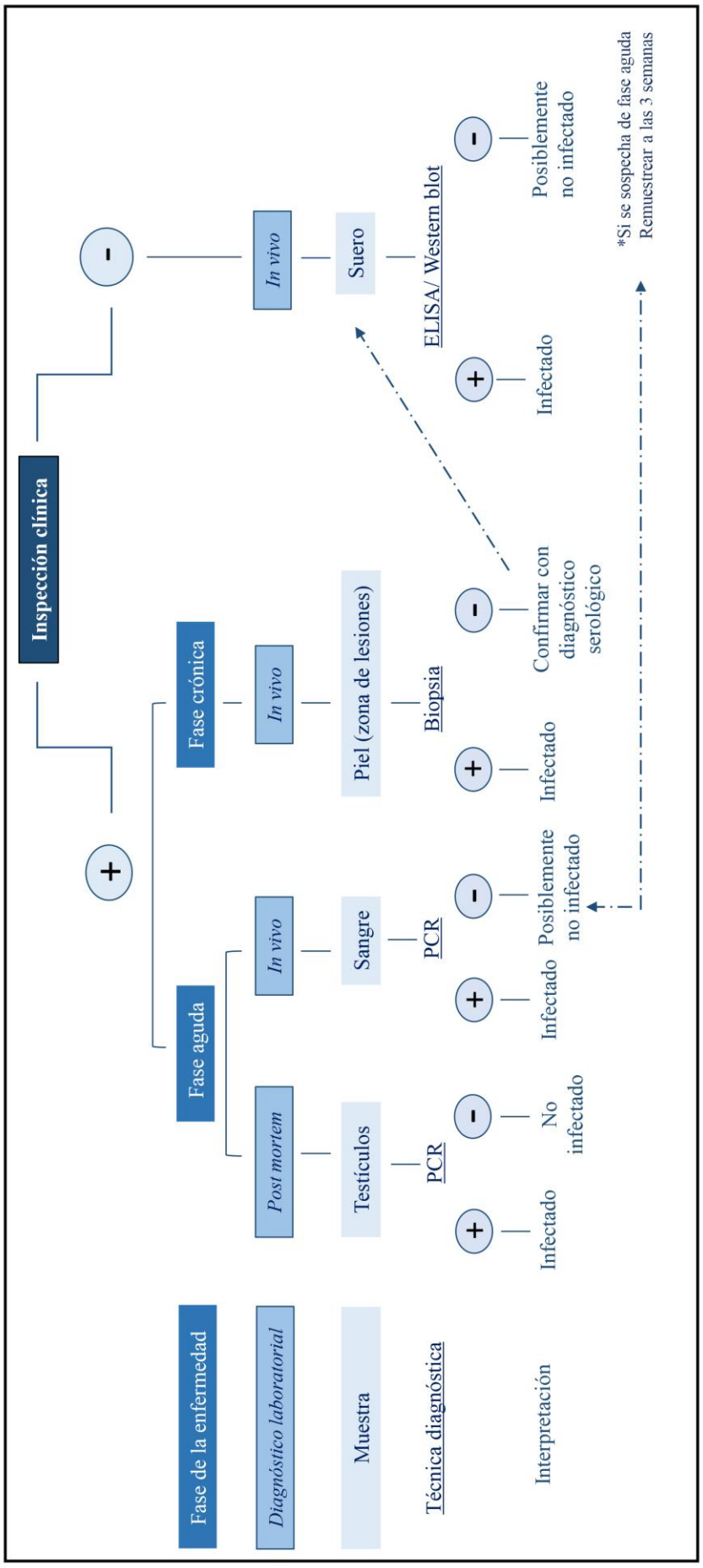


Figura 5: Abordaje diagnóstico de la besnoitiosis bovina (adaptado de Ferre y Álvarez-García, 2019).

1.8. Control

El control de la besnoitiosis bovina no es sencillo al no existir fármacos ni vacunas eficaces disponibles. El diagnóstico temprano de la enfermedad debe asociarse con medidas adecuadas de manejo en la explotación para evitar, por un lado, la entrada de la infección en rebaños libres de la enfermedad (bioseguridad) y, por otro, evitar la diseminación en aquellos rebaños infectados (biocontención) (Figura 6).

Es fundamental que antes de implementar un programa de control en las explotaciones conozcamos el estado sanitario de partida. Para ello, es recomendable realizar un análisis serológico de todo el colectivo. En rebaños libres de la enfermedad, los brotes suelen ocurrir por la introducción de animales infectados. Por tanto, se debe prestar especial atención a los sementales, ya que generalmente se intercambian y sirven a un gran número de vacas, siendo un riesgo muy elevado la introducción de un macho portador de la enfermedad (Gutiérrez-Expósito et al., 2017c). A pesar de que no existen resultados experimentales sobre la utilidad del control de vectores en la besnoitiosis bovina, el uso de repelentes (p. ej., DEET: N-dietil-3-metil-toluamida y piretroides), insecticidas (p. ej., Novaluron) y trampas interiores que se aplican en sitios de alimentación de los dípteros, podrían reducir la transmisión mecánica asociada con la actividad invernal de las moscas en el interior de los establos (Liénard et al., 2011; Taylor et al., 2012; Baldacchino et al., 2014; Lohmeyer et al., 2014; Hogsette y Kline, 2017; Sharif et al., 2019). Además, al aire libre, los aerosoles de piretroides aplicados mensualmente durante la temporada de mayor actividad de los vectores podrían reducir su número. La DEET es el repelente más eficaz contra los tábanos. Sin embargo, dado que no hay productos de larga duración disponibles, su aplicación no es práctica en el campo (Baldacchino et al., 2014).

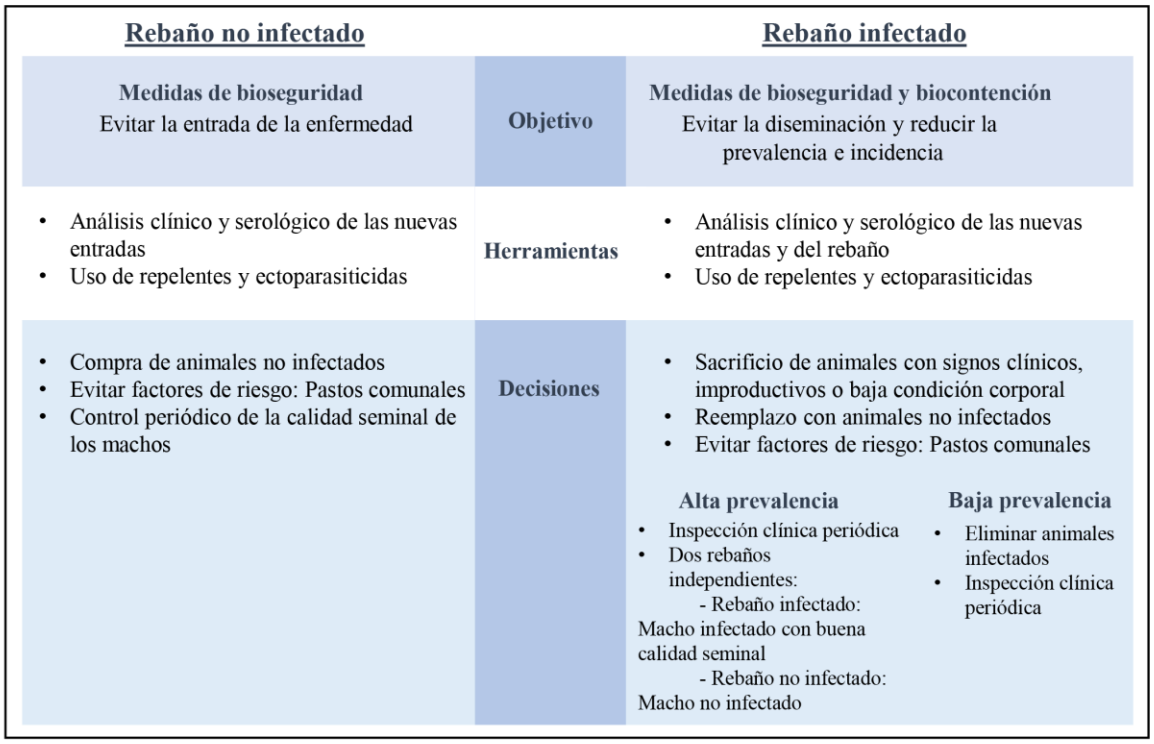
En zonas endémicas las medidas de manejo deben ir encaminadas, fundamentalmente, a evitar la trasmisión y la diseminación de la besnoitiosis (Gutiérrez-Expósito et al., 2017c; Álvarez-García et al., 2013). La posibilidad de eliminar la enfermedad en explotaciones de manejo intensivo (o semi-extensivo), donde se lleva a cabo inseminación artificial, es mucho más probable. Bastaría con ir reemplazando animales seropositivos o con signos clínicos periódicamente. Sin embargo, en condiciones de manejo extensivo, se podría conseguir una disminución de la incidencia de la enfermedad, pero la erradicación es un objetivo prácticamente imposible. Se recomienda que una vez que la infección haya entrado en un rebaño, los animales infectados sean separados de los seronegativos y se manejen dos rebaños de manera independiente. Los animales infectados deberían ir retirándose gradualmente para evitar la propagación de la enfermedad y reducirse la prevalencia. Sin embargo, mantener una estrategia conservadora parece ser la mejor opción a largo plazo. Esta consistiría en mantener un equilibrio entre el sacrificio selectivo y los parámetros productivos, ya que la seroprevalencia puede ser alta en un rebaño infectado. Sólo los animales afectados más graves e improductivos (machos estériles y hembras con baja condición corporal) deberían ser reemplazados

por animales sanos y seronegativos. Estos últimos deberán ser mantenidos en el rebaño de los animales sanos. En el caso de los sementales, es esencial realizar un control periódico de la calidad seminal ya que es posible el desarrollo de infertilidad. Los sementales seropositivos y fértiles deberían mantenerse con el rebaño infectado, en el que puede ser también útil la implantación de un protocolo de inseminación artificial. Por el contrario, los sementales seronegativos deberán estar en contacto tan sólo con los animales seronegativos (Gutiérrez-Expósito et al., 2017c) (Figura 6).

En cuanto a la terapéutica e inmunoprofilaxis, destacar que no existen actualmente fármacos disponibles frente a esta enfermedad ni vacunas autorizadas en Europa. En Sudáfrica, durante los años 70 del siglo pasado se desarrolló una vacuna con un aislado atenuado de ñu que era capaz de evitar el desarrollo de signos clínicos, aunque no prevenía de la aparición de portadores subclínicos (Bigalke et al., 1974). En Israel se vacuna de forma habitual a todos los sementales importados. Se trata de la inoculación subcutánea de un aislado atenuado procedente de un toro infectado naturalmente. Sin embargo, no se ha realizado aún ningún estudio de eficacia y se desconoce su seguridad (Pipano, 1997; Cortes et al., 2014).

Con respecto a los fármacos, numerosos estudios de cribado han mostrado resultados prometedores para el tratamiento de la besnoitiosis. Su utilidad residiría en evitar la proliferación del taquizoíto, responsable de la fase aguda de la enfermedad, para evitar la diseminación intraorgánica y el acantonamiento del parásito en quistes tisulares. La arilimidamida y algunos de los derivados de la nitozoxanida han sido valorados por Cortes et al. (2007a; 2011). Recientemente, compuestos de nueva generación como los inhibidores de la proteína quinasa dependiente de calcio (Jiménez-Meléndez et al., 2017), la curcumina (Cervantes-Valencia et al., 2018) y algunos fármacos empleados en el tratamiento de otras protozoosis (buparvacuona, diclazuril, decoquinato) (Jiménez-Meléndez et al., 2018; Müller et al., 2019) han sido testados en sistemas *in vitro* con resultados prometedores. Sin embargo, sería necesario un modelo normalizado de infección experimental para testar *in vivo* su seguridad y eficacia

Figura 6: Control de la besnoitiosis bovina (adaptación de Gutiérrez-Expósito et al., 2017c).



2. Modelos experimentales

El Consejo Nacional de Investigación de EE.UU. (NRC, "National Reserch Council") ha declarado que: *"los modelos experimentales son un componente crítico en las investigaciones biomédicas destinadas a la mejora de la salud y que, además, ofrecen ventajas que de otra forma no se podrían obtener"*. Los modelos experimentales son universalmente utilizados por la comunidad científica y facilitan resultados importantes sobre procesos biológicos que, en última instancia, deben investigarse en un organismo vivo. Se incluyen dentro de los modelos experimentales tanto a los sistemas *in vitro* (cultivos celulares, bacterias, virus y levaduras), como a los modelos *in vivo* (mamíferos y vertebrados e invertebrados terrestres y acúaticos no mamíferos); y los sistemas de simulaciones computacionales y matemáticos. Todos estos modelos han proporcionado gran cantidad de datos necesarios para el esclarecimiento de aspectos importantes de muchas enfermedades y procesos (NRC, 1998).

Esta misma institución estableció, además, una serie de criterios y características que deben cumplir los modelos experimentales y sistemas de soporte de modelos, entre los que se incluyen:

- El modelo debe ser apropiado para su(s) uso(s) previsto(s).
- El modelo específico de una enfermedad debe imitar fielmente a ésta.
- El modelo se debe desarrollar, mantener y proporcionar a un coste razonable.
- El modelo debe ser de valor para varios científicos o para múltiples propósitos.
- El modelo debe ser reproducible y fiable, por lo que los resultados pueden ser confirmados.
- El modelo debe estar razonablemente disponible y accesible.

En el desarrollo de los modelos experimentales, principalmente en aquellos que se incluyen prácticas de experimentación animal, entran en juego una serie de consideraciones éticas y de presión social que deben ser tenidas en cuenta. En este sentido, la publicación del libro "*The Principle of Human Experimental Technique*" (Russell et al., 1959) significó un gran avance, ya que proponía vías para realizar la investigación con animales de la forma más respetuosa posible. Las tres propuestas formuladas por estos autores se conocen como el principio de las tres erres (3R): "*Reemplazar, Reducir y Refinar"*. El reemplazo consiste en sustituir los animales empleados en la experimentación por equivalentes no animales (modelos matemáticos o sistemas biológicos *in vitro*) siempre y cuando esto sea posible. Reducir no es más que minimizar el número de animales empleados en un experimento. Para ello, es necesaria la realización de estudios estadísticos con el fin de averiguar el número mínimo de animales de los que debemos partir, así como para maximizar la información obtenida de cada animal empleado en la experimentación. El refinamiento consiste en el perfeccionamiento del diseño experimental y la selección del método más adecuado para minimizar

el sufrimiento o la ansiedad de los animales utilizados, así como todos los métodos de enriquecimiento ambiental para asegurar su bienestar.

En el caso concreto de la besnoitiosis bovina los modelos experimentales son esenciales para el avance del conocimiento sobre esta enfermedad. En primer lugar, porque se desconocen aún muchos aspectos relacionados con su patogenia, así como con la relación que se establece entre el parásito y el hospedador tras la infección. En segundo lugar, al no existir aún fármacos y vacunas eficaces, estos modelos son fundamentales para el desarrollo de pruebas de cribado de potenciales candidatos terapéuticos y su posterior testaje *in vivo* en pruebas de seguridad y eficacia. En este sentido, se han desarrollado varios ensayos *in vitro* para estudiar los procesos esenciales en el ciclo lítico del parásito, así como pruebas de concepto de diversos fármacos. También se han realizado diversas infecciones experimentales en animales de laboratorio, bovino y otras especies con resultados muy diversos, sin que se haya obtenido todavía un modelo *in vivo* normalizado de infección (ver sección 2.2). Un modelo *in vivo*, representaría la mejor alternativa para llevar a cabo ensayos vacunales y terapéuticos, así como para estudiar la patogenia y la respuesta inmunitaria.

2.1. Modelos *in vitro*

Actualmente, la investigación se encuentra muy influenciada por políticas de bienestar animal fomentadas por una estricta legislación europea que pretende disminuir al máximo el empleo de animales de laboratorio (Directiva 2010/63/UE, relativa a la protección de los animales utilizados para fines científicos). En este sentido, el empleo de modelos *in vitro*, como los cultivos celulares, representa una excelente alternativa para el estudio de organismos intracelulares como es el caso de los Toxoplasmatinae. Actualmente, se mantienen en cultivo celular y de forma habitual el estadio de taquizoíto de distintos aislados de *T. gondii*, *N. caninum* y *Besnoitia* spp.

Los modelos de cultivo *in vitro* de estos parásitos apicomplejos son útiles para la obtención de aislados procedentes de casos clínicos. Además, han servido para esclarecer los mecanismos moleculares que determinan la patogenia de estos protozoos formadores de quistes, así como para conocer sus características de invasión y acantonamiento. Otra utilidad de los modelos *in vitro* es la realización de pruebas de concepto de la utilidad de fármacos frente a estos parásitos (cribado, ensayos de eficacia y seguridad de fármacos) (Jiménez-Meléndez et al., 2017; 2018), estudio del ciclo lítico del parásito y caracterización *in vitro* de los diferentes aislados (Frey et al., 2013), así como identificación de factores de virulencia y de candidatos vacunales en diversos estudios ómicos (estudios de genomas, transcriptomas y proteomas) (Radke et al., 2005; Horcajo et al., 2018).

Sin embargo, existen algunas limitaciones generales de los modelos *in vitro*. No reproducen con exactitud el comportamiento de los parásitos en el hospedador (efecto del microambiente y la interacción de diferentes tejidos y células), por lo que no sustituyen a los modelos *in vivo*. Además,

se trata de modelos relativamente estáticos y presentan dificultades en el estudio de las infecciones a largo plazo. Presentan también algunas limitaciones directamente asociadas con el estudio de Toxoplasmatinae: i) incapacidad de reproducir el ciclo biológico completo del parásito (de momento no se han obtenido los estadios enteroepiteliales *in vitro*); ii) no existe un modelo de cultivo celular normalizado ni aislados parasitarios de referencia que permitan hacer comparables los diferentes estudios; iii) no existe una metodología uniforme en cuanto a los controles de calidad de los cultivos celulares; y iv) generalmente se han empleado líneas celulares inmortalizadas y procedentes de tejidos diferentes a los tejidos diana del parásito, por lo que resultaría interesante contar con líneas celulares primarias y diana del parásito (las células primarias son más relevantes para el estudio de la patogenia).

Tabla 4: Líneas celulares empleadas en cultivos *in vitro* de *B. besnoiti* (Fernández-Álvarez, 2018).

Línea celular	Tipo celular	Especie animal de origen	Tejido	Célula	Referencia
BHK	Inmortalizada	Criceto	Riñón	Fibroblasto	Neuman, 1974
BHK21	Inmortalizada	Criceto	Riñón	Epitelial	Schares et al., 2009
CRFK	Inmortalizada	Gato	Riñón	Epitelial	Göbel et al., 1985
HeLa	Inmortalizada	Humano	Cérvix	Epitelial	Neuman, 1974
MARC-145	Inmortalizada	Mono	Riñón	Epitelial	Frey et al., 2016
NA42/13	Inmortalizada	Ratón	Tejido nervioso	Neuroblasto	Schares et al., 2009
RML-15 LSTH-RA-243	Inmortalizada	Garrapata	-	Célula embrionaria	Shkap et al., 1988
Vero	Inmortalizada	Mono	Riñón	Epitelial	Cortes et al., 2007
BUVEC	Primaria	Bovino	Cordón umbilical	Endotelial	Maksimov et al., 2016
HFF	Primaria	Humano	Prepucio	Fibroblasto	Jiménez-Meléndez et al., 2017
KH-R	Primaria	Ternero	Corazón	Célula embrionaria	Schares et al., 2009

2.1.1. Modelos *in vitro* en *B. besnoiti*

Los taquizoítos de *B. besnoiti* se han mantenido en una gran variedad de líneas celulares, tanto primarias como inmortalizadas (Tabla 4). El objetivo ha sido similar al desarrollado con *N. caninum* y *T. gondii*, especialmente dirigido a la obtención de aislados y la realización de estudios farmacológicos. Sin embargo, todavía no se ha estudiado la transformación *in vitro* del estadio de taquizoíto al de bradizoíto ni se han realizado estudios ómicos.

Tabla 5: Aislados de *B. besnoiti* (adaptación de Basso et al., 2013; Álvarez-García et al., 2014b; Frey et al., 2016).

Aislado	País	Animal	Tipo de infección	Cultivo *in vitro*	Empleo en infecciones experimentales
Impala Strain	Sudáfrica	Antílope	Crónica	No	Conejo
Bb-Fuls Strain	Sudáfrica	Bovino	Aguda	Sí	Bóvido, conejo y ratón
Bb-Schoeman Strain	Sudáfrica	Bovino	Aguda	No	Conejo y ratón
Bb-Lamprechts A Strain	Sudáfrica	Bovino	Aguda	No	Bóvido, conejo y ratón
Bb-Lamprechts B	Sudáfrica	Bovino	Crónica	No	Bóvido, conejo y ratón
Bb-Wildebeest	Sudáfrica	Antílope	Crónica	Sí	Bóvido, conejo y ratón
Bb-Israel	Israel	Bovino	Crónica	Sí	Bóvido y ratón
Bb1-Evora 03	Portugal	Bovino	Crónica	Sí	No
Bb-Spain 1	España	Bovino	Crónica	Sí	No
Bb-Spain 2	España	Bovino	Crónica	Sí	No
Bb-Ger 1	Alemania	Bovino	Crónica	Sí	Cobaya, gato, perro y ratón
Bb-France	Francia	Bovino	Crónica	Sí	Ratón
Bb-Italy 1	Italia	Bovino	Crónica	Sí	No
Bb-Italy 2	Italia	Bovino	Crónica	Sí	No
Bb-IPZ-1-CH	Suiza	Bovino	Crónica	Sí	No
Bb-IPZ-2-CH	Suiza	Bovino	Crónica	Sí	No
Bb-IPZ-3-CH	Suiza	Bovino	Crónica	Sí	No

Entre los años 60 y 80 del siglo pasado se obtuvieron diferentes aislados del parásito en varios laboratorios de Sudáfrica e Israel que se emplearon, principalmente, en inoculaciones experimentales realizados en el ganado bovino. Estos aislados procedían de bovinos infectados por *B. besnoiti* en los que no se realizó ningún control sanitario. Por tanto, se desconoce si estos aislados estaban libres de

otros patógenos frecuentes en el ganado bovino. Además, la antigüedad y la falta de trazabilidad en estos aislados hacen que hoy en día no se pueda disponer de ellos. Posteriormente, se han obtenido nuevos aislados que se han mantenido en cultivos celulares. Se sabe que los aislados bovinos obtenidos en Israel y, recientemente, en Europa se aislaron directamente de ganado infectado crónicamente y dichos aislados están libres, al menos, de la infección por *Mycoplasma* spp. (Tabla 5).

Como en el caso de *T. gondii* y *N. caninum*, *B. besnoiti* tiene también un ciclo lítico con fenómenos de invasión, proliferación y egresión. Su ciclo lítico fue caracterizado *in vitro* por Frey et al. (2016) en células MARC-145 con diferentes aislados (Bb-Spain 1, Bb-Spain 2, Bb-Ger 1, Bb1-Evora 03, Bb-Israel, Bb-France). Este estudio permitió obtener un modelo *in vitro* bien caracterizado y de referencia para *B. besnoiti*. En resumen, se demostró que la invasión del parásito es más lenta que en otros apicomplejos, como *N. caninum* (Regidor-Cerrillo et al., 2011), ya que solamente el 50% de los parásitos se encontraban intracelularmente entre las 3-6 horas pi. Sin embargo, los taquizoítos de *B .besnoiti* son capaces de sobrevivir más tiempo fuera de la célula, produciéndose la invasión hasta las 24 horas pi (crecimiento asincrónico). Los taquizoítos proliferan intracelularmente hasta las 72 horas pi, momento en el cual comienza a producirse el fenómeno de la egresión (Frey et al., 2016).

Recientemente, los sistemas *in vitro* de cultivo celular también han permitido la realización de pruebas de cribado de fármacos y de potenciales candidatos terapéuticos capaces de actuar en la infección aguda por *B. besnoiti*. Se han evaluado tanto fármacos de nueva generación como fármacos ya comercializados en bovino frente a infecciones por otros apicomplejos, con resultados prometedores en modelos *in vitro* de infección de células Vero y MARC-145.

Cortes et al. (2007a) evaluaron el efecto de las nitro-tiazolidas y derivados bromados demostrando la inhibición de la proliferación de taquizoítos de *B. besnoiti* en células Vero a concentraciones de 5 y 10 µg/ml. Sin embargo, estos compuestos no impidieron la invasión de la célula hospedadora. Entre los fármacos comerciales evaluados destacan el diclazurilo, el decoquinato y la buparvacuona por su capacidad para inhibir la invasión y proliferación de los taquizoítos *in vitro*. El decoquinato y el diclazuril, empleados en el tratamiento de las infecciones por *Eimeria* spp. (coccidiosis), mostraron valores de inbición del 99% de los taquizoítos de *B. besnoiti* a concentraciones de 100 nM y 29,9 µM, respectivamente (Jiménez-Meléndez et al., 2018). La buparvacuona naptoquinona, empleada como tratamiento de la theileriosis en el ganado bovino en muchos países, afectó a la proliferación *in vitro* de los taquizoítos con una concentración inhibitoria 50 (IC50) de entre 3 y 10 nM. Sin embargo, después de una exposición prolongada (14-16 días) a la buparvacuona, los taquizoítos de *B. besnoiti* mostraron capacidad para adaptarse y reanudaron la proliferación en dosis de hasta 10 µm, aunque a un ritmo menor (Müller et al., 2019).

Además, se están explorando nuevas dianas farmacológicas para el desarrollo de fármacos de nueva generación frente a la besnoitiosis bovina. Un ejemplo de ello son los fármacos inbidores de las proteínas quinasas dependientes de calcio. Estas enzimas están conservadas en Apicomplexa, pero ausentes en las células de mamíferos y han demostrado ser eficaces frente a *T. gondii*, *N. caninum*, *Babesia* spp., *Cryptosporidium parvum*, *Plasmodium* spp. y, recientemente, frente a *B. besnoiti* en un modelo *in vitro* (Jiménez-Meléndez et al., 2018). Un trabajo reciente ha demostrado los efectos *in vitro* de la curcumina en células endoteliales de cordón umbilical infectadas con taquizoítos de *B. besnoiti*. Los ensayos de inhibición funcional mostraron una reducción de la viabilidad de los taquizoítos e indujeron efectos letales en hasta 57% de ellos (IC50 de 5,93 μM) (Cervantes-Valencia et al., 2018).

Sin embargo, sería necesario el desarrollo y la normalización de un modelo experimental *in vivo* de la infección por *B. besnoiti* para testar la seguridad y eficacia de estos compuestos.

2.2. Modelos animales

El Consejo Nacional de Investigación de los Estados Unidos sobre Modelos Animales para la Investigación en Envejecimiento *(EE.UU. National Research Committee on Animal Models for Research on Aging)* ha definido el término *"modelo animal de laboratorio"* como *"aquel en el que la normalidad biológica y de comportamiento pueden ser estudiadas, o en el cual se pueden investigar procesos patológicos espontáneos o inducidos en los cuales el fenómeno en uno o más aspectos se asemeja al humano o a otros animales"*. El uso de modelos animales en la investigación ha representado un elemento fundamental en el desarrollo de importantes avances en la prevención y tratamiento de las enfermedades. *"A pesar de los nuevos y refinados métodos alternativos, los experimentos con animales seguirán siendo esenciales en un futuro previsible para la investigación biomédica*", tal y como se incluye en la Declaración de Basilea del año 2010 elaborada con motivo de celebración de la primera conferencia *"Research at a Crossroads"*.

La elección de un modelo animal debería basarse en su adecuación como análogo a la especie de destino, la transferibilidad de la información obtenida, uniformidad, conocimiento de las propiedades biológicas, generalización de resultados y facilidad de manipulación, así como en consecuencias éticas (Arranz-Solís, 2015b). En este sentido, obviamente, el modelo ideal para una enfermedad en concreto sería la especie de destino, puesto que nos ofrecerá más precisión y unos resultados directamente extrapolables. Sin embargo, estos criterios estrictamente teóricos no siempre pueden ser considerados en la práctica debido a las restricciones concretas de cada modelo. También es importante enfatizar que, para obtener resultados fiables, la salud del animal empleado como modelo es fundamental y, por lo tanto, el interés del investigador debe ir centrado en disponer siempre de animales sanos y bien tratados (Fondecyt-Conicyt, 2009).

Por supuesto, en medicina veterinaria la utilidad de los modelos animales es incuestionable. Su utilización ha llevado al desarrollo de tratamientos y/o vacunas contra enfermedades como la rabia, ántrax, muermo, inmunodeficiencia felina, tuberculosis, fiebre de Texas, fiebre porcina clásica y diversas parásitosis, entre otras (Asociación Europea para la Investigación Animal; http://eara.eu/en/animal-research/veterinary-medicine). El empleo, normalización y refinamieno de modelos experimentales en apicomplejos como *T. gondii* y *N. caninum* ha sido fundamental para avanzar en el conocimiento de las enfermedades causadas por estos agentes, la toxoplasmosis y la neosporosis, respectivamente. Estas dos enfermedades son de notable relevancia e importancia económica en los rumiantes domésticos, así como en salud pública en el caso de la toxoplasmosis.

Puesto que *T. gondii* tiene un amplio espectro de hospedadores, se han desarrollado numerosos modelos animales en especies muy diferentes y con diferentes própositos (Dubey, 2010). Desde el primer aislamiento del parásito realizado en ratones (Sabin y Olitsky, 1937), el modelo murino ha sido ampliamente utilizado en aislamientos y estudios de virulencia, conversión taquizoíto-bradizoíto, infección congénita y recrudescencia (Dubey, 2010). Se han llevado a cabo infecciones experimentales en gatos, al ser el hospedador definitvo, para la obtención de ooquistes, así como en otras especies animales donde la toxoplasmosis tiene importancia veterinaria (ovejas, cabras, cerdos) (Dubey, 2010; Stelzer et al., 2019). Sin embargo, el principal propósito del estudio de la infección por *T. gondii* utilizando modelos animales es obtener una mejor comprensión de esta enfermedad en humanos y el desarrollo de vacunas.

En la neosporosis, el empleo de modelos murinos es una alternativa muy empleada (Cole et al., 1995; Collantes-Fernández et al., 2006). Por ejemplo, el desarrollo de modelos murinos gestantes ha permitido estudiar la trasmisión del parásito a la descendencia, por lo que se han presentado como una prueba de concepto para la evaluación de diversos candidatos terapéuticos, principalmente vacunas, antes de realizar estudios en la especie de destino (Rojo-Montejo et al., 2011; Arranz-Solís et al., 2015a; Horcajo et al., 2016). El conejo se ha utilizado principalmente para la obtención de anticuerpos, al permanecer clínicamente sanos incluso con dosis de inoculación muy elevadas (Lindsay y Dubey, 1989). Los modelos ovino y bovino, tanto gestante como no gestante, han sido desarollados y normalizados en *N. caninum* (Arranz-Solís, 2015b). Estos han permitido realizar estudios de patogenia y transmisión del parásito a la descendencia, así como evaluar nuevos candidatos terapéuticos y vacunales (Benavides et al., 2014; Sánchez-Sánchez et al., 2018).

2.2.1. Modelos animales en *B. besnoiti*

Hasta el momento, no se ha desarrollado ni consensuado un modelo animal de infección por *B. besnoiti* que reproduzca tanto la fase aguda como la crónica de la enfermedad; y que se haya normalizado cumpliendo con las características adecuadas establecidas para un modelo animal. Sin embargo, y a pesar de las limitaciones, muchas de las infecciones experimentales realizadas hasta el

momento nos han permitido conocer variables fundamentales relacionadas tanto con el parásito como con el hospedador.

Entre los años 1960 y 1980 se realizaron numerosas infecciones experimentales. Sin embargo, estas fueron desarrolladas bajo condiciones muy diferentes por lo que es muy difícil comparar los resultados obtenidos. La falta de armonización en los diseños experimentales en cuanto a los paramétros evaluados, la frecuencia de muestreo, el número de animales por grupo (en muchos casos solo existía un animal por grupo) y la duración, así como experimentos basados únicamente en la inspección clínica y la histopatología, ya que la serología y técnicas moleculares no estaban disponibles, limitan la reproducibilidad de sus resultados. A pesar de estas limitaciones, los autores describieron cuidadosamente sus resultados señalando las dificultades en reproducir la besnoitiosis (Bigalke, 1968; Basson et al., 1970; Diesing et al., 1988; Shkap y Pipano, 1993).

2.2.1.1. Modelos *in vivo* en animales de laboratorio

El desarrollo de un modelo de infección en animales de experimentación, que reproduzca tanto la fase aguda como la fase crónica de la infección, sería ideal a la hora de hacer un cribado preliminar de fármacos y/o vacunas antes de realizar estudios en la especie de destino, debido a una mayor facilidad de manejo, acortamiento del tiempo de estudio y abaratamiento de los costes. Con este objetivo, se han realizado diversas infecciones experimentales en un amplio rango de especies de roedores de laboratorio (jerbo, ratas, cricetos, diferentes estirpes de ratón) sin lograr reproducir la fase crónica de la enfermedad (Tabla 6). Únicamente los jerbos, de la especie *Meriones tristami*, han mostrado cierta susceptibilidad a la infección, por lo que fueron empleados en algunos ensayos de inmunización (Shkap, 1986; Shkap y Pipano, 1993). Los ratones defectivos en IFN-γ mostraron un curso hiperagudo de la enfermedad (debido a la importancia de la respuesta celular en la protección frente a estos parásitos). Éstos han sido empleados en ensayos de aislamiento del parásito a partir de bovinos crónicamente infectados (Schares et al., 2009; Gentile et al., 2012), siendo su utilidad como modelo muy limitada.

En cambio, el conejo ha demostrado ser más susceptible a la infección por *B. besnoiti*, tal y como se muestra en la Tabla 7. Tanto la inoculación de taquizoítos como de bradizoítos del parásito han servido para reproducir, en estos animales, signos compatibles con la fase aguda de la enfermedad; fiebre, linfadenitis, así como edemas en extremidades o escroto y conjuntivitis y fotofobia en los casos más graves (Bigalke, 1967; 1968; Basson et al., 1970; Bigalke et al., 1974; Shkap, 1986; Basso et al., 2011; Liénard et al., 2015).

Tabla 6: Infecciones experimentales con *B. besnoiti* en roedores de laboratorio.

	n[a]	Estadio inoculado	Procedencia del inoculo	Dosis	Vía	Fase aguda	Fase crónica	Detección del parásito (técnica)	Supervivencia [b] (%)	Observaciones	Referencia
Jerbo *(Meriones tristami)*	6	Tz	Cultivo celular [c]	10^7	ip	Anorexia, caquexia y muerte (6-8 días pi)	-	+, Líquido peritoneal (Frotis)	0	-	Shakp, 1986
	84	Tz	Cultivo celular [c]	10-10^7	ip	ND	-	-	56	Ensayos de inmunización	Shakp y Pipano, 1993
	84	Tz	Cultivo celular [c]	10-10^7	sc	Nódulos palpables (Punto de inoculación)	-	ND	100		Shakp y Pipano, 1993
(Meriones unguiculatus)	3	Tz	Cultivo celular [c]	$5x10^5$	sc	-	-	+, Parasitemia (ITS1- PCR)	100	Seroconversión (3 semanas pi)	Basso et al., 2011
	3	Bz	Bovino [d]	$5x10^5$	sc	-	-	-	100		Basso et al., 2011
Criceto *(Cricetus auratus)*	5	Tz	Cultivo celular [c]	10^7	ip	-	-	-	100	-	Shkap, 1986
	3	ND	Conejo/ cricetos [e]	ND	oral	Diarrea y debilidad	-	+, Peritoneo y pulmón (ND)	S	Inmunosupresión (cortisona)	Bigalke, 1968
	2	Tz	Sangre de conejo	20 ml	ip	-	-		100	-	Pols, 1960
Cobaya *(Cavia porcellus)*	5	Tz	Cultivo celular [c]	10^7	ip	-	-	-	100	-	Shakp, 1986
	16	Tz	Sangre de conejo	20-30 ml	ip/sc	Fiebre intermitente	-	+, Parasitemia (Frotis)	100	-	Pols, 1960
	3	Tz	Cultivo celular	$5x10^5$	sc	-	-	-	100	-	Basso et al., 2011
	3	Bz	Bovino [d]	$5x10^5$	sc	-	-	-	100	-	Basso et al., 2011
Ratón *(Mus musculus)*	6	ND	Sangre de bovino	10-20 ml	ip/ sc	-	-	-	100	-	Pols, 1960
	16	Tz	Sangre de conejo	10-20 ml	ip/ sc	-	-	-	100	-	Pols, 1960
	6	Tz	Cultivo celular [c]	10^7	ip	-	-	-	100	-	Shakp, 1986
Ratón BALB/C	6	Tz	Cultivo celular [c]	10^7	ip	-	-	-	100	-	Shakp, 1986
	6	Tz	Cultivo celular [c]	10^7	ip	-	-	-	100	-	Shakp, 1986
Ratón C57BL/6	6	Tz	Cultivo celular [c]	10^7	ip	-	-	-	100	-	Shakp, 1986
Ratón IFN-γ knockout	2	Bz	Bovino [d]	10^6	ip	Apatía y pelo erizado	-	+, Líquido peritoneal, piel y tejidos (qPCR)	0	Ensayos de aislamiento	Gentile et al., 2012
	6	Bz/ Tz	Bovino [d]/ ratón [f]	ND	ip	Apatía y pelo erizado	-	+, Líquido peritoneal, piel y tejidos (Histología, PCR)	0		Schares et al., 2009
Rata *(Rattus rattus)*	6	Tz	Sangre de conejo	20 ml	ip /sc	-	-	-	100	-	Pols, 1960
	8	Tz	Cultivo celular [c]	10^7	ip	-	-	-	100	-	Shkap, 1986

[a] **n:** Número total de animales inoculados. [b] El período de observación de los animales inoculados varío entre 21 y 70 días. [c] Cultivo celular mantenido en células VERO. [d] Bovino infectado crónicamente (piel). [e] Vísceras [f] lavados peritoneales. **Tz:** Taquizoítos. **Bz:** Bradizoítos. **ip:** Intraperitoneal. **sc:** Subcutánea. **ND:** No determinado. **pi:** Post-infección. **ml:** Mililitros. **S:** Sacrificado. **-:** Ausente. **+:** Positivo.

Sin embargo, la inoculación de bradizoítos ha permitido obtener los mejores resultados a la hora de reproducir la fase crónica de la enfermedad en conejos, caracterizada por la detección de quistes mediante histología, presencia de ADN del parásito en diversos tejidos y seroconversión de los animales en torno a las 2-3 semanas pi (Basso et al., 2011; Liénard et al., 2015; Sharif et al., 2019) (Tabla 7). Nuevamente, la diversidad de inóculos empleados y la falta de armonización en los experimentos realizados no han permitido establecer un modelo experimental en este animal como paso previo a estudios en la especie de destino, el bovino.

2.2.1.2. Modelos *in vivo* en bovino

Un modelo animal de infección por *B. besnoiti* en bovino, o al menos en un hospedador rumiante, que desarrollara signos clínicos tanto de besnoitiosis aguda como de crónica, sería ideal a la hora de testar posibles candidatos terapéuticos analizados previamente en sistemas *in vitro* o en animales de laboratorio. Como hemos comentado anteriormente, los resultados obtenidos en sistemas *in vitro* o en animales de laboratorio, son difícilmente extrapolables en la especie de destino. En muchas ocasiones, la respuesta inmune desarrollada contra el patógeno es muy diferente dependiendo de la especie infectada. De manera que, aunque los sistemas *in vitro* y modelos de laboratorio son muy valiosos para un cribado preliminar de fármacos, estos resultados deberían ser testados en la especie de destino como paso definitivo.

Entre los años sesenta y ochenta del pasado siglo XX, se realizaron diversas infecciones experimentales en bovinos (Tablas 8 y 9). Los resultados en cada una de las inoculaciones fueron muy variables y difícilmente comparables entre sí debido a la diversidad de los inóculos empleados, así como un diseño experimental diferente en cada una de ellas (en muchas ocasiones con solo un animal por grupo experimental). Además, la falta de un control de calidad del inóculo (que podría vehicular otros patógenos bovinos), un estado de salud previo desconocido y edades muy diferentes en los animales inoculados, dificultó la normalización y el consenso de un modelo de infección. De modo general, tal y como se indica en las Tablas 8 y 9, se reprodujo la fase aguda de la enfermedad en diversas ocasiones y con distintos inóculos (Bigalke, 1968; Basson et al., 1970; Janitschke et al., 1984; Shkap, 1986; Diesing et al., 1988). Sin embargo, para inducir la fase crónica de la enfermedad con una gravedad moderada mediante la inoculación de taquizoítos fue necesario el empleo de tratamientos inmunosupresores (animales esplenectomizados o tratamiento con glucocorticoides) (Bigalke, 1968; Basson et al., 1970). La inoculación de bradizoítos indujó la fase crónica de la enfermedad en dos ensayos. Bigalke (1968) reportó la presencia de quistes en la conjuntiva ocular al administrar bradizoítos por via intranasal o mediante picaduras de tábanos y *Stomoxys*. Posteriormente, Diesing et al. (1988) describieron la fase crónica en animales inoculados con bradizoítos y tratados previamente con corticoides.

Tabla 7: Infecciones experimentales con *B. besnoiti* realizadas en conejos.

	Procedencia del inoculo	Dosis	Vía	n [a]	Días [b]	Fase aguda	Fase crónica	Detección del parásito (técnica)	Supervivencia (%)	Observaciones	Referencia
Tz	Sangre de conejo	20 ml	ip	3	ND	-	-	-	100	-	Bigalke, 1967
	Sangre de bovino	25 ml	ip	2	ND	-	-	-	100	-	Bigalke, 1967
	Aislado de antílope	ND	ip	8	14-40	Linfadenitis y epididimitis	Lesiones cutáneas [d]	+, Aparato reproductor, miocardio y bazo (Histología)	87,5	-	Basson et al., 1970
	Cultivo celular	ND	ip	8	7-39	Linfadenitis y epididimitis	Lesiones cutáneas [d]	+, Aparato reproductor, miocardio, venas, bazo y pulmón (Histología)	100	-	Basson et al., 1970
	Cultivo celular [c]	10^7	ip	6	90	Fiebre, conjuntivitis y tumefacción en testículos	-	-	100	-	Shakp, 1986
	Cultivo celular [c]	10^4	sc	5	60	Fiebre alta † (11 días pi)	-	-	80		Bigalke et al., 1974
	Cultivo celular (Vero y riñón de mono)	10^3-10^7	sc	26	60-180	† (10-25 días pi)	-	-	38,4		Bigalke et al., 1974
	Cultivo celular (Riñón de mono)	$2x10^5$	sc	10	30	-	-	-	90	Ensayos de inmunización	Bigalke et al., 1974
	Cultivo celular [c]	10^5	sc	10	90	Fiebre alta †(14 días pi)	-	-	70		Bigalke et al., 1974
	Cultivo celular [c]	10-10^5	sc	25	60	† (21- 30 días pi)	-	+, Bazo (ND)	68		Bigalke et al., 1974
	Cultivo celular [c]	$5x10^5$	sc	3	21	Fiebre, conjuntivitis, edema en testículos	-	+, Parasitemia (ITS1-PCR)	100	Seroconversión (3 semanas pi)	Basso et al., 2011
	Cultivo celular [c]	$6x10^6$	sc	6	70	-	-	+, Parasitemia (qPCR)	100	Seroconversión (21 días pi)	Lienard et al., 2015

Bz	Bovino [e]	3-10 ml	sc	7	9-13*	Fiebre, edema en escroto, prepucio y extremidades	Quistes en piel, necrosis en escroto	+, Parasitemia (ND)	85,7	-	Bigalke, 1967
		7-10 ml	ip	4	9-20*				100	-	Bigalke, 1967
		ND	co	3	13*	-	Quiste en piel	-	100	-	Bigalke, 1968
		ND	in	2	10*	-	-	+, (ND)	100	-	Bigalke, 1968
	Aislado de antílope	ND	sc	1	20	Linfadenitis, epididimitis	Lesiones cutáneas [c]	(**)	100	-	Basson et al., 1970
		ND	sc	6	24-100	Linfadenitis	Lesiones cutáneas [c]	(**)	100	-	Basson et al., 1970
	Bovino [e]	$5x10^5$	sc	3	21*	Fiebre, conjuntivitis y edema en los testículos	-	+, Parasitemia, (ITS1-PCR)	100	Seroconversión (3 semanas pi)	Basso et al., 2011
	Bovino [e]	$6x10^6$	sc	6	70	Fiebre, fotofobia y descarga nasal	Quistes en piel y otros tejidos	+, Parasitemia y quistes (Histología, qPCR)	100	Seroconversión (14 días pi)	Lienard et al., 2016
	Stomoxys calcitrans [f]	-	P	3	152	Fiebre (7-12 días pi).	-	+, Parasitema (qPCR)	100	Seroconversión (14 días pi)	Sharif et al., 2019
Tz/ Bz	Bovino/ Conejo [g]	10-18 ml	ip	5	ND	Fiebre, edema en el escroto y prepucio	Quistes	+, Parasitemia (ND)	60 [h]	-	Bigalke, 1967
		10 ml	iv	1	ND	-	-	-	100	-	
		10 ml	sc	5	11-12*	-	-	-	60 [h]	-	
ND	Sangre de bovino infectado	30 ml	iv	1	11*	†	-	+, Parasitemia (Frotis)	0	-	Pols, 1960
		50 ml	ip	1	11*	Edema en extremidades, base de las orejas y escroto	-		0	-	
		100 ml	sc	1	14*		-		ND	-	

[a] **n:** Número total de animales inoculados. [b] Duración del ensayo (días). [c] Cultivo celular mantenido en células VERO. [d] Lesiones cutáneas en el punto de inoculación. [e] Bovino infectado crónicamente (piel).
[f] *Stomoxys* que habían picado a una vaca infectada crónicamente. [g] Homogeneizado de quistes tisulares y sangre de conejo infectado experimentalmente. [h] Muertes de 2 animales tras la inoculación, posiblemente relacionadas con la escasa calidad del inóculo. ***** Periodo de incubación. **ip:** Intraperitoneal. **sc:** Subcutánea. **iv:** Intravenosa. **P:** Picaduras. **ND:** No determinado. **pi:** Post-infección.
-: Ausente. **+:** Positivo. **ml:** Mililitros. † Muerte**.**
(**) +, Aparato reproductor, miocardio, venas, bazo y pulmón (Histología).

Tabla 8: Infecciones experimentales con taquizoítos de *B. besnoiti* en bovino (adaptación de Álvarez-García et al., 2014b).

Aislado	Procedencia del inóculo	Dosis	Vía	Sexo	Raza	Edad	Inmunosupresión	Aguda [a]	Crónica [b]	Detección del parásito	Serología (técnica)	Referencia
Lamprechts A y B	Conejo [c]	ND	Oral	Macho	Holstein	2-3 años	No	Leve	Leve	+, Quistes en conjuntiva ocular	ND	Bigalke, 1968
Fuls strain (Aislado de pase bajo)	Conejo [d]	2,7-14,1x10^7	iv	Macho	ND	3 años	No	Leve	Leve	(*)	ND	Bigalke, 1968
	Conejo [e]	ND	iv	Hembra	Hereford	ND	No	Moderado	Grave	(*)	ND	Bigalke, 1968;
	Cultivo celular	104x10^6	iv	Hembra	Hereford	7 años	Si [f]	Grave	-	+, Células endoteliales	ND	Basson et al., 1970
	Cultivo celular	984,9x10^6	iv	Macho	South Devon Afrikaner	1 año	No	Leve	Leve	(*)	ND	Basson et al., 1970
	Cultivo celular	1068x10^6	iv	Macho	Hereford	3 años	Si [f]	Moderado	Grave	(*)	ND	Bigalke, 1968
	Sangre de bovino	3,5-4l	iv	Macho	Hereford	2-3 años	No	Moderado	Moderado	(*)	ND	Basson et al., 1970
Fuls strain (Aislado de pase alto)	Cultivo celular	46,75-66,4x10^6	iv	Macho	ND	2 años	No	Leve	-	-	ND	Bigalke., 1968
	Cultivo celular	106,56x10^6	sc	Macho	ND	3 años	No	Leve	-	-	ND	Bigalke, 1968
	Conejo [d]	141,75x10^6	iv	Macho	Hereford	3 años	No	Grave	-	+, Parasitemia	ND	Bigalke, 1968;
	Sangre de bovino	4l	iv	Macho	Hereford	3 años	No	Leve	Leve	+, Quistes en piel y venas	ND	Basson et al., 1970
Bb-Israel	Cultivo celular (Vero)	10^6-7,6x10^9	iv, im, sc	Macho	Holstein	>1 año	No	Leve	-	-	+,(IFI)	Shkap, 1986
		1-2,5x10^9	iv	Macho	Holstein	>1 año	Si [j]	Leve	-	-	+,(IFI)	
	Sangre de bovino	1L	iv	Macho	Holstein	>1 año	Si [j]	Leve	-	-	+,(IFI)	
Aislado de ñu	Sangre de conejo	50 ml	iv	Macho	Hereford	3-6 años	No	-	-	-	ND	Bigalke, 1967
	Sangre de oveja	40-80 ml	sc, iv	Macho	Hereford	3-6 años	No	-	-	-	ND	Bigalke, 1967
	Cultivo celular	10^6	sc	ND	ND	3 meses	No	-	-	-	+,(IFI)	Janitschke et al., 1974
	Cultivo celular	3x10^6	iv	ND	ND	3 meses	No	-	-	-	+,(IFI)	Janitschke et al., 1974
ND	Jerbo [k]	2x10^7	iv	ND	Holstein	3-6 meses	No	-	-	-	ND	Diesing et al., 1988
				ND	Holstein		Si [j]	Leve	-	+, Parásito en piel	ND	
	Cultivo celular (MDBK)	3,7x10^8	sc	ND	Holstein		No	Leve	-	-	ND	
				ND	Holstein		Si [f]	Leve	-	-	ND	
	Cultivo celular (MDBK)	2x10^8 + 5x10^7	ip	ND	Holstein		Si [f]	-	-	-	ND	
		2x10^8 + 5x10^7	ip	ND	Holstein		No	Leve	-	-	ND	
	Cricetos [k]	4x10^7	ip	ND	ND	>1 año	Si [f/j]	Grave	-	+, Parasitemia	ND	

[a] Signos clínicos de la fase aguda: Leve (fiebre) / Moderado (Fiebre, debilidad y edema) / Grave (muerte). [b] Signos clínicos de la fase crónica: Leve (quistes visibles en conjuntiva ocular) / Moderado (hiperqueratosis, alopecia y quistes tisulares) / Grave (baja condición corporal y muerte). [c] Vísceras de conejo. [d] Homogeneizado de testículos de conejo. [e] Homogeneizado de testículos de conejo junto con sangre de un bovino infectado. [f] Esplenectomizados. [j] Tratados con corticoides antes de la inoculación. [k] Lavado peritoneal. **ip:** Intraperitoneal. **sc:** Subcutánea. **iv:** Intravenosa. **im**: Intramuscular. **ND:** No determinado. **-:** Ausente. **+:** Positivo. **ml:** Mililitros. **l:** Litros. **IFI:** Inmunofluorescencia indirecta. **MDBK**: Células epiteliales de riñon de mono. **(*)** +, Parasitemia y presencia de quistes en esclerótica, piel y venas.

Tabla 9: Infecciones experimentales con bradizoítos de *B. besnoiti* en bovino (adaptación de Álvarez-García et al., 2014b).

Especie de origen	Procedencia del inóculo	Dosis	Vía	Sexo	Raza	Edad	Inmunosupresión	Aguda [a]	Crónica [b]	Detección del parásito	Serología (técnica)	Referencia
Ñu/Impala	Quistes tisulares	1,8-5,4x10^7	sc, iv	ND	Cruzada	< 1 año	No	Leve	-	+, Parasitemia	ND	Bigalke, 1967
Bovino	Quistes tisulares	ND	sc, iv	Hembra	ND	ND	No	-	-	-	ND	Besnoit y Robin, 1912
	Quistes tisulares	ND	oral	Macho y Hembra	ND	ND	No	-	-	-	ND	Bigalke, 1968
	Quistes tisulares	2,2-4,2x10^6	in	Macho	ND	2-3 años	No	Leve	Leve	+, Quistes en conjuntiva y venas	ND	
	Quistes tisulares	2,3x10^6	sc, iv	ND	ND	2 años	No	Leve	-	-	ND	
	Picaduras de *Glossina*	ND	sc	Macho	ND	ND	No	Leve	Leve	+, Quistes en conjuntiva	ND	
		ND	sc	Macho	ND	ND	No	Moderado	-	-	ND	
	Picaduras de *Stomoxys*	ND	sc	Macho	ND	2 años	No	Leve	Leve	+, Quistes en conjuntiva	ND	
	Picaduras de 2 tábanos	ND	sc	Macho	ND	3 años	No	-	-	-	ND	
	Picaduras de 6 tábanos	ND	sc	ND	ND	ND	No	Moderado	Leve	+, Quistes en conjuntiva y piel	ND	
	Quistes tisulares	2x10^7	ip	ND	Holstein	3-6 meses	Sí [c]	Moderado	Moderado	+, Tejido conectivo	ND	Diesing et al., 1988
		2x10^7	ip	ND	Holstein	3-6 meses	No	Leve	-	-	ND	
		1,4x10^7	sc, iv	ND	Holstein	3-6 meses	Sí [c]	Moderado	Moderado	+, Tejido conectivo	ND	
		1,5x10^7	ip	ND	Holstein	3-6 meses	No	-	-	-	ND	
		3x10^8	sc, iv	ND	Holstein	3-6 meses	Sí [c/d]	Grave	-	+, Parasitemia	ND	
		2x10^8	sc	Macho	ND	Adulto	Sí [c/d]	Moderado/Grave	-	+, Parasitemia	ND	
		2x10^8	iv	Macho	ND	Adulto	Sí [c/d]	Grave	-	+, Parasitemia	ND	
		2x10^8	ip	Macho	ND	Adulto	Sí [c/d]	Moderado/Grave	-	+, Parasitemia	ND	
		2x10^7	ip	ND	ND	Adulto	Sí [c/d]	Grave	-	+, Parasitemia	ND	
	Quistes tisulares	3x10^7	sc	Macho	Holstein	>1 año	No	Leve	-	-	+, (IFI)	Shkap, 1986

[a] Signos clínicos de fase aguda: Leve (fiebre) / Moderado (Fiebre, debilidad y edema) / Grave (muerte). [b] Signos clínicos de fase crónica: Leve (quistes visibles en conjuntiva ocular) / Moderado (hiperqueratosis, alopecia y quistes tisulares) / Grave (baja condición corporal y muerte). [c] Tratados previamente con corticoides. [d] Esplenectomizados. **ip:** Intraperitoneal. **sc:** Subcutánea. **iv:** Intravenosa. **in**: Intranasal **ND:** No determinado. **-:** Ausente. **+:** Positivo. **IFI:** Inmunofluorescencia indirecta.

La inmunosupresión de los animales indujo el desarrollo de signos clínicos. Sin embargo, partir de animales inmunodeprimidos no parece ser la mejor opción para el desarrollo de un modelo animal, ya que los hace más susceptibles a infecciones ambientales.

2.2.1.3. Modelos *in vivo* en otras especies

Como se muestra en la Tabla 10, se han inoculado con *B. besnoiti* especies de animales muy diferentes. El objetivo de estas infecciones fue, generalmente, esclarecer el ciclo biológico completo del parásito, ya que el hospedador definitvo no se conoce. Para ello, diversas especies de carnívoros, reptiles y aves fueron alimentadas con tejido procedente de bovinos infectados crónicamente que contenían quistes del parásito (Pols, 1960; Diesing et al., 1988; Basso et al., 2011). Sin embargo, hasta el momento, ninguno de estos animales ha sido identificado como hospedador definitvo en el ciclo biológico de *B. besnoiti*. Solo Peteshev et al. (1974) reportaron la eliminación de ooquistes por gatos infectados y que, posteriormente, fueron capaces de infectar a terneros y cabras. Sin embargo, la dificultad para reproducir nuevamente este experimento con las condiciones similares a las descritas ha hecho que se cuestionen seriamente los resultados obtenidos por estos autores.

De las especies de roedores inoculadas, diferentes de las incluidas en las especies de laboratorio (Tabla 6), únicamente los topillos (*Microtus arvalis*) mostraron cierta susceptibilidad a la infección, comprobándose la distribución intraorgánica del parásito *(*Basso et al., 2011). Otros animales como gallos, caballos y pequeños rumiantes (ovejas) no parecen ser susceptibles (Pols, 1960). Este mismo autor, reportó signos clínicos característicos de las fases aguda y crónica de la enfermedad en cuatro cabras inoculadas por vía intravenosa con sangre de conejo infectado experimentalmente. Sin embargo, la falta de un control de calidad en el inóculo y del estado sanitario previo de los animales dificulta la interpretación de estos resultados.

2.2.2. Modelos *in vivo* en otras especies de *Besnoitia*

En la Tabla 11 se muestran algunas de las infecciones experimentales realizadas con otras especies de *Besnoitia* distintas a *B. besnoiti*. Entre los años sesenta y setenta del siglo pasado, la descripción de nuevas especies de *Besnoitia* hizo que las inoculaciones experimentales en distintos animales fueran fundamentales para entender, entre otros aspectos, su ciclo biólogico, modo de transmisión y rango de hospedadores (Senaud et al., 1974; Frenkel, 1977; Fayer y Frenkel, 1979; Smith y Frenkel, 1984). Algunas de las infecciones experimentales realizadas en animales de laboratorio mostraron la susceptibilidad de éstos a aquellas especies de *Besnoitia* que tienen al gato como hospedador definitivo (*B. darlingi, B. wallacei, B. oryctofelisi, B. neotomofelis*) (Dubey et al., 2002; Dubey y Lindsay, 2003; Dubey et al., 2003b; Dubey y Yabsley, 2010). En estas especies, las infecciones de gatos realizadas por vía oral demostraron la eliminación de ooquistes no esporulados en las heces de estos animales, así como esquizontes y diversas formas compatibles con la

reproducción sexual del parásito en el intestino (Frenkel, 1977, Smith y Frenkel 1984; Dubey y Lindsay, 2002; Dubey y Yabsley, 2010). En cambio, infecciones similares realizadas en gatos con *B. besnoiti* y *B. caprae* no reportaron resultados similares (Tabla 12).

Con respecto a *B. caprae*, diversos experimentos han intentado establecer un modelo animal para esta enfermedad (Tabla 11). Para ello, distintas especies de roedores y rumiantes se han inoculado, principalmente, con bradizoítos del parásito, obteniendose resultados similares a los observados en *B. besnoiti* (Njenga et al., 1993; Ng'ang'a y Kasigazi, 1994; Oryan et al., 2010; Namazi et al., 2011; Oryan et al., 2014). La única diferencia reportada entre infecciones experimentales realizadas con *B. besnoiti* o *B. caprae* fue con respecto a la susceptibilidad de los conejos a la infección. En un solo trabajo (Ng'ang'a y Kasigazi, 1994), los conejos inoculados no fueron susceptibles a la infección por bradizoítos de *B. caprae*, a diferencia de lo que ocurre con *B. besnoiti*. La inoculación de este mismo estadio, procedente de quistes contenidos en tejido parasitado de cabras infectadas naturalmente, produjo signos clínicos de fase aguda (fiebre, edema) y de la fase crónica (quistes en conjuntiva ocular, dermis, fascia, túnica albugínea, ollares, cornetes nasales y pezones de las ubres) en cabras sanas (Njenga et al., 1993; Ng'ang'a y Kasigazi, 1994). Oryan et al. (2010) desarrollaron un modelo en embrión de pollo para esta enfermedad, al igual que se hace para el estudio de diversos virus, aunque este modelo no se ha vuelto a emplear (Tabla 11).

Tabla 10: Infecciones experimentales con *B. besnoiti* en otras especies.

Orden Nombre común / Nombre científico		n	Duración[a]	Estadio	Origen	Dosis	Vía[b]	Detección del parasito (técnica)	Observaciones	Referencia
Carnívoros										
Gato doméstico/	*Felis silvestris catus*	3	ND	Bz	Bovino	ND	oral	+, Ooquistes (Flotación)	13-16 días pi	Peteshev et al., 1974
		2	21	Bz	Bovino	ND	oral	-	-	Diesing et al., 1988
		2	21	Tz	Cultivo celular[c]	$5x10^6$	oral	-	-	Basso et al., 2011
		3	21	Bz	Bovino	$2x10^7$	oral	-	Seroconversión (3 semanas pi)	Basso et al., 2011
		11	46	Bz	Bovino	$3x10^8$	oral	-	Seroconversión (23-46 días pi)	Marcén-Seral, 2011
Perro/	*Canis lupus familiaris*	1	90	Tz	Conejo[d]	200 ml	iv	-	-	Pols, 1960
		1	21	Bz	Bovino	ND	oral	-	-	Diesing et al., 1988
		3	21	Tz	Cultivo celular[c]	$5x10^6$	oral	-	-	Basso et al., 2011
		3	21	Bz	Bovino	$2x10^7$	oral	-	-	Basso et al., 2011
Caracal/	*Caracal caracal*	2	21	Bz	Bovino	ND	oral	-	-	Diesing et al., 1988
Chacal de lomo negro/	*Canis mesomelas*	2	21	Bz	Bovino	ND	oral	-	-	
Gato de la jungla/	*Felis chaus*	2	21	Bz	Bovino	ND	oral	-	-	
Guepardo/	*Acinonyx jubatus*	2	21	Bz	Bovino	ND	oral	-	-	
León/	*Panthera leo*	1	21	Bz	Bovino	ND	oral	-	-	
Leopardo/	*Panthera pardus*	3	21	Bz	Bovino	ND	oral	-	Ooquistes tipo *Cystoisospora*[e]	
Zorro del cabo/	*Vulpes chama*	1	21	Bz	Bovino	ND	oral	-	-	
Mangosta negra/	*Atilax paludinosus*	1	21	Bz	Bovino	ND	oral	-	-	
Mangosta rayada/	*Mungos mungo*	2	21	Bz	Bovino	ND	oral	-	-	
Jineta/	*Genetta genetta*	2	21	Bz	Bovino	ND	oral	-	-	
Roedores										
Rata de arena/	*Psammomys obesus*	8	30	Tz	Cultivo celular	10^7	ip	+, Parasitemia (Microscopía)	† (6-8 días pi)	Shakp 1986
Topillo/	*Microtus arvalis*	3	21	Tz	Cultivo celular[c]	$5x10^5$	sc	+, Parasitemia/tejidos (ITS1-PCR)	Seroconversión (3 semanas pi)	Basso et al., 2011
		4	21	Bz	Bovino	$5x10^5$	sc			Basso et al., 2011
Hiracoideos										
Damán roquero/	*Procavia capensis*	4	42	Tz	Sangre de conejo	40 ml	ip	-	-	Pols, 1960
Équidos										
Caballo/	*Equus ferus caballus*	2	90	Tz	Conejo[d]	600 ml	iv	-	-	Pols, 1960

Rumiantes										
Oveja/	*Ovis aries*	4	5- 7*	Tz	Sangre de conejo	300-500 ml	iv	-	Fiebre, anorexia	Pols, 1960
Cabra/	*Capra aegagrus*	4	6	Tz	Sangre de conejo	300-500 ml	iv	+, Parasitemia (ND)	(**)	Pols, 1960
Reptiles										
Culebra bastarda/	*Malpolon monspessulanus*	2	21	Bz	Bovino	ND	oral	-	-	Diesing et al., 1988
Culebra negra/	*Dolichophis jugularis*	3	21	Bz	Bovino	ND	oral	-	-	
Serpiente gato árabe/	*Telescopus dhara*	2	21	Bz	Bovino	ND	oral	-	-	
Serpiente látigo/	*Hemorrhois ravergieri*	2	21	Bz	Bovino	ND	oral	-	-	
Víbora bufadora/	*Bitis arietans*	2	21	Bz	Bovino	ND	oral	-	-	
Víbora de palestina/	*Daboia palaestinae*	8	21	Bz	Bovino	ND	oral	-	-	
Aves										
Gallo/	*Gallus gallus domesticus*	6	42	Tz	Sangre de conejo	60 ml	ip, sc, iv	-	-	Pols, 1960
		6	42	Tz	Sangre de conejo	70 ml	sc, iv	-	-	Pols, 1960
Buitre africano/	*Gyps africanus*	2	21	Bz	Bovino	ND	oral	-	-	Diesing et al., 1988

n: Número total de animales inoculados. [a] Duración del ensayo (días). [b] Las inoculaciones por vía oral se realizaron, principalmente, para identificar un posible hospedador definitivo.
[c] Aislado Bb- GER1. [d] Suspensión de hígado, bazo y sangre de conejo infectado. [e] Los ooquistes, al ser empleados como inóculo, no reprodujeron signos de besnoitiosis en los bovinos infectados con ellos.
†: Muerte**. *** Periodo de incubación. **ip:** Intraperitoneal. **sc:** Subcutánea. **iv:** Intravenosa. **Tz:** Taquizoítos. **Bz:** Bradizoítos. **ml:** Mililitros. **ND:** No determinado. **pi:** Post-infección. **-:** Ausente. **+:** Positivo.
(**): Las cabras inoculadas mostraron signos característicos de la besnoitosis aguda como fiebre, edema, lacrimeo ocular y descarga nasal. Además, mostraron quistes y alopecias difusas al final del ensayo.

Tabla 11: Infecciones experimentales realizadas con otras especies de *Besnoitia*.

Besnoitia	Especie inoculada	n	Días [a]	Estadio inoculado	Origen del inóculo	Dosis	Vía	Resultados más relevantes	Referencias
B. darlingi	Zarigüella	3	83	Bz	Ratón [b]	ND	oral	Orejas, lengua, ln. mesentéricos, tracto gastrointestinal, riñón, glándula adrenal y corazón [h]	Smith y Frenkel, 1984
	Zarigüella	2	124	Ooq	Gato [b]		oral		
	Zarigüella	1	92	Tz	Zarigüeya [c]		ip	Orejas, lengua, hígado, mesenterio y bazo [h]	Smith y Frenkel, 1984
	Ratón [d]	1	11	Bz	Zarigüeya		ip	† Exudado peritoneal [g]	
	Ratón [d]	2	12	Bz	Zarigüeya		sc	† Hígado y pulmón [g]	
	Ratón [e]	1	154	Tz	Cultivo celular		sc	-	Dubey et al., 2002
	Ratón [e]	15	11-154	Bz	Quistes de Zarigüeya		ip	Exudado peritoneal [g]. Corazón y lengua [h]	
	Ratón [e]	8	11-121	Ooq	Gato [b]		oral	Corazón, hígado, pulmón y bazo [h]	
B. oryctofelisi	Jerbo	5	13*	Ooq	Gatos [b]	Incontables	oral	† (50% con enteritis, peritonitis) Hígado, intestino, pulmones, ln. meséntéricos y corazón [g]	Dubey y Lindsay, 2003
	Jerbo	5	49*	Ooq	Gatos [b]	$10\text{-}10^4$	oral	Corazón, pulmón, ln. mesentéricos, músculo y cerebro [h]	
	Jerbo	5	53*	Tz	Ratón [c]	ND	sc	Corazón, pulmón, ln. mesentéricos, músculo y cerebro [h]	
	Ratón [d]	3	7-12	Tz	Jerbo [b]	ND	ip	† Peritonitis	
	Ratón [d]	3	7-12	Tz	Jerbo [b]	ND	ip	† Peritonitis	
	Conejo	2	34-37	Ooq	Gato [b]	2500-25000	oral	Sanos. Ojo, fascia y músculo [h]	
	Ratón [e]	20	30	Ooq	Gato [b]	$10\text{-}10^4$	oral	† (20% de los animales)	
	Ratón [d]	20	18	Ooq	Gato [b]	$10\text{-}10^4$	oral	† (18 días pi) Enteritis, necrosis en ln. linfáticos y hepatitis.	
B. wallacei	Ratón	ND	60	Ooq	Gato [b]	ND	oral	Ojo, intestino y peritoneo (40-60 días pi) [h]	Frenkel, 1977
	Rata	ND	56-393				oral	Intestino, mesenterio, hígado y pulmón [h]	
	Ternero	2	30-91				oral	Aumento de la temperatura (1-4 dias pi)	Fayer y Frenkel, 1979
B. jellisoni	Criceto	ND	ND	Tz	ND	ND	sc	Desarrollo de hipercortisolismo adrenal [i]	Frenkel, 1977
	Ratón	20	63	Bz	Ratón [b]	ND	ip	Líquido peritoneal (4-5 días pi) [g]. Hígado, bazo, intestino y ciego (3-9 semanas pi) [h/ i]	Senaud et al., 1974
B. neotomofelis	Ratón [d]	16	6-89	Tz/Bz/Ooq	Cultivo celular/ ratón y gato [b]	ND	sc	† (6-18 días pi). Enteritis, ulceras intestinales, neumonitis y hepatitis [h]	Dubey y Yabsley 2010
	Ratón [e]	198							

B. caprae	Conejo	4	7-56	Bz	Cabra	$4x10^9$	sc	-	Njenga et al., 1993
	Conejo	4				$4x10^9$	iv	-	
	Conejo	4				$4x10^9$	ip	-	
	Criceto	6				$3x10^9$	sc	-	
	Criceto	6				$3x10^9$	ip	-	
	Criceto	6				$3x10^9$	sc	-	
	Cobaya	6				$4x10^9$	ip	-	
	Rata	4				$3x10^9$	sc	-	
	Rata	4				$3x10^9$	iv	-	
	Rata	4				$3x10^9$	ip	-	
	Ratón	6				$2,5x10^9$	sc	-	
	Ratón	6				$2,5x10^9$	ip	-	
	Cabra	6				$8x10^9$	sc	(**)	
	Cabra	6				$8x10^9$	iv	(**)	
	Toro	2	56-60			$20x10^9$	sc	-	
	Toro	2	56-60			$20x10^9$	iv	-	
	Rata	20	60-120	Bz	Cabra	10^6	sc, ip	-	Ng`ang`a y Kasigazi, 1994
	Ratón	20				10^6	sc, ip	-	
	Conejo	15				10^6	sc, ip	-	
	Oveja	10				10^8	sc, ip	-	
	Cabra	4				10^8	sc, ip	Conjuntiva ocular, piel, músculo esquelético, ln. mesentéricos, testículos y pezones (25-45 días pi) [h]	
	Ratón [f]	25	40	Bz	Aislado *B. caprae*-1	10^3-10^6	ip	(***)	Oryan et al., 2010
		25	40	Bz	Aislado *B. caprae*-2	10^3-10^6	ip	-	
	Embrión de pollo	42	21-50	Tz	Cultivo celular (VERO)	10^3-$2x10^7$	Cavidad alantoidea	† (5-12 días pi). Al nacimiento paresía, hiperemia en la piel y pérdida de plumas. Neumonía e inflamación en hígado y corazón (50 días pi)	Oryan et al., 2010
	Ratón [f]	25	60	Tz	Ratón [f/ c]	$12,5x10^3$-$2x10^5$	ip	† (3-10 dias pi). Líquido peritoneal [g]. Alopecia y lesiones de piel en la parte ventral del cuerpo (60 dias pi)	Namazi et al., 2011
	Ratón [f] Ratón no consanguíneo [f] Ratón C57 BL/6	30	60	Tz	Ratón [f/ c]	$12,5x10^3$-$2x10^5$	ip	† (5-8 días pi). Lavado peritoneal [g]	Oryan et al., 2014

n: Número total de animales inoculados. [a] Días de duración del ensayo. [b] Animales infectados experimentalmente. [c] Lavados de líquido peritoneal. [d] Ratones IFN-ɣ knockout [e] Ratones Swiss Webster.
[f] Ratones BALB/c. [g] Detección de taquizoítos. [h] Detección de quistes. [i] Tratados posteriormente con sulfadiazinas y glucocorticoides. * Periodo de incubación. **ip:** Intraperitoneal. **sc:** Subcutánea.
iv: Intravenosa. **Ooq:** Ooquistes. **Tz:** Taquizoítos. **Bz:** Bradizoítos. **pi:** Post-infección. **-:** Ausencia de signos clínicos. **ln:** Linfonódulos. † Muertes.
(**) Fiebre, anorexia y ligero edema. Detección de quistes en conjuntiva ocular, dermis, fascia, túnica albugínea, ollares, cornetes nasales y pezones (25-40 días pi).
(***) Animales inoculados con dosis altas: †4-9 días pi. Neumonía, peritonitis, hiperemia y congestión del miocardio, bazo e intestino.
Animales inoculados con dosis bajas: Inflamación crónica en miocardio, bazo, pulmones y enteritis hemorrágica.

Tabla 12: Infecciones experimentales realizadas con *Besnoitia* spp. en gatos por vía oral.

Besnoitia spp.	n	Edad	Duración [a]	Estadio	Procedencia	Dosis	Periodo de prepatencia [a]	Periodo de patencia [a]	Observaciones	Referencia
B. darlingi	14	ND	39	Bz	Zarigüeya	ND	9-14	3-13	-	Smith y Frenkel, 1984
	2		39	Oq	Gatos		-	-	-	Smith y Frenkel, 1984
	5		ND	Bz	Zarigüeya		13-21	ND(**)	Esquizontes en intestino, hígado y mesenterio	Dubey et al., 2002
B.oryctofelisi	7	64-70 [a]	4-11	Bz	Jerbos	ND	9-11	ND(**)	(***)	Dubey y Lindsay., 2003
B. wallacei	11	Jóvenes	ND	Bz	Ratones	ND	12-15	5-12	Esquizontes en intestino e hígado (4-14 días pi)	Frenkel, 1977
	4	ND		-	Tejidos de gato [c]		-	-	-	Frenkel, 1977
	13	ND		Bz	Ratones y ratas		12-16	ND(**)	-	Ngabga, 1993
B. neotomofelis	14	ND	2-69	Bz	Cricetos	ND	11-27	ND(**)	(****)	Dubey y Yabsley, 2010
	2	ND	2-69	Bz	Ratones	ND	-	-	-	Dubey y Yabsley, 2010
B. caprae	8	ND	30	Bz	Cabra [d]	-	-	-	-	Ng`ang`a y Kasigazi, 1994
	2				Cabra [d]	10^6	-	-	-	
	2				Cabra [d]	-	-	-	-	
	2				Ratas y ratones	ND	-	-	-	
B. besnoiti (*)	2	ND	21	Bz	Bovinos [d]	ND	-	-	-	Diesing et al., 1988
	2	19-48 [b]	21	Tz	Aislado Bb-GER1	$5x10^6$	-	-	-	Basso et al., 2011
	3	ND	21	Bz	Bovino [d]	$2x10^7$	-	-	Seroconversión (2 gatos; 3ª semana pi)	Basso et al., 2011
	11	3-4[b]	46	Bz	Bovino [d]	$3x10^8$	-	-	Seroconversión (3 gatos; 23-46 días pi)	Marcén-Seral, 2011

n: Número total de animales inoculados. [a] Días. [b] Meses. [c] Tejidos (hígado, pulmón, intestino, corazón, linfonódulos) de gatos que habían eliminado ooquistes. [d] Animales infectados crónicamente.
O**q:** Ooquistes. **Tz:** Taquizoítos. **Bz:** Bradizoítos. **pi:** Post-infección. **-:** Ausencia. **ND:** No determinado.
(*) Peteshev et al. (1974) reportaron la eliminación de posibles ooquistes de *B. besnoiti* en gatos tras la ingestión de téjidos de bovino infectado crónicamente.
Sin embargo, estos hallazgos son cuestionables al no haber sido reproducidos posteriormente en infecciones similares bajo condiciones más controladas (Basso et al., 2011).
(**) Se detectaron escaso número de ooquistes en las heces.
(**) Detección del parásito en linfonódulos mesentéricos, glándula adrenal, cerebro, corazón, riñón, hígado, pulmón, páncreas, músculo y bazo.
(***) Detección de taquizoítos y quistes en submucosa intestinal, músculo intersticial y linfonódulos.

CAPÍTULO III

JUSTIFICATION AND OBJECTIVES

Justification and objectives

Bovine besnoitiosis, caused by the apicomplexan protozoan *Besnoitia besnoiti*, is a chronic and debilitating disease that causes local and systemic clinical signs in the infected animals. Males may develop infertility or even sterility and dams may occasionally abort, being responsible for considerable economic losses, mainly in cattle industry (Álvarez-García et al., 2013; Gutiérrez-Expósito et al., 2017c). The disease spreads rapidly and it is considered to be re-emerging in Europe (European Food Safety Authority, 2010). This is mainly due to the fact that the control and prevention relies only on diagnostic and management measures, since no chemotherapeutics are available and no vaccines are licensed in Europe (Álvarez-García, 2013).

In this respect, it should be noted that several proof-of-concept studies performed in *in vitro* systems have shown that new generation drugs and commercially available drugs are effective against parasite invasion and proliferation (Cortes et al., 2011; Jiménez-Meléndez et al., 2017; 2018; Cervantes-Valencia et al., 2018; Müller et al., 2019). However, these promising therapeutic tools need to be tested in an *in vivo* model able to reproduce clinical signs characteristic of acute and chronic besnoitiosis. The use of laboratory animals is of advantage in terms of low costs, easy handling and litter size (López-Pérez et al., 2006). However, in *B. besnoiti*, several laboratory rodents (hamster, gerbils and various strains of white mice) have not been susceptible to the infection (Shkap et al., 1987; Basso et al., 2011). In contrast, gamma-interferon knockout mice are very susceptible to the acute infection (Schares et al., 2009), although they are not recommended as an appropriate laboratory model due to the rapid onset of the acute stage and sudden death. Rabbits showed susceptibility to experimental infection by developing clinical signs of the acute phase of the disease, but the results were variable with respect to the chronic stage (Liénard et al., 2015). Moreover, the authors suggested that *B. besnoiti* adaptation to a heterologous host was difficult since cattle is the target species.

Between the 1960s and 1980s, a considerable effort was invested to develop an experimental model of bovine besnoitiosis with inconclusive results. These results were due to the diversity of the inocula employed (parasite isolate, infection dose, quality control of the inoculum) and variable experimental designs (route of administration, time of infection, age and previous health status of the inoculated animals) in the absence of serological and molecular tests. In general, acute besnoitiosis (fever and lymphadenopathy) has been detected on several occasions in infected animals (Bigalke, 1968; Bigalke et al., 1970; Basson et al., 1974). By the contrary, specific clinical signs of the acute (e.g. oedema and anasarca) and chronic phase (tissues cysts) were only reported in infected adult animals (Basson et al., 1970), as well as in immunosuppressed animals that had been infected with bradyzoites (Diesing et al., 1988). Accordingly, the lack of a *B. besnoiti* cattle standardized model impairs comparisons among experiments and results.

In this scenario**, the general aim of the present Doctoral Thesis was to develop a bovine experimental model for *B. besnoiti* infection.** To this end, the two known asexual stages of the parasite (tachyzoites and bradyzoites) were inoculated in cattle following a standardized experimental desing. Other parasite- (dose, inoculation route) and host- (age) dependent factors that may determine the outcome of the infection were also studied (Álvarez-García et al., 2014b). Moreover, a high quality control of the inoculum and a good health status of the animals were properly checked. First, a new *B. besnoiti* isolate (Bb-Spain 3) was obtained from a young calf with chronic besnoitiosis. Bb-Spain 3 tachyzoites were maintained in *in vitro* culture cells until these were intravenously inoculated in bulls and calves with different doses. Next, tachyzoites were inoculated by different administration routes and, finally, bradyzoites from a chronically infected bull were inoculated by intravenous, subcutaneous and intradermal route. Herein, histological findings in experimentally infected calves were analysed in different target organs. Additionally, these experimental infections provided valuable serum samples that were useful for the development of novel serological tools for the diagnosis of early *B. besnoiti* infection.

- **Objective 1**

Inoculation of *Besnoita besnoiti* tachyzoites in catte: Influence of parasite dose, administration route and animal age.

- Sub-objective 1.1. *Bovine chronic besnoitiosis in a calf: Characterization of a novel* Besnoitia besnoiti *isolate from an unusual case report.*

The aim of this sub-objective was to obtain and characterize the first *B. besnoiti* isolate from a calf origin in an *in vitro* system and molecular genotyping. Moreover, the first case report of chronic bovine besnoitiosis in a calf younger than six months of age was thoroughly described. After the post-mortem examination of the animal, an exhaustive tissue collection was carried out, and data on lesions and intra-organic parasite distribution were obtained by means of histopathological and PCR techniques.

- Sub-objective 1.2. *Effect of parasite dose and host age on the infection with* Besnoitia besnoiti *tachyzoites in cattle*.

The aim was to study the effect of parasite dose and host age on the infection dynamics with *B. besnoiti* tachyzoites in cattle and their impact on both the acute and chronic phases of the disease. Accordingly, two independent experimental infections were performed in calves and bulls following an exhaustive clinical monitoring and avoiding the limitations of previous trials (isolate, experimental design and diagnostic tests employed). Calves were intravenously inoculated with three doses (10^8, 10^7, and 10^6 tachyzoites) and bulls were infected with 10^6 tachyzoites by the same inoculation route. Exhaustive clinical monitoring and regular samplings were carried out, employing complementary

serological, histological and molecular tools in order to determinate the clinical outcome, immune response and intraorganic distribution of the parasite.

- Sub-objective 1.3. *The route of* Besnoitia besnoiti *tachyzoites inoculation does not influence the clinical outcome of the infection in calves.*

Herein, the influence of administration route on *B. besnoiti* tachyzoites inoculation was investigated. Calves were infected with 10^6 tachyzoites by the subcutaneous and intradermal route following the experimental design previously established with the aim of developing macroscopic clinical signs and lesions characteristic of chronic besnoitiosis. Again, the clinical outcome, immune response and intraorganic distribution of the parasite were studied.

- **Objective 2**

Inoculation of *Besnoitia besnoiti* bradyzoites in calves: Key factors in the appearance of clinical signs characteristic of chronic besnoitiosis and description of histological findings in target tissues.

- Sub-objective 2.1. *A model for chronic bovine besnoitiosis: Parasite stage and inoculation route are key factors.*

The objective was to investigate the outcome of the infection in cattle inoculated with a different invasive stage (bradyzoite stage) to that used in the previous objective. For this purpose, 10^6 bradyzoites were inoculated in calves by different routes of administration: intravenous, subcutaneous and intradermal routes. Clinical evaluation and serological monitoring were perfomed in the infected animals. Moreover, a large amount of tissue samples was analysed to parasite detection by histological and molecular tools.

- Sub-objective 2.2. *Histological findings in experimentally infected male calves with chronic besnoitiosis.*

Accordingly, the aim of this study was to describe the histological findings associated with *B. besnoiti* infection in experimentally infected male calves with chronic besnoitiosis. Microscopic lesions together with size and distribution of tissue cysts were exhaustively analysed in samples from reproductive and respiratory tracts as well as skin from different regions including the scrotum.

- **Objective 3**

Development and usefulness of novel serological tools for the diagnosis of early *Besnoitia besnoiti* infection.

- Sub-objective 3.1. *Progress on the diagnosis of bovine besnoitiosis: Added value of IgM detection and low avidity index as indicators of acute disease.*

The aim of this sub-objective was to improve the serological diagnosis of early bovine besnoitiosis. First, an IgM ELISA was developed and standardized. Next, specific IgM levels and IgG avidity kinetics were determined using a well-defined bovine sera panel from experimentally and naturally infected cattle.

CAPÍTULO IV

RESULTADOS (PUBLICACIONES)
RESULTS (PUBLICATIONS)

❖ Objetivo 1: Inoculación de taquizoítos de *Besnoitia besnoiti* en bovinos: Influencia de la dosis parasitaria, ruta de inoculación y edad de los animales.

La besnoitiosis bovina es una enfermedad crónica y debilitante del ganado vacuno que ocasiona cuantiosas pérdidas económicas asociadas a baja condición corporal, problemas de bienestar y fallo reproductivo en las hembras y los sementales afectados. En 2010, la EFSA alertó sobre su reaparición y, desde entonces, la enfermedad se ha propagado por toda la región Mediterránea, Europa Central y del Este, norte de Bélgica e Irlanda. Actualmente, el control y la prevención de la besnoitiosis bovina se basan únicamente en el diagnóstico precoz y la aplicación de medidas de manejo adecuadas, ya que no existen fármacos ni vacunas disponibles en Europa. Se han evaluado varios fármacos en sistemas *in vitro* con resultados prometedores, aunque todavía no se ha desarrollado un modelo *in vivo* de infección donde comprobar su seguridad y eficacia. Se han inoculado algunas especies de laboratorio sin obtener resultados satisfactorios y los estudios en modelo bovino, se han realizado bajo condiciones experimentales tan diferentes que es muy difícil establecer comparaciones entre ellos. En este objetivo de la Tesis Doctoral se ha intentado desarrollar un modelo bovino normalizado de infección por *B. besnoiti* inoculando taquizoítos del parásito y estudiando los efectos de la dosis parasitaria, la ruta de administración y la edad de los animales inoculados en el desarrollo de la infección.

En primer lugar, se obtuvo y se caracterizó un nuevo aislado de *B. besnoiti* (que se denominó Bb-Spain 3) procedente de una ternera infectada con besnoitiosis crónica. Este aislado, cuyo genotipo y compartamiento en cultivo celular (baja invasión y proliferación y desarrollo asincrónico) fue muy similar al de otros aislados españoles y europeos, se empleó en las infecciones experimentales, puesto que era capaz de causar enfermedad en animales jóvenes y, su reciente aislamiento, aseguraba escasa adaptación al cultivo celular. Además, se describió por primera vez y en detalle el caso clínico de un animal infectado con besnoitiosis bovina y menor de seis meses de edad. Este mostró las lesiones y la distribución intraorgánica del parásito descritas previamente en animales adultos con infección crónica.

A continuación, en un primer ensayo, tres grupos de terneros compuestos por tres animales cada uno se inocularon individualmente por vía endovenosa con tres dosis diferentes (10^8, 10^7, 10^6) de taquizoítos del aislado Bb-Spain 3, respectivamente, incluyéndose además un grupo testigo no infectado. Además, para estudiar el efecto de la edad en el resultado de la infección, se inocularon por la misma vía seis novillos con 10^6 taquizoítos del mismo aislado. Se realizó un seguimiento clínico y serológico de todos los animales para valorar la respuesta inmunitaria humoral y celular. A los 70 dias pi, en el caso de los terneros, y a los 115 días pi, en el de los novillos, se sacrificó a los animales para valorar las lesiones y la presencia del parásito en los tejidos. Se logró reproducir la fase aguda de la enfermedad observándose signos clínicos de carácter leve-moderado, pero no se observaron signos macroscópicos de la fase crónica (p.ej. quistes tisulares) ni en los terneros ni en los novillos, por lo que la edad de los animales inoculados parece no afectar al desarrollo de la infección. La distribución intraórganica del

parásito en los tejidos analizados fue escasa, encontrándose mayor cantidad de tejidos parasitados en los terneros inoculados con 10^6 taquizoítos.

Tras este ensayo se realizó otra inoculación con el mismo diseño experimental. En este caso, se utilizaron nueve terneros frisones (de mejor manejo y menor coste que los novillos) y se establecieron aleatoriamente tres grupos con tres animales cada uno. Dos grupos se inocularon con 10^6 taquizoítos del aislado Bb-Spain 3 por vía subcutánea e intradérmica respectivamente y el tercer grupo no se inoculó, actuando como testigo negativo de la infección. La dosis fue elegida según los resultados obtenidos en el experimento anterior. Se realizó un seguimiento clínico y serológico de los terneros para valorar la respuesta inmunitaria humoral y celular. A los 70 dias pi fueron sacrificados para valorar las lesiones y la presencia del parásito en los diferentes tejidos. Los animales inoculados desarrollaron signos clínicos de la fase aguda con gravedad de carácter leve-moderado (fiebre esporádica y linfadenomegalia). Sin embargo, no desarrollaron signos clínicos característicos de la fase crónica. La distribución intraorgánica del parásito en los tejidos fue escasa, sin detección histológica de quistes.

Por todo ello, se concluye que la dosis, la edad y la vía de inoculación de los taquizoítos de *B. besnoiti* no parecen influir en el desarrollo de la infección en bovino.

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Bovine chronic besnoitiosis in a calf: Characterization of novel *Besnoitia besnoiti* isolate from an unusual case report

Diezma-Díaz, C.[a], Jiménez-Meléndez, A.[a], Fernández, M.[b], Gutiérrez-Expósito, D.[a], García-Lunar, P.[a], Ortega-Mora, L.M.[a], Pérez-Salas, J.A.[c], Blanco-Murcia, J.[c], Ferre, I.[a], Álvarez-García, G.[a,*]

[a] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040-Madrid, Spain.

[b] Instituto de Ganadería de Montaña (CSIC-ULE). 24346. León, Spain.

[c] Animal Medicine and Surgery, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.

* Corresponding author: Gema Álvarez García. Tel.: +34-913944095. E-mail address: gemaga@vet.ucm.es

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Abstract

Bovine besnoitiosis, caused by the apicomplexan *Besnoitia besnoiti,* is a chronic and debilitating disease characterized by cutaneous and systemic manifestations that primarily affects adult beef cattle. Previous studies have reported that clinical besnoitiosisis is rare in calves. However, we isolated *B. besnoiti* from a chronically infected calf for the first time. The identity of the *Besnoitia* species was determined after parasite isolation and molecular genotyping. According to the results obtained *in vitro* the new isolate, named as Bb-Spain 3, was characterized in a reproducible *in vitro* model and was categorized as a low invader and low prolific isolate with a slower lytic cycle compared to Bb-Spain 1 isolate. Specific traits that differentiate isolates obtained from adult animals from those infecting calves were not found. Next, we described the first case report of chronic besnoitiosis in a female calf less than 6 months-old with a low body condition. The disease was confirmed by the presence of specific anti-*B. besnoiti* antibodies and parasite detection in the skin. At post-mortem examination, tissue samples were collected for histological, immunohistochemical and molecular analyses. DNA-parasite was detected in 31 different calf's tissues, being the most highly parasitized tissues the skin and the respiratory and reproductive tracts. In addition, the parasite was also present in heart, eyes, lymph nodes and brain. The high parasite load, a wide intra-organic parasite distribution and the presence of both viable and degenerated cysts, were indicative of a rapid progression of the disease. This case report underlines the need to include the inspection of young animals in besnoitiosis control.

Keywords: *Besnoitia besnoiti*; bovine besnoitiosis; Bb-Spain 3 isolate; calf; *in vitro* behaviour; case report.

1. Introduction

Bovine besnoitiosis, caused by the cyst forming apicomplexan parasite *Besnoitia besnoiti* (Besnoit and Robin, 1912), is a chronic and a debilitating disease that primarily affects beef cattle and has a negative impact on productive and reproductive parameters, welfare and causes occasional deaths. The European Food Safety Authority in 2010 (EFSA, 2010) was alerted on the re-emergence of this disease in Europe from areas where the disease was traditionally endemic (French Pyrenees, the Alentejo region in Portugal and the northeaster part of Spain). At present, the disease has already reached Ireland and Eastern countries (Hornok et al., 2014; Vanhoudt et al., 2015; Ryan et al., 2016). Unfortunately, there are still gaps in the epidemiology of the disease, and this lack of knowledge hampers its control, which solely relies on diagnosis and management measures in the absence of either drugs or vaccines. In particular, the definitive host and transmission routes remain to be elucidated (Álvarez-García et al., 2013).

It has been postulated that *B. besnoiti* could have a heteroxenous life cycle, where cattle, antelopes, red deer and roe deer act as intermediate hosts (Arnal et al., 2016; Gutiérrez-Expósito et al., 2016). In the intermediate host, two different asexual parasitic stages develop. First, the tachyzoites invade the vascular endothelium of blood vessels and are responsible for the acute stage of the disease. During the acute stage of the disease, the infected animals develop non-specific clinical signs that may go unnoticed, such as fever, nasal and ocular discharge, depression, lameness, orchitis and subcutaneous oedema. Next, tachyzoites switch into bradyzoites as a mechanism of immune response evasion, which pack inside thick-walled cysts mostly found in subcutaneous tissues and mucous membranes during the chronic stage of the disease. Consequently, chronically infected cattle develop hyperkeratosis, alopecia, atrophy of the testes, as well as pathognomonic tissue cysts in the sclera conjunctiva or the *vestibulum vaginae* (Álvarez-García et al., 2014b; Gollnick et al., 2015).

Parasite and host dependent factors may determine the outcome of the infection (Álvarez-García et al., 2014b), such as the isolate. Recently, Frey et al. (2016) has studied and compared seven *B. besnoiti* isolates, from six countries and two continents, and reported different *in vitro* characteristics (invasion and proliferation) but no obvious difference between them with regard to virulence. Gutiérrez-Expósito et al. (2016) using microsatellite analysis, could only find variation in a unique MS marker in one isolate (Bb-Italy 2) that differed from 9 other, homogeneus, *B. besnoiti* isolates. This may be due to the fact that all compared isolates had been obtained from adult cattle clinically affected. Until now, *B. besnoiti* isolates have not been obtained from affected young animals, which may allow us to address if there are specific traits that differentiate isolates obtained from adult animals from those infecting younger animals.

Whether animal age have influence in the infection remains to be clarified. There are only two recent reports of the disease in calves. Hornok et al. (2014) reported the first case of besnoitiosis in Hungary and found three seropositive calves with respiratory disorders. More recently, Ryan et al. (2016) detected scleral tissue cysts in 41.9% of the calves present in an affected dairy herd in Ireland. The calves tested by ELISA proved to be seronegative. In the past, several authors (Bigalke, 1968; Alzieu, 2007) have concluded that the clinical incidence of besnoitiosis is very low in animals under one year of age. Indeed, clinical signs are more often observed among 2 to 4-year-

old adults and rarely occur in calves under 6 months of age (Janitschke et al., 1984), this finding is also supported by recent reports demonstrating an increase of prevalence rates with age (Gutiérrez-Expósito et al., 2017a; Fernández-García et al., 2010).

In the present study, firstly we obtained and characterized the first *B. besnoiti* isolate from a calf origin in an *in vitro* system. Next, we described the first case report of chronic bovine besnoitiosis in a calf younger than 6 months of age. After the post-mortem examination of the animal, an exhaustive tissue collection was carried out, and data on lesions and intra-organic parasite distribution were obtained by means of histopathological and PCR techniques.

2. Materials and methods

2.1. Case report and herd samplings

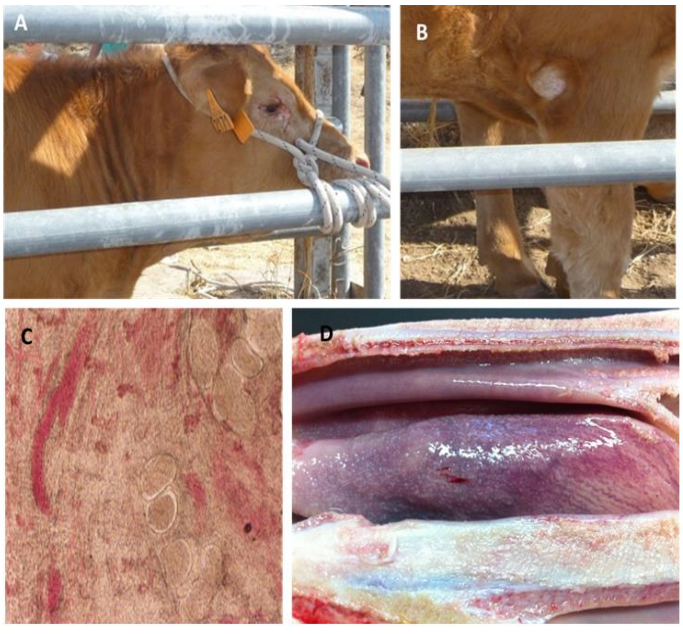
In August 2013, local veterinarians reported three suspicious clinical cases of chronic bovine besnoitiosis located in Central Spain (north-eastern part of Toledo province), where the presence of the disease had not previously been confirmed. The affected animals were an adult Limousin cow and a calf from a closed beef cattle herd. This herd was composed of 176 animals raised under extensive conditions where natural mating was the rule. In addition, a Charolais cow in a nearby farm that practised similar management measures was also affected.

The adult animals showed compatible clinical signs of chronic besnoitiosis, such as hyperkeratosis, skin folding in the neck and limbs, nodules and cracks in the udders and tissue cysts that could be seen by the naked eye in the sclera conjunctivae (Supplementary figure). The 4-month-old Limousin calf also showed signs of alopecia in the periorbital region and muzzle (Figure 1). Two months before, the animal had displayed non-specific clinical signs compatible with the acute stage of bovine besnoitiosis, such as fever, depression and anorexia.

Sera from these animals were collected and examined for specific antibodies by ELISA. Biopsies from the skin in the tarsal region in the calf and from the *vestibulum vaginae* in the adult cow were collected and squashed between trichinelloscopy plates to visualize tissue cysts by direct microscopic examination. The serological analysis was repeated in the calf at 7 and 8 months of age. Subsequently, it was donated to the Veterinary Medical Teaching Hospital (Complutense University of Madrid) for further examination.

Due to poor body condition, the calf was sacrificed at 8 months of age. The animal was sedated with xylazine hydrochloride (Rompun®; Bayer) and

Figure 1: Clinical signs, gross lesions and parasitological findings in the calf infected with *B. besnoiti*. A and B: Clinical signs compatible with chronic besnoitiosis (alopecic in the periorbital region and limbs). **C**: Microscopic tissue cysts visualized in a skin biopsy. **D**: Macroscopic tissue cysts in the tongue and nasal turbinates.



immediately euthanized by an intravenously overdose of embutramide and mebezonium iodide (T61®; Intervet) in the ruminant medical facilities at the Veterinary Medical Teaching Hospital. All experimental procedures complied with current EU legislation (Directive 2003/65/CE and 2010/63/EU).

A complete and systematic post-mortem study was carried on the carcass, and tissue samples from 35 different locations were taken (Table 1). Tissues were fixed in neutral buffered 10% formalin solution for histopathology and immunohistochemistry studies and frozen at -80ªC for PCR analyses. In addition, an epiglottis sample was also collected for parasite isolation in cell culture.

2.2. Parasite isolation and *in vitro* assays

The presence of *B. besnoiti* tissue cysts in the calf was confirmed in samples from the epiglottis after tissue compression on a trichinelloscopy plate and visualization under the microscope (Nikon eclipse 50*i*). The tissue was homogenized using a Potter Elvehjem homogenizer (Sigma-Aldrich®) in a solution consisting of PBS (phosphate buffer saline) with 2% antibiotic (Penicillin/Streptomycin + Amphotericin B (Lonza®)). Bradyzoites released from the tissue cysts were observed at 40x in a light microscope, and aliquots of $4x10^7$ bradyzoites were inoculated onto fresh monolayers of Marc-145 cells in T75 flasks (Nunc®; Thermofisher Scientific). All isolates were maintained on this type of cell, employing the same procedure described by (Fernández-García et al., 2009b). Invaded bradyzoites were observed as soon as 48 hours post infection (hpi). Tachyzoites were maintained and passaged every 3 to 5 days in confluent cell cultures. This newly obtained isolate was named Bb-Spain 3. Tachyzoites from Bb-Spain 1 and the newly obtained Bb-Spain 3 isolates were grown in Marc-145 cells and purified as described by Frey et al. (2016). In order to avoid adaptation of the parasites to the cell line employed, isolates with a low passage number were included in the experiments (Bb-Spain 1 from 10 to 16 and Bb-Spain 3 from 6 to 10 passage number). Bb-Spain 1 was included as an internal control of reproducibility in invasion and proliferation assays. All isolates used for *in vitro* assays tested negative to *Mycoplasma* spp. infection by PCR (Venor™GeM Mycoplasma Detection Kit; Minerva Biolabs) and bovine viral diarrhea virus (BVDV) by quantitative real-time PCR (qPCR) (Hoffman et al., 2006). Foetal calf serum used in all the experiments was previously checked for the absence of either specific IgG against *B. besnoiti*, *Neospora caninum* and *Toxoplasma gondii* by IFAT or BVDV RNA by qPCR (Fernández-García et al., 2009b).

2.2.1. Parasite invasion rate

For invasion assays, 10^5 MARC-145 cells/well were grown to confluence in P24 cell culture plates (Nunc®; Thermofisher Scientific) incubated at 37°C with 5% CO2. The next day, 10^3 tachyzoites of each isolate (infection rate 1:100) were added to the cell cultures. Four washes with PBS were performed at 4, 6, 8 and 24 hpi to discard non-invaded tachyzoites. One ml of fresh culture medium was added, and the plates were incubated at 37°C with 5% CO2. In addition, non-washed plates were kept until fixation. After 72 hpi, IFAT (see section 2.6.) was carried out in order to count the total number of invasion events (parasitophorous vacuoles and/or lysis plaques) per well according to the procedure established by Frey et al. (2016). Three replicates of invasion assays were repeated in three independent experiments for each isolate.

2.2.2. Proliferation kinetics, tachyzoite yield and doubling time determination

For the proliferation assay, P24 cell culture plates with confluent Marc-145 cells (10^5 cells/well), maintained with DMEM 10% FCS (HyClone®; Thermofisher Scientific), were used. The monolayers were infected with 10^6 purified tachyzoites/well (infection rate 10:1) suspended in 1 ml DMEM 5% FCS. After 4 hours, the wells were washed three times with DMEM and 5% FCS, and the infected monolayers were further cultured at 37 °C and 5% CO2. At 4, 8, 24, 48 and 72 hpi, the supernatants were discarded, and the parasites were harvested following the manufacturer's instructions included in the DNeasy blood and tissue Kit (Qiagen®). DNA was extracted and stored at -20 °C until further qPCR analysis (see section 2.5.). Proliferation assays were carried out in triplicates and were repeated in three independent experiments for each isolate. To account for variations in the DNA content of the samples, the number of tachyzoites per µl was normalized using the DNA concentration per µl determined using a Biotek multiplate reader (Biotek ®), and the results were finally expressed as tachyzoites per nanogram of DNA (tachyzoite yields, TY).

In parallel, to study the proliferation kinetics of both isolates, replicates of cell cultures in coverslips were infected as described above and labelled using a double immunostaining. Three coverslips were photographed for each condition using an inverted fluorescence microscope (Nikon Eclipse TE 200, Chiyoda, TYO, JP).

The doubling time (Td) was defined as the period of time required for a tachyzoite to duplicate during the exponential multiplication period, excluding the lag phase (when there is not parasite multiplication) and the egression phase.

2.3. ELISAs

All sera were analysed by SALUVET in-house ELISA based in lyophilized tachyzoites of *B. besnoiti* as antigen (García-Lunar et al., 2017) to discriminate between seropositive and seronegative animals. The optical density was converted into the RIPC (Relative Index Percent) using the formula described by (Fernández-García et al., 2010). An animal with an RIPC ≥ 17.34 was considered positive.

To discriminate between acute and chronic infection, the calf's sera collected at 4, 5, 7 and 8 months of age were also tested by an avidity SALUVET in-house ELISA. The test was essentially carried out as described above but included one additional incubation step with 6 M urea in the tested samples and PBS-Tween in the controls after the incubation step with sera (Schares et al., 2013). The cut-off to discern between low and high avidity values (acute and chronic stages, respectively) was estimated using a panel of 11 sera from naturally infected and seropositive cattle based on the results obtained by SALUVET in-house ELISA. Four sera samples came from animals that showed clinical signs compatible with the acute stage of the disease, and 7 sera samples came from seropositive chronically infected animals with tissue cysts. Serial dilutions of the sera were performed starting from 1:100 to 1:102,400, and the avidity indexes were calculated, and the cut-off was established at 50.8, according to Schares et al. (2013).

2.4. Histopathology (HP) and immunohistochemistry (IHC)

After five days of fixation, tissue samples were dehydrated using a graded series of alcohols and were embedded in paraffin. Tissue sections of 5µm were cut from each sample and stained with

haematoxylin and eosin (H/E) for the histopathological evaluation. The immunohistochemical labelling of the parasites was performed using deparaffinised sections from the collected tissues. Primary in-house rabbit polyclonal antibodies against *B. besnoiti* tachyzoites were used at a 1:3000 dilution following the same protocol described by Frey et al. (2013). Parasite cysts found in the histological sections were counted when there were less than 30 or were subjectively classified as between 30 and 50 or more than 50.

On selected sections, the inflammatory cells infiltrating the area adjacent to the tissue cysts were characterized by immunohistochemical labelling. Primary antibodies specific for T-lymphocytes (rabbit anti-human CD3 polyclonal antibody) and monocytes/macrophages (mouse anti-human CD68 monoclonal antibody and mouse anti-human myeloid/histiocyte-antigen monoclonal antibody; clone MAC387) were used following the procedure described by Frey et al. (2013).

2.5. DNA extraction, ITS1-PCR and qPCR

The DNA extraction of the tissue samples were complete dusing Maxwell® 16 Instrument (Promega) with the Maxwell® 16 Tissue DNA Purification Kit (Promega) (Frey et al., 2013). The DNA content from each sample was adjusted to 40 ng/µl, and it was measured with a NanoPhotometer® (Implen).

The products of the DNA extraction process were tested for the specific detection of *Besnoitia* spp. by ITS-1 rDNA PCR (Cortes et al., 2007b). The forward primer ITS1F (50-TGACATTTAATAACAATCAACCCTT-30) and the reverse primer ITS1R (50-GGTTTGTATTAACCAATCCGTGA-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 were visualized after electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The positive control was DNA extracted from *in vitro* cultured tachyzoites of *B. besnoiti*, and PCR grade water was used as the negative control.

The qPCR assay for the detection of *Besnoitia* spp. DNA from the ungulates was performed according to Frey et al. (2016). In each PCR, 10-fold serial dilutions of genomic DNA corresponding to 0.1-10,000 Bb-Spain 1 (Fernández-García et al., 2009b) tachyzoites were included. To quantify the amount of DNA, dilutions of DNA extracted from the liver of a cow corresponding to 100, 20, 4, and 1 ng/µl were included. The cycling conditions were 10 min at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Fluorescence emissions were measured during the 60°C step. A dissociation stage was added.

2.6. Indirect fluorescent antibody test (IFAT)

IFAT was carried out on infected MARC-145 monolayers at 72 hpi. The protocol used was the same as that previously described by Frey et al. (2016). The total number of invasion events per well (IR; invasion rate) was counted using a fluorescence microscope (Nikon eclipse TE200) at 200X magnification. Three categories of invasion outcomes were distinguished: small parasitophorous vacuoles (PVs), large PVs, and lysis plaques. Vacuoles filled with tachyzoites forming a rosette and with individually distinguishable tachyzoites were regarded as small PVs. When the PV was packed with tachyzoites that were not individually discernible, a large PV was recorded. A lysis plaque was identified as soon as the host cell lysed, manifesting in an

accumulation of multiple infected cells with just a few tachyzoites infecting each cell (with the infected cells typically being located around a central space with no cells at all and with extracellular tachyzoites) (Frey et al., 2016).

2.7. Data analysis

To assess differences in time points in invasion and proliferation assays for each isolate, and between isolates (Bb-Spain 1/ Bb-Spain 3), ANOVA and Mann-Whitney *U* test were performed, respectively. We represented dT for each isolate as the average value obtained from all of the determinations that revealed a linear regression, $R^2 \geq 0.95$ (Jiménez-Pelayo et al., 2017). Doubling time was determined by applying non-linear regression analysis and an exponential growth equation as described by (Regidor-Cerrillo et al., 2011). Finally, a chi-square test was employed to address possible differences regarding the different invasion events between both isolates.

Statistical analysis was performed with GraphPad statistic software (San Diego, CA, USA).

3. Results

3.1. Bb-Spain 3 *in vitro* behaviour

At 24 hpi, the maximal IRs of Bb-Spain 1 and Bb-Spain 3 isolate was 3% and 2-3%, respectively ($P>0.05$) (Figure 3A). However, there were no statistically significant differences for Bb-Spain 1 and Bb-Spain 3 among the different time points studied (Figure 3A, ANOVA; $P<0.05$). Nevertheless, there was an increase in the number of parasites that had invaded longer than 24 hpi (Figure 3A).

Regarding parasite proliferation results evidenced that there was no multiplication of the parasite until 24 hpi, establishing a lag phase no longer than 24 hpi (Figure 3B and 4). Growth curves obtained for both isolates fitted an exponential growth pattern (Figure 3B, $R^2>0.95$) and both isolates showed a significant increase in tachyzoite numbers between 24 and 48 hpi ($P<0.05$, ANOVA, Mann-Whitney *U* test) (Figure 3B and 4). Significant differences were found when mean tachyzoite yields (tachyzoites/ng DNA) for both isolates at 72 hpi were compared ($P<0.05$, Mann-Whitney test). Doubling times during the exponential growth period were 12.74 ± 4.66 and 10.45 ± 2.37 hours for Bb-Spain 1 and Bb-Spain 3 isolates, respectively.

Egress of tachyzoites after lysis of their host cells was observed by light microscopy between 48 and 72 hpi (Figure 4). After 72 hpi, the infected cultures exhibited small PVs, large PVs and lysis plaques in both isolates (Figure 3C). These three events were simultaneously present and homogeneously distributed over the cell monolayer for Bb-Spain 1 and Bb-Spain 3 isolates. In Bb-Spain 1, the predominant event was the formation of lysis plaques (65.5% of the events) *versus* large and small PVs (14% and 20.4%, respectively). For isolate Bb-Spain 3, the different invasion events were similarly represented (32.1% small PVs, 30.9% large PVs, and 37% lysis plaques) and differed significantly from Bb-Spain 1 isolate ($P<0.05$, ANOVA) (Figure 3C).

3.2. Specific anti-*B. besnoiti* IgG antibodies

Anti-*B. besnoiti* antibodies were detected in the calf at 4 months of age (RIPC=165.5), in the adult cow of the same farm (RIPC=27.5), and simultaneously in the Charolais cow from the nearby farm (RIPC= 61.4). Specific antibody levels remained high in the calf at all samplings analysed at 6, 7 and 8 months of age (RIPC=152.7, 140.5, 166.6, respectively), until it was slaughtered. The avidity indexes tested in those same samples were 48, 73, 74 and 60.

3.3. Lesions, histopathology, parasite distribution and burden in positive samples.

3.3.1. Gross lesions

Macroscopic tissue cysts were detected in the ocular conjunctiva, epiglottis and the nasal turbinate of the calf (Figure 1). Tissue cysts were visualized through the trichinelloscopy plates in the biopsies collected from the tarsal region in the calf and from the *vestibulum vaginae* in the adult cow (Figure 1).

3.3.2. Microscopic lesions

Microscopic lesions were mostly related to the presence of tissue cysts and were found in the skin and oral mucosa. They were characterized by a multifocal infiltration of lymphocytes, macrophages, plasma cells and neutrophils in the epithelium, where there was also hydropic degeneration of the keratinocytes and lamina propria-submucosa of the tongue. A similar infiltration was found in the dermis of the skin from the tarsus, nipple and the perineal area. In the vagina, there were a few small foci of mononuclear inflammatory cells in the propria-submucosa in the absence of tissue cysts.

3.3.3. HP and IHC

Parasite tissue cysts were detected in 15 different locations. Cysts were observed in the tissue sections from skin samples (neck region, upper-eyelid, carpal, tarsal, ear pinna, thigh region, nipple and perineal area), the respiratory system (tongue, muzzle, nasal turbinate, nasal sinus and pharynx), the reproductive tract (vulva) and the ocular conjunctiva (Table 1). The skin and the upper respiratory track (muzzle and nasal turbinates) were the most parasitized locations.

Parasite tissue cysts were classified into three categories, according to Langenmayer et al. (2015c): a) developed tissue cyst, b) developed tissue cyst with pericystic inflammation, and c) lysed cyst with necrotic debris and pericystic inflammation (Table 1 and Figure 2).

A subjective evaluation of the immunohistochemical labelling of cell populations in the inflammatory lesions showed that the infiltrate was mainly formed by CD3+

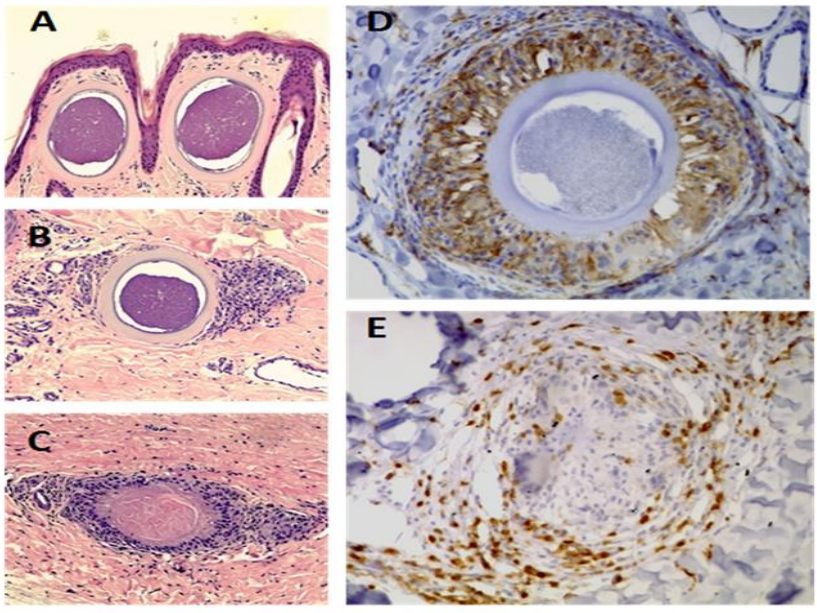


Figure 2: ***Besnoitia besnoiti*** **tissue cysts in the skin sections. H/E; A**: Viable tissue cyst with no surrounding inflammatory cells. **B**: Viable tissue cyst surrounded by pericystic inflammatory infiltrate. **C**: Lysed tissue cyst, with necrotic debris inside the capsule, in the lamina propria with pericystic inflammatory-infiltrate. **Inflammatory infiltrate. IHC; D**: Recent recruited macrophages (positive labelling for myeloid/histiocyte-antigen. Clone MAC387 antibody) close to the hyaline capsule. **E**: T lymphocytes within the infiltrate (rabbit anti-human CD3 polyclonal antibody).

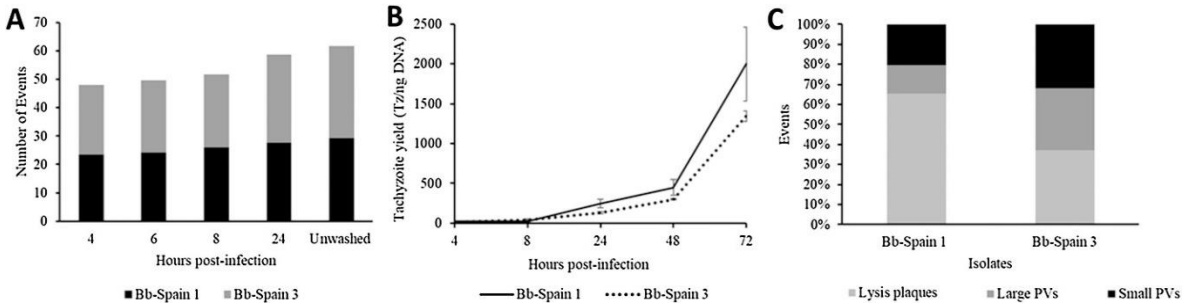


Figure 3: *In vitro* behaviour of Bb-Spain 1 and Bb-Spain 3 isolates. A: Number of invasion events at different time points post-infection; **B**: Growth measured as tachyzoite yield as a function of time in a proliferation assay; **C**: Proportions of invasion outcomes counted per isolate.

lymphocytes. Macrophages were mostly positive to the labelling for myeloid/histiocyte-antigen (MAC387 clone) and negative for CD68 expression (Figure 2). The host cells of the parasite cysts were negative to all antibodies employed.

3.3.4. PCR detection and quantification

As shown in Table 1, 30 tissues samples were positive by PCR. From the 16 locations where parasite cysts were found in the histological examination, 13 of them were also positive by PCR analysis, whilst no *Besnoitia* spp. DNA was detected in the vulva, the skin from the neck, and the ocular conjunctiva, tissues where parasite tissue cysts were evidenced by HP.

On the other hand, *Besnoitia* spp. DNA was amplified in samples where no tissue cysts were found by histological examination (atrium, ventricle and aorta, as well as trachea, bronchi and lungs). The reproductive tract from cervix to ovaries and skin samples from the different areas were also PCR positive (Table 1).

Regarding the lymphatic system, only the bronchial lymph node and tonsils yielded positive results. Remarkably, parasite DNA was detected in the brain. Other assessed tissues, such as the ocular sclera and the auditory canal, were also positive. Finally, the parasite was not detected in the kidney, liver and the digestive track.

The amount of tachyzoites/ng cow DNA in the ITS1-PCR positive samples are shown in Table 1. The highest parasite loads corresponded to skin samples from the tarsal, carpal and thigh regions and the upper-eyelid, followed by the perineal skin. Low parasite loads were detected in the respiratory and reproductive tracks, but for the ovaries, the load was relatively higher.

4. Discussion

Bovine besnoitiosis is rare in calves and cases of chronic disease have not been described up to date. Thus, the control of this re-emergent disease focus solely on adult cattle. In the present study, we have obtained and characterized the first isolate from calf origin and we have described this unusual case report in a calf younger than 6 months. The identity of *Besnoitia* species present in the calf was determined after parasite isolation (named as Bb-Spain 3 isolate) and molecular genotyping. The newly obtained Bb-Spain 3 isolate had been already genotyped by Gutiérrez-Expósito et al. (2016) (named as skin biopsy 3). Six microsatellite sequence loci were sequenced and they showed identical microsatellite patterns compared with other *B. besnoiti* isolates from different countries. Herein *in vitro* behaviour of the new isolate was compared with Bb-Spain 1 reference isolate. We have evidenced again that *B. besnoiti* shows limited invasion capacity (low invasion rates), requires

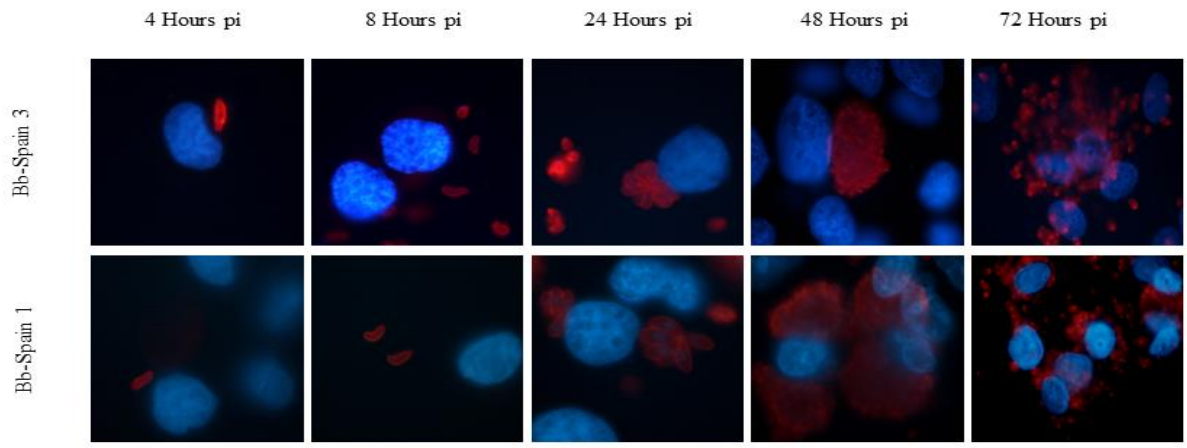


Figure 4: Microscopic follow up of invasion events over 72 hpi in Marc-145 cell cultures infected with Bb-Spain 1 and Bb-Spain 3 isolates.

prolonged time to invade host cells and shows high extracellular survival periods that may extend up to 24 hpi regardless the isolate, in contrast to *N. caninum* or *T. gondii* (Naguleswaran et al., 2003; Dellarupe et al., 2014). Moreover, Bb-Spain 1 isolate showed similar results as those obtained by Frey et al. (2016), where Bb-Spain 1 isolate was classified as a low invader (3.2% IR) and low prolific isolate. Accordingly, this *in vitro* culture system represents a useful and reproducible tool for further assays including the study of *B. besnoiti* intra-species variability or parasite drug screenings.

Bb-Spain 3 behaved similarly to Bb-Spain 1 and was classified in the same category like other European isolates (Bb-Spain 2 and Bb-Italy 2) with IR around 3%. Both isolates showed a growth curve that fitted an exponential growth up to 72 hpi, a lag phase and doubling times in accordance to other Spanish isolates (Frey et al., 2016), despite some inherent variability. However, the significantly different TYs suggest that Bb-Spain 3 has a slower lytic cycle and is less prolific than Bb-Spain 1, which was corroborated by immunofluorescence findings being lysis plaques less frequent. Similar to other *B. besnoiti* isolates, the new isolate showed non-synchronic growth pattern at 72 hpi, since PVs and lysis plaques coexisted. However, different percentages of invasion outcomes were found. Bb-Spain 3 isolate produced more PVs, whereas Bb-Spain 1 isolate showed more lysis plaques. In agreement, Frey et al. (2016) reported that lysis plaques represented up to 80% of the invasion outcomes at 72 hpi for Bb-Spain 1 isolate.

Biological diversity is another remarkable difference between the closely related *N. caninum* or *T. gondii* and *B. besnoiti*, that relies on extended genetic variability (Regidor-Cerrillo et al., 2013; Verma et al., 2015) and a correlation between *in vitro* phenotypic traits and *in vivo* virulence (Regidor-Cerrillo et al., 2011; 2014; Jungersen et al., 2002). In contrast, genetic homogeneity is characteristic of all *B. besnoiti* isolates and no *in vitro* parameter was found indicative of the parasite virulence in cattle as previously reported by Frey et al. (2016).

The present study also represents the first thorough description of a case report of chronic bovine besnoitiosis in a calf less than 6 months of age. It has already been demonstrated that clinical cases in young cattle rarely occur in animals under 1 year of age (Pols, 1960). These findings might be

Table 1: Parasite intra-organic distribution by histopathology (HP) and PCR

	PCR			HP		
Tissue[a]	**ITS-1rDNA**	**qRT**[b]	**Results**	Viable cysts (VC)	VC+ Imflammation	Degenerated cysts
Circulatory system						
Aorta	Positive	ND[d]	Negative			
Ventricle	Positive	ND[d]	Negative			
Atrium	Positive	ND[d]	Negative			
Respiratory system						
Tongue	Positive	2.8	Positive	20	2	
Muzzle	Positive	0.2	Positive	>50	>50	>50
Nasal turbinates	Positive	5.6	Positive	>50	>50	>30
Nasal sinus	Positive	241.8	Positive	5		
Pharynx	Positive	ND[d]	Positive	1	1	2
Epiglottis	Positive	6.3	Negative			
Larynx	Negative		Negative			
Trachea	Positive	0.2	Negative			
Bronchi	Positive	0.3	Negative			
Lungs	Positive	0.2	Negative			
Reproductive system						
Vulva	Negative		Positive		2	5
Vagina	Negative		Negative			
Cervix	Positive	0.1	Negative			
Uterus	Positive	ND[d]	Negative			
Uterine horns	Positive	ND[d]	Negative			
Oviducts	Positive	0.1	Negative			
Ovaries	Positive	98.7	Negative			
Skin						
Neck	Negative		Positive	5	20	20
Upper-eyelid	Positive	234.6	Positive	>50	>50	>50
Carpal	Positive	1558.7	Positive	>30	>50	>50
Tarsal	Positive	4210.2	Positive	>50	>50	>20
Ear pinna	Positive	0.2	Positive	9		
Thigh	Positive	1224.4	Positive	5	20	20
Udder	Positive	ND[d]	Negative			
Nipple	Positive	0.1	Positive	1	2	10
Perineal	Positive	109.2	Positive	1	8	9
Lymphatic system						
Bronquial lymph node	Positive	ND[d]	Negative			
Tonsils	Positive	ND[d]	Negative			
CNS						
Brain	Positive	ND[d]	Negative			
Other organs						
Kidney	Negative		Negative			
Liver	Negative		Negative			
Ocular conjunctiva	Negative	ND[d]	Positive	15	2	
Ocular sclera	Positive	ND[d]	NA[c]			
Auditory canal	Positive	ND[d]	NA[c]			

[a] Tissues that tested negative: Lymphatic system (Retropharyngeal, Axillary, Mandibular, Subscapularis, Mesenteric, Traqueal, Iliac, Popliteal, Portal, Inguinal, Ileocaecal lymph nodes and Parotid, Thymus and Spleen) and Digestive system (Esophagus, Abomasum, Reticulum, Duodenum, Jejunum, ileum, caecum and rectum).

[b] N° tachyzoites/ng cow DNA by quantitative real-time PCR (qPCR).

[c] NA: Not available.

[d] ND: Not detected by qPCR

explained by i) the lower risk of young animals being exposed to the parasite by the horizontal transmission route, ii) a lower receptivity of a young calf due to passive immunity acquired from the dam, and iii) innate protective immunity as it apparently occurs in *Babesia* spp. infections in young cattle (Bock et al., 2004; Zintl et al., 2005). However, these clinical cases might be more frequent that originally thought. Hornok et al. (2014) reported three seropositive calves under 6

months of age showing respiratory signs. In addition, Ryan et al. (2016) mentioned that exposure to besnoitiosis occurs in animals before six months of age, which was evidenced by the detection of 41.9% of calves with scleral tissue cysts. More recently, a systemic chronic besnoitiosis in a juvenile roe deer was reported, and molecular findings verified *B. besnoiti* identity (Arnal et al., 2016). However, up to now, all reported severely chronically infected animals with skin lesions and wide parasite dissemination corresponded to adult animals (Álvarez-García et al., 2014b).

Herein, the clinical signs compatible with the disease (alopecia, folding and thickening of the skin), tissue cysts detection, specific anti-*Besnoitia* spp. antibodies (also corroborated by western blot; data not shown), as well as the avidity maturation of the IgGs, led us to confirm chronic besnoitiosis in a calf, similar to previous descriptions in adult animals (Álvarez-García et al., 2014a; Gollnick et al., 2015). This severe case of chronic besnoitiosis was characterized not only by macroscopic lesions but also by wide intra-organic parasite distribution evidenced by HP and PCR techniques. Skin samples at different locations showed a high parasite load, followed by the upper respiratory tract, vulva and ocular conjunctiva. These locations are predilection sites for *B. besnoiti* that show tropism for the connective tissue of the superficial skin layers, scleral, conjunctiva and mucous membranes of the upper respiratory and distal genital tracts (Pols, 1960; Nobel et al., 1981; Manuali et al., 2011; Gentile et al., 2012; Frey et al., 2013). Skin lesions, along with the high parasite load detected, evidenced a dermatotropic tropism of the parasite. Remarkably, *B. besnoiti*-DNA was also detected in the lower respiratory tract (bronchi and lungs), reproductive system (cervix, uterus, both oviducts and ovaries), circulatory system (heart and aorta), tonsils, the bronchial lymph nodes and brain. Similarly, Basso et al. (2013) and Langenmayer et al. (2015c) found *B. besnoiti* in these locations in adult cattle. In particular, we corroborated for the second time that *B. besnoiti* may be present in the CNS in cattle. The parasite stage present remains to be elucidated. Interestingly, Arnal et al. (2016) recently detected tissue cysts in the brain of a roe deer, which had affected the grey and white matter. The cyst size was smaller compared to those located in the target locations. The authors suggested that an immune response could have limited cyst growth, which was supported by the inflammatory response found in the brain cortex, considering that tissue cyst development is rather synchronous. The parasite load was low in the remaining PCR positive tissues. It is possible that either intracellular tachyzoites or tissue cysts were present in these highly vascularised tissues since endothelial cells are the primary target cells for initial parasite replication (Basson et al., 1970). Moreover, previous studies showed that these tissues do not harbour any or very seldom contain singular cysts (McCully et al., 1966; Langenmayer et al., 2015c). The low parasite load detected in these locations may explain the discordant results obtained between the histologic and more sensitive PCR techniques (Schares et al., 2011b).

Another distinct feature of chronic bovine besnoitiosis was the detection of three different type of cysts found herein, and reported by other authors in chronically and sub clinically infected adult cattle (Frey et al., 2013; Langenmayer et al., 2015c). Viable cysts without any signs of pericystic infiltrates, cysts with marked pericystic inflammation and degenerated cysts were found. Previous studies in *Besnoitia* spp. infections agreed that viable tissue cysts might show variable host immune reactions. These reactions vary from an

absent inflammatory response to an intense granulomatous response (Nobel et al., 1981). Langenmayer et al. (2015c) reported apparently degenerated cysts, which appeared from 30 days post-infection onwards (Basson et al., 1970), surrounded by inflammatory cells. According to this author, the lysis of degenerated cysts could occur when pericystic inflammation breaks the hyaline layer. Frey et al. (2013) suggested that in these cases, bradyzoites could reach the bloodstream, leading to parasite exposure to the immune system and the development of high levels of specific antibodies as detected herein. Several authors have recently characterized the inflammatory infiltrate surrounding the tissue cysts in chronically infected cows; it was composed of numerous T-lymphocytes, recently recruited macrophages and fewer eosinophils, which agree with the present findings (Frey et al., 2013; Langenmayer et al., 2015c).

High avidity antibodies were also indicative of the establishment of a chronic infection, despite having an avidity index lower than the established cut off in the first sampling. Schares et al. (2013) claimed that the avidity maturation of IgG is slower than cyst development.

Our hypothesis is that this calf might have been infected through horizontal transmission, as it usually occurs in adult cattle, either by direct contact with the infected adult cow or by hematophagous arthropod bites. The serological analyses in the calf ruled out the vertical transmission since colostral antibodies decrease after 2 months post-infection (Shkap et al., 1994) and the calf's mother was seronegative (data not shown). We ignore whether any immunosuppressive factor could have favoured the outcome of the infection in the calf. An association between BVDV infection and other protozoan infections (e.g. *N. caninum*) have been reported in cattle (Waldner, 2005; Vanleeuwen et al., 2009). In the present study, BVDV infection was discarded in an ear notch sample of the calf (data not shown). However, other immunosuppressive factors were not excluded.

5. Conclusion

In summary, we have isolated *B. besnoiti* from a calf for the first time. Genotyping or *in vitro* behaviour differences of the new isolate were not found with respect other Spanish isolates obtained from adult cattle. Moreover, this is the first exhaustive description of a severe clinical case of chronic bovine besnoitiosis reported in a calf less than 6 months of age. Lesions, clinical signs, parasite load and intra-organic distribution were characteristic of a severe chronic besnoitiosis and demonstrated the rapid progression of the disease (Nobel et al., 1981; Frey et al., 2013; Gollnick et al., 2015). Further studies should elucidate the molecular mechanisms that may underlay the disease progression. This case report shows the need to include the inspection of young animals in the control of the disease.

Acknowledgements

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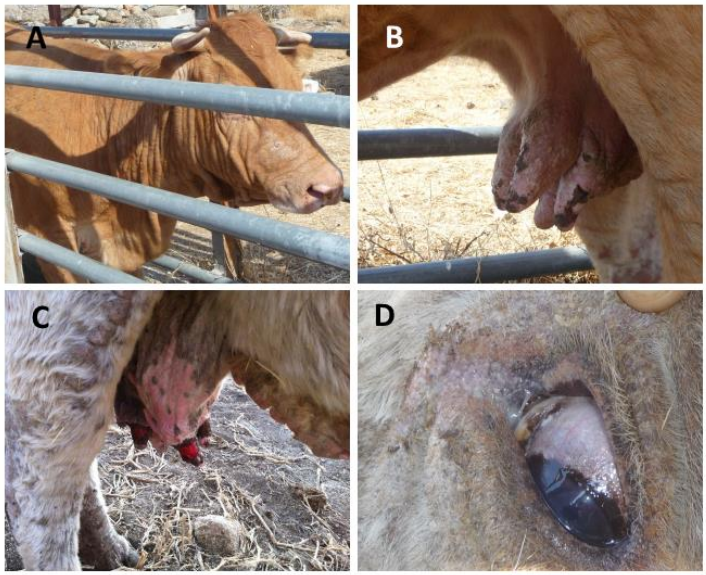
Appendix A



Supplementary Figure: Chronic bovine besnoitiosis in adult cows. A and B: Adult Limousin cow from the same herd of the calf. **C and D:** Adult Charolais cow from a nearby farm. Hyperkeratosis, skin thickening and folding **(A)**. Nodular thickening and ulcerative dermatitis in the nipples **(B, C)**, and pathognomonic tissue cysts in the ocular conjunctiva **(D)**.

Effect of parasite dose and host age on the infection with *Besnoitia besnoiti* tachyzoites in cattle

Diezma-Díaz, C.[1], Jiménez-Meléndez, A.[1], Re, M.[1, 2], Ferre, I.[1], Ferreras, M.C. [3], Gutiérrez-Expósito, D.[1, 3], Rojo-Montejo, S.[1,4], Román-Trufero A.[4], Benavides-Silván, J.[3], García-Lunar, P.[1], Calleja-Bueno, L. [2], Blanco-Murcia, J.[1,2], Osoro, K. [4], Ortega-Mora, L.M.[1], Álvarez-García, G.[1*]

[1] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.

[2] Animal Medicine and Surgery Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.

[3] Livestock Health and Production Institute (ULE-CSIC), 24346 León, Spain.

[4] Regional Service for Research and Agri-Food Development (SERIDA), Animal Production Systems, 33300 Villaviciosa, Spain.

* Corresponding author: Gema Álvarez García. Tel.: +34-913944095. E-mail address: gemaga@vet.ucm.es

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Abstract

Bovine besnoitiosis is continuing to spread in Europe. Therefore, the development of ruminant animal models of infection is urgently needed to evaluate therapeutic and prophylactic tools. Herein, we studied the effect of parasite dose and host age on the infection dynamics with *Besnoitia besnoiti* tachyzoites in cattle in two independent experimental infections.

In experiment A, twelve 3-month-old male calves were inoculated intravenously with either three different doses of tachyzoites (G1: 10^{8}; G2: 10^{7}; G3: 10^{6}) or with PBS (G4). In experiment B, six 14-month-old bulls were inoculated with 10^{6} tachyzoites based on results obtained in experiment A. In both trials, clinical signs compatible with acute and chronic besnoitiosis were monitored daily; blood and skin samples were collected regularly for 70-115 days post-infection (pi). Finally, animals were euthanized, and tissues were collected for lesion and parasite detections.

Infected animals developed mild-moderate signs compatible with acute besnoitiosis. Lymphadenopathy and fever were observed in both calves (from 12 hours until seven days pi) and bulls (from six days until nine days pi). Seroconversion was detected at 16-19 days pi, and antibody levels remained high. Infected animals did not developed characteristic clinical signs and macroscopic lesions of chronic besnoitiosis. However, successfully, parasite-DNA was detected in a reduced number of target tissues: conjunctiva, ocular sclera, epididymis, skin of the scrotum and carpus in calves (n=10, 6 of which belonged to G3), and pampiniform plexus and testicular parenchyma in bulls. Remarkably, one tissue cyst and mild microscopic lesions were also detected.

In summary inoculated animals developed the acute besnoitiosis and chronic infection was evidenced by microscopic findings. However, our results suggest that tachyzoite dose and host age are not key variables for inducing clinical signs and macroscopic lesions characteristic of chronic besnoitiosis. Thus, a further refinement of this model should evaluate other parasite-and host-dependent variables.

Keywords: *Besnoitia besnoiti*, calf, bull, experimental infection, tachyzoites.

1. Introduction

Besnoitia besnoiti is a cyst-forming apicomplexan parasite responsible for bovine besnoitiosis. At present, the disease is considered to be re-emerging in Europe by the European Food Safety Authority due to an increased number of cases and the geographic expansion of besnoitiosis into cattle herds in several Western and Central European countries (European Food Safety Authority, 2010; Álvarez-García, 2016). The control and prevention of this disease relies only on diagnostic and management measures, since no chemotherapeutics are available and no vaccines are licensed in Europe (Álvarez-García et al., 2013). Promising drugs have been tested *in vitro* (Cortes et al., 2011; Jiménez-Meléndez et al., 2017). However, an appropriate ruminant experimental model has not been developed yet; this model is a requirement to test the safety and efficacy of these potential prophylactic and therapeutic tools.

In this regard, only *B. besnoiti* tachyzoite and bradyzoite stages have been used so far for the inoculation of animals. *Besnoitia besnoiti*-oocysts are not available since the definitive host is unknown (Basso et al., 2011). Several laboratory rodents (hamster, gerbils, and various strains of white mice) have been infected with *B. besnoiti* (Shkap et al., 1987; Basso et al., 2011). Although gamma-interferon knockout mice are very susceptible to the acute infection (Schares et al., 2009), they are not recommended as an appropriate laboratory model due to the rapid onset of the acute stage and death. Rabbits showed susceptibility to experimental infection by developing clinical signs of the acute phase of the disease (Liénard et al., 2015), but the results were variable with respect to the chronic stage (Bigalke, 1968; Basson et al., 1970). The disease was successfully replicated in these animals through subcutaneous inoculation of bradyzoites (6 x 10^6) (Liénard et al., 2015) resulting in mild clinical signs and the formation of cysts in the leg dermis, nasal mucosa, eyelid and penis. However, the authors suggested that the establishment of *B. besnoiti* and its adaptation to a heterologous host was difficult.

Between the 1960s and 1980s, there were a few attempts to establish a reproducible *in vivo* model for bovine besnoitiosis, but failed due to the difficulty in inducing the characteristic clinical signs (Álvarez-García et al., 2014b). Unfortunately, all the inoculations were carried out under different experimental conditions (e.g., different ages of the infected animals, unknown previous health status and use of immunosuppressive therapies). Moreover, the inocula were obtained from different sources, such as blood or tissue cysts from cattle with chronic besnoitiosis (Bigalke, 1967), blood from acutely infected rabbits (Basson et al., 1970) or from a blue wildebeest-strain maintained in cell culture (Bigalke et al., 1974). In infected animals, fever and lymphadenopathy have been detected on several occasions (Bigalke, 1968; Basson et al., 1970; Bigalke et al., 1974). However, specific clinical signs of the acute phase, such as oedema and anasarca, and of the chronic phase (tissues cysts) were only reported in infected adult animals (Basson et al., 1970), as well as in immunosuppressed animals that had been infected with bradyzoites by Diesing et al. (1988). This heterogeneity in the experimental assay parameters does not allow for the development of an experimental model of besnoitiosis in cattle. Additionally, most results have been based on clinical inspection and histopathology, since molecular tests were not available and serological tests were rarely employed, which is crucial since immunity to re-infections has been reported (Bigalke, 1968; Basson et al., 1970; Janitschke et al., 1984; Shkap et al., 1987).

Nevertheless, relevant information concerning pathogenesis has been derived from these trials. It is known that both tachyzoite and bradyzoite stages are infective for cattle (Pols, 1960; Bigalke, 1967; 1968; Diessing et al., 1988). We expect that the parasite dose is important in determining the severity of the infection. Moreover, the incubation period may depend on the route of infection. Information regarding age suggests that the disease is mainly present in older animals due to longer periods of exposure (Álvarez-García et al., 2014b). However, the disease was recently confirmed in a 4-month-old calf (Diezma-Díaz et al., 2017), demonstrating that animals younger than 6 months old can also be chronically infected.

The aim of the present study was to study the effect of parasite dose and host age on the infection dynamics with *B. besnoiti* tachyzoites in cattle and their impact on both the acute and chronic phases of the disease. Accordingly, two independent experimental infections were carried out in calves and bulls. We addressed the limitations of previous trials by: i) the employment of a well characterized *B. besnoiti* isolate; ii) checking the viability and quality of the inoculum; and iii) checking the animal health status prior to inoculations. Moreover, exhaustive clinical monitoring and regular samplings were carried out, employing complementary serological, histological and molecular tools.

2. Materials and methods

2.1. Ethics statement

All our experimental procedures were approved by the Animal Welfare Committee of the Community of Madrid, Spain following proceedings described in Spanish and EU legislations (PROEX 92/14, Law 32/2007, R.D. 53/2013), and Council Directive 2010/63/EU.

Animals were housed in clinical facilities belonging to the Faculty of Veterinary Sciences of Complutense University of Madrid (Number of register: ES280790000101).

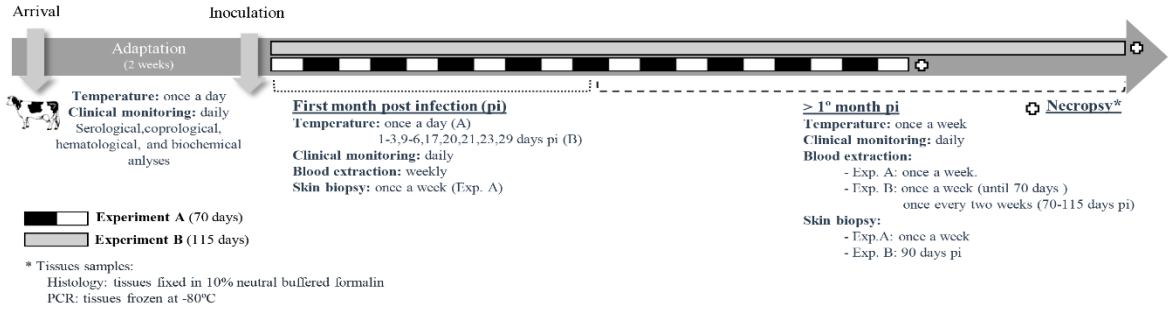
2.2. Animals, experimental design, clinical monitoring and collection of samples

The experimental design followed in both trials is shown in Figure 1. The same clinical and laboratory parameters were evaluated with slight differences in their periodicity.

Experiment A

The calves used in this study came from a single Holstein Friesian dairy herd located in Madrid province and were free from relevant infectious (tuberculosis and brucellosis) and parasitic diseases of dairy cattle. Twelve 2-month-old male calves were selected after assessing the absence of specific antibodies against *B. besnoiti* and other closely related sarcocystidae parasites (*Neospora caninum* ; *Toxoplasma gondii* and *Sarcocystis* spp.) by western blot. Prior to the inoculation and to guarantee the health status of the animals, the calves were vaccinated against Bovine Respiratory Syndrome (Cattle Master ®). The absence of gastrointestinal parasites was checked by means of coprological analyses. Additionally, haematological and biochemical analyses were also carried out.

Figure 1: Timeline, clinical monitoring and sample collection in experimentally infected calves (A) and bulls (B).



Score	Category	Acute besnoitiosis	Chronic besnoitiosis
0	Absence of infection	None	None
1	Mild	Local lymphadenopathy	Systemic lymphadenopathy and/or congestive conjunctiva and/or conjunctival cysts (cat. 1) and/or ocular secretion †
2	Moderate	Fever and/or systemic lymphadenopathy and/or cough/nasal secretion and/or congestive conjunctiva †	Systemic lymphadenopathy and conjunctival cysts (cat. 2) and/or ocular secretion
3	Severe	Oedema, orchitis, lameness, anorexia	Systemic lymphadenopathy and conjunctival cysts (cat. 3) and/or ocular secretion and/or skin lesions †

Table 1: Clinical score employed and based on the severity of the clinical signs compatible with acute and chronic phases of bovine besnoitiosis monitored throughout the experimental infections.

†At least the detection of two clinical signs

Calves were randomly allocated into four different groups of three animals each. Group 1 (G1) was inoculated intravenously with 10^8 tachyzoites, group 2 (G2) with 10^7 tachyzoites and group 3 (G3) with 10^6 tachyzoites by the same route of administration. Group 4 (G4), used as a non-infected control group, was inoculated with PBS and MARC-145 cells, where tachyzoites are routinely propagated (see section 2.3 for the inoculum). Rectal temperatures and other clinical signs characteristic of either acute or chronic phase infections were monitored periodically along the experimental period (Figure 1). Any temperature above 39.5 °C was considered as a fever. A clinical scoring system was established to classify the severity of the outcome of the infection (Table 1). The number of tissue cysts in the ocular conjunctiva was estimated and assigned to the following categories: 1: 1-10 cysts; 2: 11-20 cysts; 3: 21->30 cysts in both eyes (Langenmayer et al., 2015c).

A criterion for the humanitarian slaughtering of the animals consisting of one day of total anorexia and prostration was established.

Blood samples were collected regularly until the end of the experiment by jugular veni-puncture into 5 mL Vacutainer tubes (Becton Dickinson and Company, Plymouth, UK) with and without ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Haematological and biochemical parameters were assessed in blood samples at 7 days prior to infection and at 4, 7, 14 and 21 days post-infection (pi). For biochemical and serological analyses, the blood samples that were collected in vacutainers without anticoagulant were allowed to clot and were centrifuged (1,200 *x g* for 10 minutes) to obtain serum samples (stored at −80°C until use). For haematological analyses and detection of parasitaemia, the blood samples that were collected in vacutainers containing EDTA were immediately

processed to obtain peripheral blood mononuclear cells (PBMC).

Once a week, two skin biopsies from each animal were collected from the inner thigh using 6-8-mm-diameter biopsy punches to perform *B. besnoiti* ITS-1 PCR and histopathology. At the end of the experiment, the animals were sedated with xylazine hydrochloride (Rompun ®; Bayer, Mannheim, Germany) and immediately euthanized by an intravenously overdose of embutramide and mebezonio iodide (T61®; Intervet, Salamanca, Spain). Post-mortem examination of the calves was carried out immediately after euthanasia and tissue samples were collected for PCR and histopathological analyses. Tissues collected were as follows: reproductive system (testis, epididymis head, body and tail, vasa deferential, bulbourethral gland, prostatic gland, seminal vesicles and penis), respiratory tract (nostrils, nasal turbinates, larynx, pharynx, epiglottis, trachea, bronchi and lungs), digestive tract (tongue), 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), as well as other organs (ventriculum, atrium, ocular conjunctiva, sclerotic, distal fascia-tendon from the rear leg and hoof corium).

Experiment B

Six 14-month-old young bulls (B1-B6) of the Asturiana de la Montaña breed were included in this trial. Health status monitoring of the animals and laboratory analyses were done as in experiment A. The animals were inoculated with 10^6 tachyzoites through jugular venipuncture (see section 2.3). The experimental design in experiment B was similar to A. The periodicity of clinical monitoring and skin biopsies and blood samplings are shown in Figure 1. Bulls were euthanized at 115 days pi. Tissues from the reproductive tract (testicular parenchyma, epididymis, pampiniform plexus and penis), respiratory tract (nostrils, epiglottis and trachea), skin (scrotum, carpus, tarsus, elbow, perineum and pinna), spleen and ocular conjunctiva were collected for PCR and histopathological analyses.

2.3. Parasites

Tachyzoites from the Bb-Spain 3 isolate were inoculated in both experiments. This isolate was obtained from a calf with chronic besnoitiosis and showed similar *in vitro* traits as other Spanish isolates (Diezma-Diaz et al., 2017). Tachyzoites were propagated in a MARC-145 cell monolayer (passage number = 4) according to a previously described protocol (Fernández-García et al., 2009b) and were free of bovine viral diarrhoea virus (BVDV) and *Mycoplasma* spp. Tachyzoites were recovered from the cell monolayer when the majority of the parasites were still intracellular. The tachyzoites were then counted using a Neubauer chamber and parasite viability was determined using trypan blue exclusion and a plaque invasion assay by counting lysis plaques and parasitophorous vacuoles (Rojo-Montejo et al., 2009).

To prepare the inocula, tachyzoites were resuspended in phosphate buffer saline (PBS) at the required dose (10^6, 10^7 and 10^8 tachyzoites in a final volume of 2 ml) and were administered to animals within 1 hour of harvesting from cell culture. MARC-145 cells were also counted using a Neubauer chamber to determine the number of cells per dose. These were resuspended in PBS and inoculated in G4.

2.4. Biochemical and haematological analyses

Biochemical and haematological analyses were performed according to procedures previously described (Langenmayer et al., 2015a). They were

analysed by comparing values from infected animals with bovine reference values, taking into account that values may vary due to age and environmental factors (George et al., 2010).

2.5. IFN-γ innate responses

IFN-γ levels were measured in sera by the Bovine IFN-γ ELISA development kits (Mabtech AB, Stockholm, Sweden), following manufacturer's recommendations. Colour reaction was developed by the addition of 3, 3′, 5, 5′-tetramethylbenzidine substrate (Sigma–Aldrich, Madrid, Spain) and incubated for 5–10 minutes in the dark. Reactions were stopped by adding 2N H_2SO_4. Then, plates were read at 450 nm. The cytokine concentrations were calculated by interpolation from a standard curve generated with recombinant cytokines provided with the kits.

2.6. Humoral immune responses

Besnoitia besnoiti-specific IgG were determined by ELISA using lyophilized *B. besnoiti* tachyzoites as antigen (García-Lunar et al., 2017). Serum samples were analysed and the value of the optical density (OD) was converted into a relative index percent (RIPC) using the following formula: RIPC = (OD_{405} sample – OD_{405} negative control)/ (OD_{405} positive control – OD_{405} negative control) x 100. An RIPC value ≥17.34 indicates a positive result.

Besnoitia besnoiti specific IgG1 and IgG2 serum isotypes were also determined by ELISA. Briefly, ELISA was performed as described above using a 1:100 dilution of sera samples and anti-bovine IgG1 or IgG2 peroxidase-conjugated as secondary antibody (1:1000; Bio-Rad, Hercules, California, USA). Sera from naturally infected and non-infected cattle were used as positive and negative controls, respectively. For each plate, the values of the optical density read at 405 nm wavelength (OD405) were converted into the corresponding RIPC value as described above.

2.7. DNA extraction and PCR determinations

Whole blood collected in a heparin tube was used to obtain PBMC. An aliquot of 800 µL of blood was gently mixed for 5 minutes with 960 µL of cold lysing solution (0.83 % NH_4Cl + 2.06 % Tris) at room temperature. After a centrifugation for 30 seconds at 12,000 x *g*, the supernatant was decanted, and the process was repeated until the liquid was clear red. The pellet was stored at -80ºC. Genomic DNA was extracted from PBMC and tissue samples using the Maxwell® 16 MouseTail DNA Purification Kit (Promega, Wisconsin, USA). The DNA content of each sample was measured using a NanoPhotometer® (Implen, Munich, Germany) and adjusted to 40 ng/µl. ITS-1 PCR was performed as described previously (Cortes et al., 2007b).

2.8. Histopathology and immunohistochemistry

Tissue samples and biopsies were fixed in 10% neutral buffered formalin and conventionally processed through a graded alcohol series before being embedded in paraffin wax. Four µm thick sections were obtained and stained with haematoxylin and eosin (H/E).

The immunohistochemical labelling was performed on those samples with histological lesions consistent with *B. besnoiti* infection and those where PCR-positive results were obtained. A primary in-house rabbit polyclonal antibody against *B. besnoiti* tachyzoite antigens (Gutiérrez-Expósito et al., 2012) was used for this purpose at 1:4000 dilution. Briefly, an enzymatic digestion was carried out with trypsin, the primary antibody was incubated overnight at 4 ºC and, later on, a polymer-based detection system was used (EnVision+ System Labelled Polymer-HRP anti-rabbit; Dako, Glostrup, Denmark). The

reaction was developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB Peroxidase Substrate Kit; Vector Laboratories, California, USA). The sections were counterstained with Mayer's haematoxylin. The specificity of the technique was assessed by omitting the primary antibody and also using preimmune rabbit serum.

2.9. Statistical analysis

Rectal temperatures and serology were analysed using a two way ANOVA repeated measures test followed by a Tukey post-test. Statistical significance for all analyses was assessed with $p < 0.05$. All statistical analyses were carried out using GraphPad Prism 6.01 software (San Diego, CA, USA).

3. Results

3.1. Clinical signs and clinical score

From the first day pi onwards, the mean temperatures of the infected calves were significantly higher than the uninfected group ($p < 0.001$) (Figure 2). However, there were no significant differences between infected groups ($p > 0.05$; two-way repeated measures ANOVA, Tukey post-test). All infected animals presented with fever from one day pi onwards until the end of the first week. The onset of fever was earlier in the higher-dose infected group (G1). These animals showed temperatures higher than 39.6 ºC at 12 hours pi. The highest temperature values were observed in one animal from G1 at one and three days pi (40.8 ºC and 40.3 ºC, respectively). The remaining calves from G1 also developed high temperatures up to 40.3 ºC. A febrile response was also observed in all calves from G2 for as long as 7 days pi. The highest values from G2 were observed in one animal at one and three days pi (40.3 ºC and 40.7º C, respectively). In G3, all animals developed fever. The highest values were observed in two animals at 4 days pi (40.1 ºC and 40 ºC). Animals from the uninfected control group (G4) had temperatures below 39.5 ºC throughout the experimental period.

The only temperatures higher than 39.5 ºC at 8 days (n = 3) and 9 days pi (n = 1) were detected in 4 out of 6 infected bulls (Figure 2).

All calves from G1, G2 and G3 developed lymphadenopathy at 4 days pi in at least two of the three lymph nodes (submandibular, pre-scapular and pre-crural) examined. Lymphadenopathy remained longer in G1, for as long as 34 days pi vs. 30 days pi in G2 and G3. Respiratory signs were also detected sporadically in calves from infected groups. Most animals coughed and had respiratory distress with an increased expiratory effort, at least once during the first month pi, which is characteristic of diseases that affect the lower respiratory tract.

Other signs characteristic of the acute stage of the disease, such as oedema, orchitis or lameness, did not develop in any of the infected animals. The outcome of the acute infection was classified as a signs (orchitis, oedemas or lameness) were found. *In vivo* clinical signs characteristic of the chronic stage, such as skin lesions, were not detected.

All infected bulls developed lymphadenopathy in one or both lymph nodes (only precrural or pre-scapular were monitored in bulls). This enlargement was observed at three days pi in five out of six bulls and after six days in all infected animals. Lymphadenopathy was maintained until 55 days pi but was more evident within the first 24 days. Respiratory signs such as cough and nasal discharge 'mild-moderate infection' according to the clinical score presented in Table 2A, since no severe clinical

Figure 2: Mean rectal temperatures (+SD) recorded during the first month post-infection. The cut off for fever was ≥39.5° C.

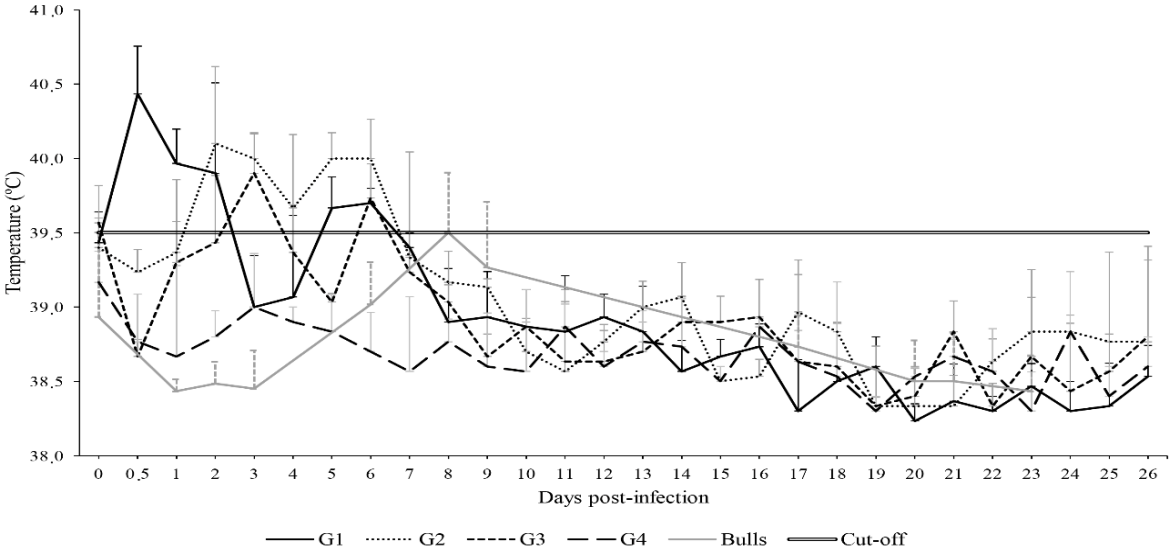


Table 2: Clinical score estimated in calves **(A)** and bulls **(B)**.

A	Group	Days post-infection										
		0-2	3-6	7-9	10-13	14-16	17-20	21-23	24-27	28-30	31-34	34-70
C1	1	2	2	2	2	2	1	1	1	0	1	0
C2	1	2	2	2	2	2	2	2	1	1	1	0
C3	1	2	2	2	2	2	1	1	1	1	1	0
C4	2	2	2	2	2	2	2	1	1	1	0	0
C5	2	2	2	2	2	1	1	1	1	1	0	0
C6	2	2	2	2	2	1	1	1	0	1	0	0
C7	3	2	2	2	2	2	2	1	0	1	0	0
C8	3	0	2	2	1	2	1	1	1	1	1	0
C9	3	0	2	2	2	2	2	1	0	1	0	0
C10	4	0	0	0	0	0	0	0	0	0	0	0
C11	4	0	0	0	0	0	0	0	0	1	0	0
C12	4	0	1	0	0	0	0	1	1	1	0	0

B	Days post-infection																	
	0	1	2	3	6	8	9	17	20	21	23	29	31	35	42	48	55	>64
B1	0	1	1	2	2	2	2	2	2	2	1	1	1	2	1	1	1	0
B2	0	1	1	1	2	2	2	2	2	1	1	1	1	1	1	1	1	0
B3	1	1	1	2	2	2	2	2	2	1	1	1	1	1	1	1	1	0
B4	0	1	1	2	2	2	1	2	2	1	1	1	1	1	1	1	1	0
B5	0	0	0	0	1	1	1	2	1	1	1	1	1	1	1	1	1	0
B6	1	1	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	0

C: Calf (1-12); B: Bulls (1-6)

were observed mainly between eight and 20 days pi. Respiratory distress with an increased expiratory effort was observed in all animals between 17 and 20 days pi. Similar to the calves, the bulls did not develop other signs of severe acute stage or chronic phase of the disease, and the outcomes of the infections were classified as mild-moderate based on the clinical score shown in Table 2B.

3.2. Kinetics of IFN-γ innate responses

Maximum serum IFN-γ levels were observed at seven days pi in G1 and at four days pi in G2 and G3 and were significantly higher compared to the control group ($p < 0.05$; two-way ANOVA). At 4 days pi, IFN-γ levels were significantly higher in G2 and G3 ($p < 0.05$; Tukey post-test) compared to G1 and bulls. IFN-γ levels decreased to basal values in all three infected groups from day 10 pi and remained low onwards (Figure 3A)

In bulls, IFN-γ levels peaked at 8 days pi and were significantly higher compared to calves ($p < 0.05$;

Tukey post-test). Levels decreased to basal values at 16 days pi (Figure 3A).

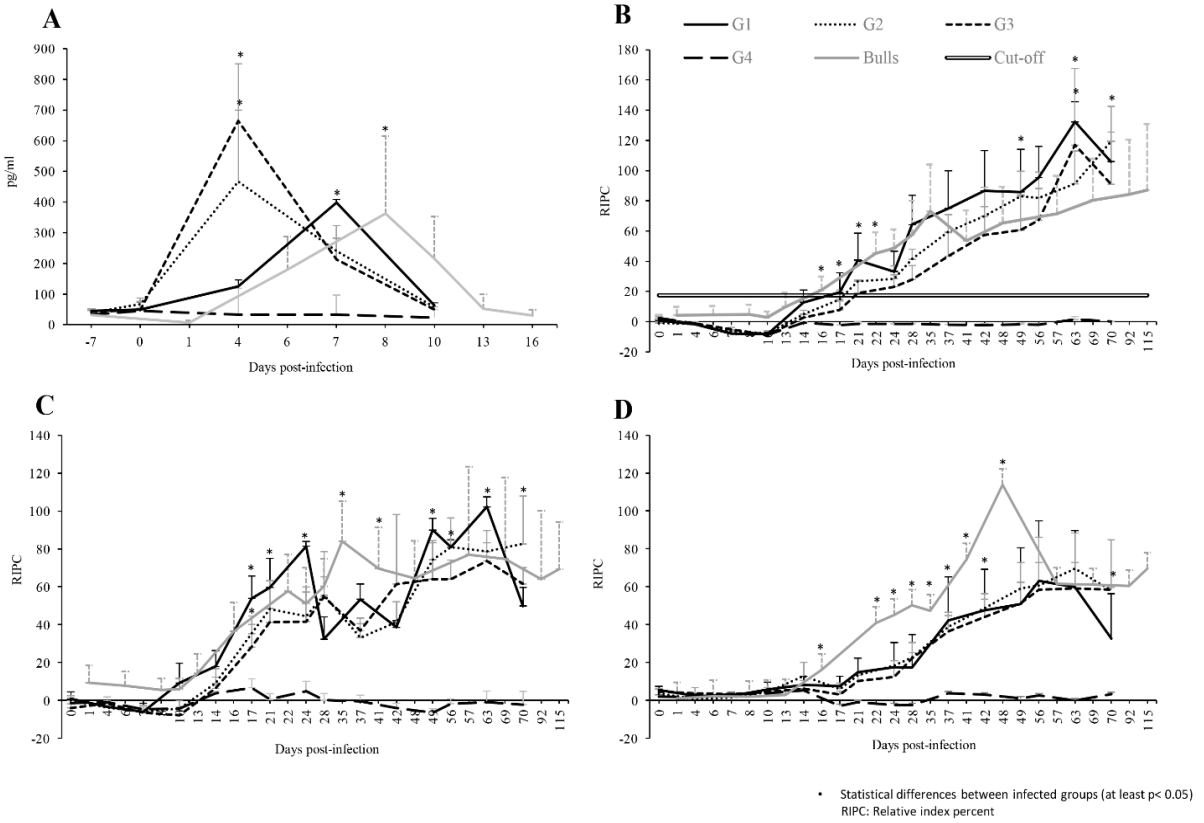
3.3. Humoral immune responses

After infection, seroconversion was detected at 16 days in bulls, 17 days in G1 and G2 and at 21 days in G3. The antibody levels were significantly higher in infected calves than in G4 from 17 days pi onwards ($p < 0.05$; two-way repeated measures ANOVA, Tukey post-test).

In calves, the higher dose-infected group (G1) showed significantly higher antibody levels compared with the lower dose-infected group (G3) starting at 17 days and remained higher from 24 days pi onwards ($p < 0.05$; two-way repeated measures ANOVA, Tukey test). However, there were no significant differences between G1 and G2 ($p > 0.05$; two-way repeated measures ANOVA, Tukey post-test). The antibodies kinetics were similar in calves and bulls with a few significant differences. The antibody levels in bulls were significantly higher than in the G4 from 16 to 70 days pi ($p < 0.05$; two-way repeated measures ANOVA, Tukey post-test) (see Figure 3B).

Significantly higher levels of IgG1 were detected in the infected groups compared to uninfected animals from 17 days pi ($p < 0.01$; two-way repeated measures ANOVA, Tukey post-test) (Figure 3C). Infected calves, G1 had significantly higher levels than the other infected groups between 21 and 24 days pi ($p < 0.01$) and higher than G3 at 56 and 63 days pi ($p < 0.05$). The levels in G2 were significantly higher than G3 at 56 days pi, and higher than the other infected groups at the end of the trial ($p < 0.05$). IgG1-levels in bulls were significantly higher than infected calves between 37-41 days pi, ($p < 0.05$).

Figure 3: Immune responses measured in calves and bulls. **A:** Innate IFN-γ responses measured in serum samples; **B:** *B. besnoiti* –specific IgG responses; **C:** Mean Relative Index (+SD) IgG1 antibody levels; **D:** Mean Relative Index (+SD) IgG2 antibody levels.



In calves, IgG1 levels were higher than IgG2 levels throughout the whole trial. Although IgG2 levels were detected approximately 21 days pi, significant differences between infected animals and the negative control group were not detected until 37 days pi (two-way repeated measures ANOVA, Tukey post-test $p < 0.05$). Since IgG2 levels decreased in G1, significant differences were only observed between G1 and the other two infected groups in day 70 pi ($p < 0.01$). An increase in IgG1-IgG2 levels was observed in bulls at 16 days pi and levels remained high until the end of the experiment. IgG2 levels were always lower than IgG1 except for 41 and 48 days pi, when IgG2 levels reached their highest values (Figure 3D). IgG2 levels were higher in bulls than in infected calves between 16 to 48 days pi ($p < 0.001$).

3.4. Detection of *B. besnoiti* DNA in blood and tissues by PCR

Parasitaemia was detected sporadically in calves from G1 and G2 until seven days pi. Parasitaemia was observed in two bulls between 10 and 24 days pi. However, the parasite was not detected in skin biopsies in any of the groups (Table 3).

After necropsy, ten tissue samples from calves were PCR positive with the highest number of PCR positive tissues (skin, reproductive and eyes samples) in G3. In the experiment B, only two tissue samples (testicular parenchyma and pampiniform plexus) from the same animal were positive by PCR (Table 3).

3.5. Histopathology

Macroscopic lesions were not detected at necropsy. In contrast, compatible and characteristic microscopic lesions of chronic infection were found. Vasculitis was found in infected calves, particularly, in the skin (neck, tarsus, carpus, pinna and perineum), reproductive tract (testis, epididymis, vasa deferens and bulbourethral gland) and other locations (nostrils, tonsils, prescapular lymphnode, lung and hoof corium) (Supplementary Figure1A). This lesion was characterized by the infiltration of lymphocytes, plasma cells, macrophages and eosinophils within the arteriole and venule walls and hyaline deposits at the arteriolar wall. Granulomatous vasculitis with foreign body giant cells in the testicle and reticular dermis of the perineum was observed in four positive-PCR

Table 3: Detection of *Besnoitia besnoiti*-DNA in blood and tissues.

Groups		Blood: days post-infection (animals)	Tissues (animals)
Calves	G1	4 (C2, C3); 7 (C1,C2,C3)	Carpal (C2) and scrotum skin (C3)
	G2	7 (C5, C6)	Proximal vas deferens (C5), epididymis body (C5) and scleral (C6)
	G3	Negative	Epididymis tail (C9), ocular conjuctiva (C7, C8), scleral (C8) and carpal skin (C7)
	G4	Negative	Negative
Bulls		10 (B3, B6), 13 (B6), 16 (B6), 24 (B3)	Parenchyma testicular (B2) and pampiniform plexus (B2)

C: Calf (1-12); B: Bulls (1-6)

animals: one from G1 (C3), 2 from G2 (C5, C6) and one from G3 (C9) (Table 3).

Focal lymphoplasmacytic inflammatory infiltrates with the presence of eosinophils and macrophages were found in all infected animals. These infiltrates, often perivascular, were more numerous in lamina propria of the respiratory mucosa (larynx and nostrils), in skin dermis (scrotum, tarsus and neck) as well as in the stroma of genital organs (epididymis). Regarding other lesions in the male genital tract, C3 from G1 and C9 from G3 (both positive by PCR) showed testicular degeneration characterized by the formation of multinucleated giant cells in the lumen of seminiferous tubules.

A tissue cyst was only found in one animal (C2) from G1 in carpal skin (Supplementary Figure 1B). This tissue cyst was located in the deep dermis near vessels, surrounded by inflammatory cells (lymphocytes, macrophages and eosinophils accompanied by fibrinous necrosis of the muscular of the arterioles.

In bulls, mild local inflammation was detected in most of the tissue samples from skin, reproductive and respiratory tract. These foci were formed mainly by lymphocytes and, to a lesser extent, macrophages and plasma cells. They were located at the skin, dermis and lamina propria of the respiratory mucosa. Specifically, these lesions were mainly found at the trachea and scrotum (all animals), epiglottis and skin of the elbow, carpus and thigh (five out of six animals) and skin of the tarsus (four out of six animals). In addition, the spleen of two bulls showed mild lymphoid hyperplasia. Regarding the genital tract, three animals showed foci on non-purulent inflammatory infiltrate adjacent to vessels at the epididymis and one of them (B2) also at the pampiniform plexus.

All immunohistochemistry labelled samples were negative for *B. besnoitii* antigen but for the carpal skin sample from C2G1.

4. Discussion

An *in vivo* ruminant model of besnoitiosis is a major need. Thus, we studied the effect of parasite dose and host age on the infection dynamics with *B. besnoiti* tachyzoites in cattle with the ultimate goal of developing a reproducible experimental model of *B. besnoiti* infection in cattle. We have successfully reproduced the acute phase of the disease and microscopic findings evidenced the chronic infection. Indeed infected animals without detectable clinical signs and macroscopic lesions characteristic of the chronic phase are more frequently found than clinically affected animals in endemically infected herds (Gollnick et al., 2018). However, to test potential drugs or vaccines, an ideal experimental bovine model should be able to reproduce the acute stage followed by the characteristic clinical signs of the chronic stage of the disease. Fulfilling this requirement is not an easy task based on the variable results obtained by others in the past, where the absence of a well standardized experimental design hampered the ability to infer conclusions about the key variables and, consequently, to reproduce the results (Álvarez-García et al., 2014b).

To address the previous limitations, we carried out two experimental infections where we examined the tachyzoite dose and host age as parasite- and host-dependent variables, respectively. Three key elements were carefully controlled to ensure reproducible results: i) the employment of a well-characterized *B. besnoiti* isolate; ii) the viability and quality of the inocula; iii) the animal health status prior to infections.

We chose the Bb-Spain 3 isolate for several reasons: first, it was obtained from an animal younger than

four months with marked skin lesions and, second, its recent isolation rules out the possibility of *in vitro* adaptation that might compromise its ability to form tissue cysts *in vivo*. Moreover, it has shown similar *in vitro* behaviour compared to other Spanish isolates (Diezma-Díaz et al., 2017). We also carefully monitored: i) the inocula to ensure that the tachyzoites were free of BVDV and *Mycoplasma* spp. infections; ii) the animal health status prior to inoculations; and iii) the numerous clinical signs and laboratory parameters measured by complementary techniques.

On the other hand, we selected males for these experiments, even though both sexes may be equally infected (Álvarez-García et al., 2014b). However, bulls are at higher risk of acquiring and transmitting the infection since they serve a large number of cows and they appear to show more acute signs and a higher mortality rate (Álvarez-García et al., 2014b; Gazzonis et al., 2017). Moreover, testicles are target organs for *B. besnoiti* replication and persistence (Pols, 1960; Kumi-Diaka et al., 1981) and recent studies have provided evidence for the serious consequences of male sterility for herd fertility due to *B. besnoiti* infection (Esteban-Gil et al., 2016; Gazzonis et al., 2017).

Herein, we successfully reproduced the acute stage of the disease. Calves and young bulls developed fever followed by lymphadenopathy and respiratory signs. All infected calves developed fever during the first week pi and the incubation period lasted less than 12 hours, which agrees with the less than two-day incubation period established previously using different intravenous inocula (Bigalke, 1968). However, the incubation period was longer in bulls. Four out of six inoculated bulls showed temperatures higher than 39.5º C at 8-9 days pi. Respiratory signs of infection were observed in bulls later and the lymphadenopathy was maintained longer. It is possible that a longer incubation period leads to a delay in the appearance of acute clinical signs. This delay could also have influenced on parasitaemia, since the parasite was detected in blood until seven days pi in calves vs. 24 days pi in bulls. These findings agree with previous studies that detected proliferative organisms in blood smears from the 3rd to the 12th day after the beginning of the febrile reaction in experimentally infected cattle (Basson et al., 1970). However, this finding was not corroborated in natural infections (Gollnick et al., 2015), where examinations of blood smears from naturally infected cows during the acute phase did not reveal either free or intracellular tachyzoites in peripheral blood. However, the sensitivity of blood smears is lower than PCR, which might explain these negative results. Our analyses of haematological and biochemical parameters showed very little relevant changes, which is in agreement with the mild to moderate outcome of the infection in both calves and bulls. We only observed an increase of serum globulin fractions that was correlated with seroconversion in infected animals. There is only one previous study where haematological and biochemical alterations in sera were studied in *B. besnoiti* seropositive cows during disease progression of naturally acquired bovine besnoitiosis (Langenmayer et al., 2015a). Disturbances in Aspartate Aminotransferase or Creatine Kinase activities, and typical alterations of chronic inflammatory diseases such as hyper-(gamma)-globulinemia or reduced erythrocyte were described by these authors.

No relevant differences were observed during the acute stage regarding the parasite dose and host age variables studied. It has been suggested that tachyzoite dose plays an important role in determining a severe vs. a mild infection (Álvarez-García et al., 2014b). In the present study, this variable influenced only the occurrence of fever and

the incubation period, but not the clinical score of the disease. Higher temperature values were detected in the group inoculated with the highest tachyzoite dose, similar to previous observations (Basson et al., 1970), but no relevant differences in the severity of the acute phase were observed among groups. It is tempting to hypothesize that doses higher than 10^8 tachyzoites would not significantly exacerbate clinical signs since relevant differences in the outcome of the infection were not observed among the three infected groups. In four experimental infections carried out in the past, tachyzoite doses higher than 10^8 were inoculated in cattle (Bigalke, 1968; Basson et al., 1970; Diessing et al., 1988, Álvarez-García et al., 2014b). Acute phase was observed in all inoculated animals with mild-moderate severity, but for one immunosuppressed cow that died during this phase (Basson et al., 1970). However, severe clinical signs of chronic phase were only reported in one bull that had been splenectomiced previously (Basson et al., 1970). We must carefully consider these results since the inoculations were carried out under different experimental conditions. Moreover, a high number of zoites are not likely to be involved in natural infections with

B. besnoiti, where horizontal transmission seems to be the main route of parasite transmission, either by cyst rupture during direct contact or by blood suckling arthropods bites (Álvarez-García et al., 2013). In fact, in naturally infected herds where animals might be exposed to similar infective doses, only a few animals develop characteristic clinical signs of bovine besnoitiosis, which may be due not only to parasite dose but also to other parasite and host-dependent factors. Regarding host age, according to previous experimental infections (Bigalke, 1970; Bigalke et al., 1974), the incubation period, as well as the onset and duration of the febricula were highly variable in adult animals and were probably influenced by the different sources of the inocula. Herein, the inocula employed in both experiments were the same so that the most feasible explanation for the delay in the onset of clinical signs observed in bulls compared to calves might be the maturity of the immune system. The immune system maturation progresses in calves from birth until 6 months, when innate and adaptive immune responses reach values close to the ones developed by adults (Chase et al., 2008).

After the acute stage of the disease, the chronic stage of the infection was evidenced by parasite detection and microscopic lesions in target tissues despite no macroscopic lesions characteristic of the chronic stage of the disease, such as hyperqueratosis or alopecia, were detected *in vivo*. In the present study, vasculitis was the most relevant lesion present in skin, genital and respiratory tract in all infected groups. Despite being a non-specific lesion, target locations where vasculitis was found suggested that this might be a consequence of endothelial damage produced by the parasite during the acute phase. Similar lesions have been described under natural infection (Langenmayer et al., 2015c). Focal degenerative lesions in testicle of the calves were observed only in relation to vasculitis that probably contributed to mild degenerative changes in the germinal epithelium. However, the lesions observed in the reproductive tract were compatible with normal testicular function. Remarkably, in this study, a tissue cyst was located at the reticular dermis of the carpus region, a less frequent location than the papillary dermis (Langenmayer et al, 2015c). As expected, a higher parasite intraorganic distribution was found by PCR compared with histopathological results. PCR positive tissues were predilection sites for *B. besnoiti*, that show tropism for the connective tissue of the superficial skin layers, scleral, conjunctiva and mucous membranes of distal genital tract (Nobel et al., 1981; Manuali et al., 2011;

Gentile et al., 2012; Frey et al., 2013). The DNA detected is expected to belong to the bradyzoite stage according to the infection dynamics as it was detected at 70-110 days pi during the chronic phase of the disease (Álvarez-García et al., 2014b). Slight differences were found among infected groups since a higher number of PCR-positive samples (six out of the 10) belonged to calves inoculated with 10^6 tachyzoites. It appears that inoculation with a lower dose of the parasite could have caused less stimulus of immune response, facilitating the evasion of the parasite and this could have allowed a higher early intra-organic distribution. This fact could be in agreement with what happens in natural conditions where low parasite doses might be inoculated under natural conditions through either direct contact or via haematophagous vectors as mentioned above (Álvarez-García et al., 2013). Based on the similar results between infected calves, the dose of 10^6 tachyzoites was chosen for the inoculation of bulls, where only two testicular samples were PCR-positive. Regarding host age, three-six month old Holstein Friesian calves were experimentally infected with *B. besnoiti* bradyzoites in the past and animals only developed tissue cysts and skin lesions under immunosupresive treatment regardless the route of infection employed (Diesing et al., 1988). When tachyzoites were used as inocula only one out of eight infected animals develop a few cysts without skin lesions in agreement with the results obtained in the present study.

Herein, efficient immune responses elicited by immune competent animals were likely to have cleared most parasites, which is evidenced by the low parasite load detected by histology and correlated with mild lesions despite the high doses of parasites and the route of inoculation employed and reported by others (Bigalke, 1968; Álvarez-García et al., 2014b). Accordingly, in both calves and bulls, the infection was controlled, and the immune response kinetics were similar in all infected groups. After infection, an innate IFN-γ response was developed during the first week pi, followed by an acquired immune response evidenced by seroconversion at 2-3 weeks pi and the maintenance of high antibody levels until the end of the experiment. IgG1 levels increased simultaneously with total IgG levels followed by an increase of IgG2 levels a few days later. Similar immune response kinetics have been reported in experimentally infected cattle with closely related apicomplexan parasites such as *Neospora caninum* (Regidor-Cerrillo et al., 2014). The basis of an effective immune response that governs the control of *B. besnoiti* infection remains to be clarified. However, a few remarkable findings were obtained. First, we corroborated that high antibody levels are neither predictive of the outcome of the infection nor protective against the infection, since no relevant differences were observed among groups regardless tachyzoite dose and host age. In contrast, it has been suggested that cell-mediated immune responses may play a key role in the control of the infection (Álvarez-García et al., 2014b). Indeed, *B. besnoiti*-induced neutrophil extracellular trap formation was recently demonstrated as an important innate immune response mechanism of PMN acting against *B. besnoiti* (Caro et al., 2014) that might be influenced by parasite dose and host age based on the results obtained. It was also reported that *B. besnoiti* infection triggers early innate immune responses in endothelial cells (Maksimov et al., 2016). Although the information we obtained is limited to IFN-γ and IgG2 responses, the results are in agreement with this hypothesis. The late IFN-γ response displayed by G1 and G2 calves and bulls, together with the higher IgG2 levels observed in bulls might be responsible for a better control of the infection. This mild dose-dependent modulation of the immune response has been previously reported by (Rojo-

Montejo et al., 2012), who suggested that when a high parasite dose is administered, a large number of tachyzoites may remain extracellular and stimulates the immune response more efficiently, whereas lower parasite doses might facilitate parasite immune evasion. Further works should elucidate the roles played by the immune T-cells repertoire, the Th1/Th2 balance and other cytokines, among others, in the pathogenesis and control of *B. besnoiti* infection.

In summary, infected animals developed clinical signs compatible with the acute stage and microscopic lesions characteristic of the chronic stage of the disease. Neither parasite dose nor host age seem to be relevant parasite- and host-dependent factors when tachyzoites were inoculated, since they did not significantly influenced the outcome of the infection. Moreover, we have set the stage for carrying out further controlled experimental infections in bovines to refine the present experimental model and induce chronic clinical signs. Thus, other variables such as alternative inoculation routes or parasite stages (e.g., bradyzoites) should be investigated in order to be able to reproduce clinical signs and macroscopic lesions characteristic of chronic besnoitiosis.

Acknowledgements

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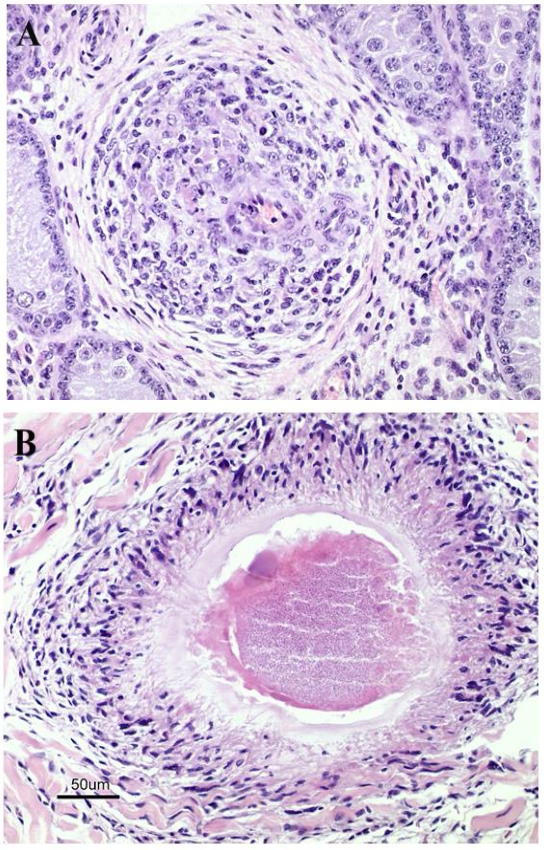
Appendix A

Supplementary figure: Hematoxylin and eosin: **A:** Vasculitis lesion in testicle sample from one calf (C5) of G2. **B:** Tissue cyst associated with vasculitis in carpal skin sample. Note the pericystic inflammatory infiltrate (Calf G1C2).



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

Diezma-Díaz, C.[1], Ferre, I.[1], Re, M.[1, 2], Jiménez-Meléndez, A.[1], Tabanera, E.[2], González-Huecas, M.[2], Pizarro-Díaz, M.[2], Yanguas-Pérez, D. [1], Brum, P. L.[3], Blanco-Murcia, J.[1, 2], Ortega-Mora, L.M.[1], Álvarez-García, G.[1*]

[1] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.

[2] Animal Medicine and Surgery Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.

[3] Laboratory of Microbiology and Parasitology, UNIPAMPA, Federal University of Pampa, Dom Pedrito, Brazil.

* Corresponding author: Gema Álvarez García. Tel.: +34-913944095. E-mail address: gemaga@vet.ucm.es

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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.

Keywords*: Besnoitia besnoiti*, tachyzoites, subcutaneous route, intradermal route, calves.

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., 2014b; Gutiérrez-Expósito et al., 2017b). 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 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., 2014b), 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 3-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 -80ºC 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-TGACATTTAATAACAATCAACCCTT-30) and the reverse primer ITS1R (50-GGTTTGTATTAACCAATCCGTGA-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 (> 39.5ºC) 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 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 (Figure 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 (Figure 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 ($P < 0.001$) (Figure 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) (Figure 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 ($P= 0.018$) (Figure 1B).

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).

As shown in Figure 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) (Figure 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) (Figure 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.

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

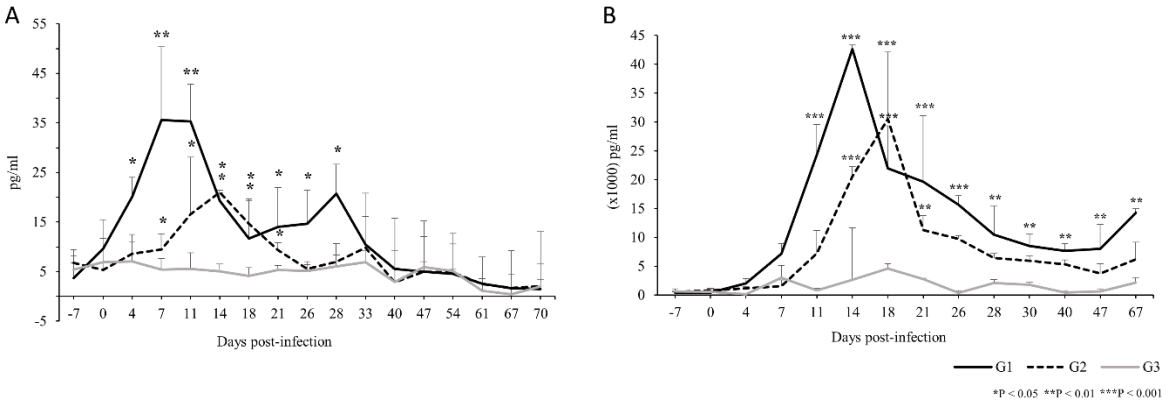
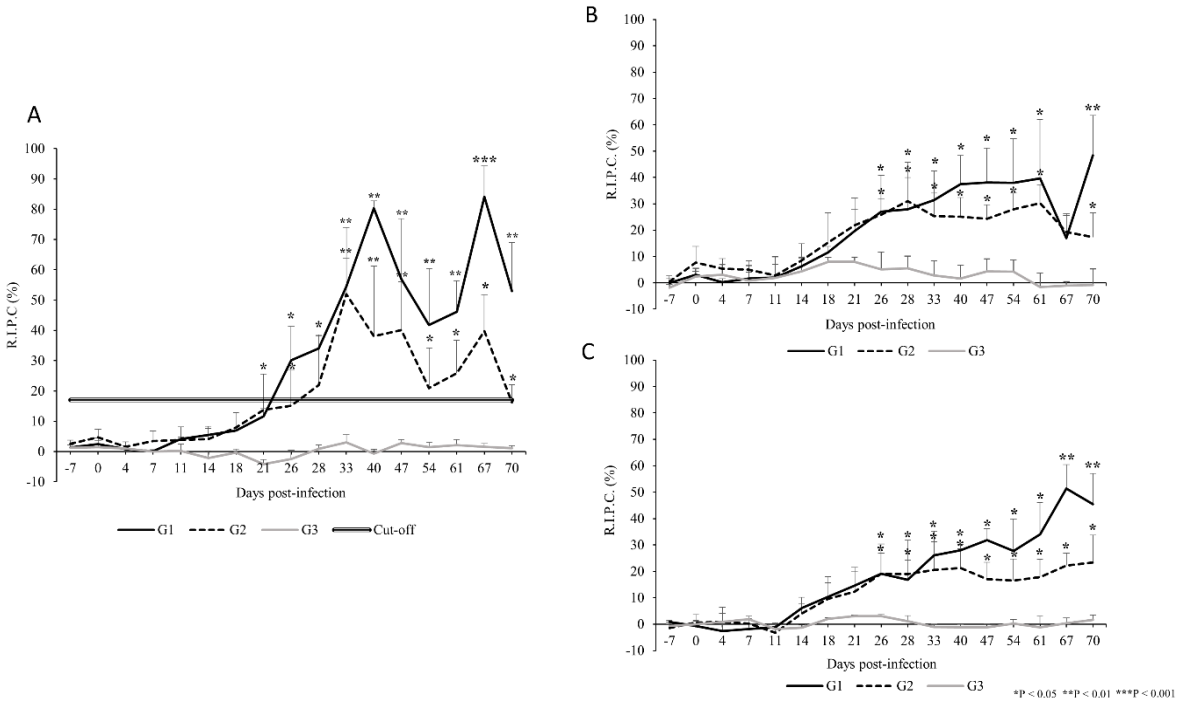


Figure 2: Humoral immune response: A: *Besnoitia besnoiti*–specific IgG responses; **B**: IgG1 antibody levels; **C**: IgG2 antibody levels. Mean Relative Index (+Standard Deviation) (R.I.P.C: Relative index per cent).



4. Discussion

The route of inoculation is a host-dependent variable that has an impact on microbial infection outcome (Benavides 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 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., 2014b).

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 animal 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 (Benavides 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., 2014b).

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 x 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 x 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., 2014b).

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-γ 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.

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❖ Objetivo 2: Inoculación de bradizoítos de *Besnoitia besnoiti* en terneros: Factores clave en la aparición de los signos clínicos característicos de la besnoitiosis crónica y descripción de los hallazgos histológicos en los tejidos diana.

Diversos fármacos, tanto de nueva generación como ya comercializados para otras patologías en el ganado vacuno, han mostrado en modelos *in vitro* resultados prometedores para el tratamiento de la besnoitiosis bovina. Sin embargo, en la actualidad, se necesitaría un modelo *in vivo* de infección experimental bien normalizado para evaluar su seguridad y eficacia, así como la de otros candidatos terapéuticos. Con los estudios realizados en el objetivo 1 de la presente Tesis Doctoral, se comprobó que la inoculación de distintas dosis de taquizoítos por diferentes rutas (intravenosa, subcutánea e intradérmica) en terneros da lugar al desarrollo de signos clínicos propios de la fase aguda de la enfermedad y, únicamente, hallazgos microscópicos de la fase crónica sin detectarse los signos clínicos macroscópicos característicos de ésta (quistes conjuntivales patognomónicos y lesiones cutáneas). Del mismo modo, la inoculación de taquizoítos por vía intravenosa en novillos produjó los mismos signos clínicos. Por ello, en este objetivo se refinó el modelo utilizado anteriormente en terneros, valorando en este caso el otro estadio parasitario infectante para los bovinos, el bradizoíto, administrado también por diferentes vías de inoculación.

Se inocularon 12 terneros frisones, estableciéndose aleatoriamente cuatro grupos con tres animales cada uno. Tres grupos se inocularon con 10^6 bradizoítos por vía intravenosa, subcutánea o intradérmica respectivamente y un grupo no inoculado actuó como testigo negativo de la infección. Los bradizoítos se obtuvieron de quistes tisulares presentes en la mucosa traqueal de un animal con signos clínicos y lesiones características de una infección crónica que fue sacrificado en un matadero autorizado. Se llevó a cabo un control de calidad exhaustivo del inóculo, valorando su capacidad de infección en cultivo celular, así como descartando la contaminación por *Mycoplasma* spp. y la presencia del virus de la diarrea vírica bovina. Se realizó un seguimiento clínico y serológico de los terneros para valorar la respuesta inmunitaria humoral y celular. A los 3 meses post-infección (pi) fueron sacrificados para la detección de lesiones y presencia del parásito en sus tejidos. Los animales inoculados, tanto por vía intravenosa, subcutánea como intradérmica, desarrollaron signos clínicos evidentes de la fase aguda (fiebre, linfadenomegalia) y de la fase crónica (quistes en conjuntiva ocular) de la besnoitiosis. Sin embargo, la ruta intradérmica causó signos clínicos más graves. Se detectaron un mayor número de quistes en la conjuntiva ocular (a partir de los 43 días pi) y lesiones en piel (49 días pi) en los terneros inoculados por esta vía, así como mayor distribución intraorgánica del parásito, detectada tanto por técnicas histológicas como moleculares. Histológicamente, los tejidos con mayor número quistes fueron la piel, incluido el escroto (40,8% de las muestras positivas), los ollares y los cornetes nasales. Los quistes tisulares, con un diámetro promedio de 181,2 µm, estaban completamente desarrollados. Sin embargo, estos no alcanzaron el tamaño máximo reportado por otros autores en infecciones naturales,

posiblemente como consecuencia del control de la infección por la respuesta inmune del hospedador. Las lesiones microscópicas se detectaron principalmente en muestras de piel, pero también de muestras obtenidas de tracto reproductivo y respiratorio superior, aunque no se asociaron necesariamente con la presencia de quistes tisulares. Coexistieron lesiones compatibles tanto con la fase aguda (trombo, edema e inflamación) como con la fase crónica (lesiones cutáneas, hiperqueratosis y glándulas sudoríparas dilatadas) de la enfermedad. Con respecto al aparato reproductor, que fue estudiado en detalle por la importancia de la esterilidad que puede desarrollarse en los sementales infectados, se observó con mayor frecuencia daño vascular e inflamación en la piel de escroto, seguido del parénquima testicular, epidídimo y plexo pampiniforme. Los hallazgos histológicos mostraron una besnoitiosis crónica subclínica en los terneros infectados experimentalmente. Ésta, posiblemente, no habría afectado la fertilidad de los animales durante su vida adulta, ya que la estructura de los túbulos seminíferos y el epitelio germinal de los testículos no mostraron alteraciones relevantes.

En conclusión, el estadio parasitario (bradizoíto) y la ruta de inoculación (intradérmica) fueron variables determinantes en el establecimiento de la infección crónica. Aunque el empleo de bradizoítos como inóculo presenta limitaciones con respecto a su obtención y mantenimiento en cultivo celular, no cabe duda que este estudio sienta las bases para el desarrollo de un modelo experimental de infección por *B. besnoiti* en el cual se puedan testar futuros candidatos terapéuticos.

A model for chronic bovine besnoitiosis: Parasite stage and inoculation route are key factors.

Diezma-Díaz, C.[1], Ferre, I.[1], Re, M.[1, 2], Jiménez-Meléndez, A.[1], Tabanera, E.[2], Pizarro-Díaz, M.[2], González-Huecas, M.[2], Alcaide-Pardo, M. [1], Blanco-Murcia, J.[1, 2], Ortega-Mora, L.M.[1], Álvarez-García, G.[1*]

[1] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.

[2] Animal Medicine and Surgery Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.

* Corresponding author: Gema Álvarez García. Tel.: +34-913944095; E-mail address: gemaga@vet.ucm.es.

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Abstract

In this work, an experimental model for chronic besnoitiosis in bovine was developed and characterized. Using a previously stablished calf model, two new variables (parasite stage and inoculation route) were combined and used. Twelve Holstein Friesian 3-month-old male calves were randomly divided into four groups of three animals each. Bradyzoites were obtained from a chronically infected bull and used for inoculation via three different inoculation routes. Three groups were inoculated with 10^6 bradyzoites by intravenous (G1), subcutaneous (G2) and intradermal (G3) routes, and a non-infected control group (G4) was inoculated with PBS. The trial lasted for 90 days and included daily clinical monitoring as well as weekly skin biopsies and blood sampling. Sera were obtained to analyse both cellular and humoral responses. Once the calves were euthanized, tissues from the skin, eyes, respiratory and reproductive tracts, among others, were collected to study presence of the parasite.

Clinically, the infection was classified as mild to moderate for the acute stage since all infected calves showed lymphadenopathy from four days post-infection (pi) and fever from one week pi until 24 days pi. However, the most relevant results were achieved during the chronic stage that was classified as moderate to severe. In fact, pathognomonic conjunctival cysts were observed in all infected calves from 40 days pi onwards and were more abundant in G3. Moreover, one calf from this group developed skin lesions (49 days pi). The microscopic tissue cysts and *Besnoitia*-DNA were detected primarily in skin, reproductive tract and respiratory tissue samples and parasite load was higher in G3.

In conclusion, the parasite stage (bradyzoite) and the inoculation route are key factors that influence the outcome of an infection. In particular, the intradermal route led to more severe clinical signs of the chronic phase in the inoculated calves.

Keywords: *Besnoitia besnoiti*, *in vivo* model, chronic besnoitiosis, bradyzoites, inoculation routes.

1. Introduction

Bovine besnoitiosis, caused by the apicomplexan protozoan *Besnoitia besnoiti*, is a chronic and debilitating disease that causes local and systemic clinical signs (Bigalke, 1968) and is responsible for considerable economic losses, mainly in cattle (Álvarez-García et al., 2013). Currently, this transboundary disease poses a serious concern to the cattle industry in beef cattle in some EU areas since fertility rates are notably reduced in infected herds (Gazzonis et al., 2017). Moreover, the disease is spreading in several European countries, mainly in association with animal trade (European Food Safety Authority, 2010; Gentile et al., 2012; Basso et al., 2013; Hornok et al., 2014; Vanhoudt et al., 2015; Ryan et al., 2016).

Unfortunately, control of the disease is only possible through management measures coupled to diagnosis since chemotherapeutics are not available and no vaccines are licensed in Europe. Drugs are promising options to fight against the tachyzoite stage responsible for the acute stage. Regarding the chronic stage of the disease, therapeutic options are unlikely to be successful, due to the limited accessibility to the bradyzoites packed inside tissue cysts, which are responsible for the lesions developed during this phase (Gutiérrez-Expósito et al., 2017c). Remarkably, *in vitro* models have provided proof-of-concept of putative effective drugs, such as arylimidamides (Cortes et al., 2011), bumped kinase inhibitors (BKIs) (Jiménez-Meléndez et al., 2017) and commercially available drugs, such as decoquinate, diclazuril (Jiménez-Meléndez et al., 2018), curcumin (Cervantes-Valencia et al., 2018) and naphthoquinone buparvaquone (Müller et al., 2019).

Currently, *in vivo* testing is an essential step in drug and vaccine efficacy trials. The availability of well-characterized experimental models is needed to obtain comparable, robust and reliable data (Joachim et al., 2018). Unfortunately, in bovine besnoitiosis several laboratory animals offer serious limitations for this purpose and preclinical drug screening can only be achieved in *in vitro* systems. Various rodent species are resistant to the disease (Shkap et al., 1987), while others, including rabbits, are characterized by a rapid onset of the acute stage of the disease and sudden death and show difficulty in reproducing the chronic phase (Liénard et al., 2015). Ideally, an *in vivo* experimental model of *B. besnoiti* in bovine should be developed. In the 1960s and 1980s, numerous experimental infections were conducted in cattle. Unfortunately, a reproducible model was not obtained due to variable inoculants and experimental designs (Bigalke, 1968; Basson et al., 1970; Bigalke et al., 1974; Diesing et al., 1988).

Recently, we developed a standardized cattle model of besnoitiosis. Several parameters were evaluated in a bovine model using tachyzoites as the challenge parasite stage. In a first trial, different tachyzoites doses were intravenously inoculated into calves and bulls. Infected animals developed clinical signs compatible with the acute stage and microscopic lesions characteristic of the chronic stage (Diezma-Díaz et al., 2018). In a second trial, 10^6 parasites were inoculated by a subcutaneous and intradermal route to mimic parasite transmission under natural conditions by blood-sucking arthropods and direct contact, with similar results (Acevedo et al., 2005; Diezma-Díaz et al., 2019a). Accordingly, host (age and inoculation route)- and parasite- dependent factors, such as parasite dose, are not key variables when inducing macroscopic clinical signs of chronic besnoitiosis after cattle inoculation with the tachyzoite stage.

Thus, the objective of this study was to investigate the outcome of the infection in cattle inoculated with a different invasive stage (bradyzoite stage) to that used in previous occasions. For this purpose, different routes of administration (intravenous, subcutaneous and intradermal routes) were assayed.

2. Materials and methods

2.1. Ethics statement

All experimental procedures were approved by the Animal Welfare Committee of the Community of Madrid (Spain) following proceedings described in Spanish and EU legislation (PROEX 92/14, Law 32/2007, R. D. 53/2013) and in Council Directive 2010/63/EU.

Animals were housed in clinical facilities belonging to the Faculty of Veterinary Sciences of the Complutense University of Madrid (Registration number: ES280790000101).

2.2. Animals, experimental design, clinical monitoring and sampling

The twelve 3-month-old calves used in this study came from a Holstein Friesian dairy herd located in Madrid Province; they were free from bovine besnoitiosis and bovine viral diarrhoea (BVD). Calves were selected after assessing the absence of specific antibodies against *B. besnoiti* and other closely related coccidian parasites (*Neospora caninum* and *Toxoplasma gondii*) (Diezma-Díaz et al., 2018). In addition, prior to inoculation, a vaccination protocol against the causative agents of bovine respiratory disease (bovine parainfluenza, bovine viral diarrhoea, infectious bovine rhinotracheitis and bovine respiratory syncytial virus) and enterotoxaemia (*Clostridium perfringens* types A, B, C and D, C. *chauvoei*, C. *novyi*, C. *septicum*, C. *tetani*, C. *sordellii*, and C. *haemolyticum*) was performed to guarantee the health status of the animals. Upon arrival, animals were randomly allocated into four different groups (G1, G2, G3 and G4) composed of three calves each, and a quarantine-adaptation period began (Diezma-Díaz et al., 2018).

The inoculum consisted of 10^6 bradyzoites (see section 2.3) diluted in 2 ml PBS administered through three different inoculation routes: intravenous inoculation by single jugular venipuncture (G1), subcutaneous inoculation in the left prescapular area (G2) and intradermal inoculation in the thigh area (G3), with the dosage subdivided into ten inoculation points, five of them on each side of the animal and administered with an intradermal Dermojet HR syringe (Akra DermoJet®, Pau, France). Moreover, a non-infected control group was inoculated with PBS intravenously (G4).

The experimental design, clinical monitoring, samplings (Figure 1A) and clinical score for the acute (from 1 to 42 days post-infection) and chronic phase (from 43 to 90 days post-infection) (Table 1) were established in the previous trials (Diezma-Díaz et al., 2018; Diezma-Díaz et al., 2019a). Fever, lymphadenopathy, cough, nasal and ocular secretion, congestive ocular conjunctive, as well as general oedema, were monitored during the acute phase, along with ocular conjunctival cysts, orchitis and skin lesions during the chronic phase. Blood samples to evaluate immune responses 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 the assay. Five millilitres of peripheral blood in Vacutainer tubes (Becton Dickinson and Company, Plymouth, UK), with and without anticoagulant, were obtained by jugular venipuncture. Vacutainers without anticoagulant were centrifuged (1,200 x *g* for 1

min) to obtain serum samples, and vacutainers with ethylenediaminetetraacetic acid (EDTA) and with lithium heparin were processed to obtain peripheral blood mononuclear cells (PBMCS) and to proceed with peripheral blood stimulation, respectively. Once a week, skin biopsies from each animal were collected from the inner part of the thigh using 6-mm-diameter dermal biopsy punch (Spengler, Issoudun, France) to perform PCR. A criterion for the humanitarian slaughtering of the animals consisting of one day of total fasting and prostration was established (Diezma-Díaz et al., 2018). At 90 days pi, calves were sedated with xylazine hydrochloride (Xilagesic 2%®, Calier, Barcelona, Spain) and immediately euthanized by an intravenously overdose of embutramide and mebezonium iodide (T61®, Intervet, Salamanca, Spain). Post-mortem examination of the calves was carried out immediately after euthanasia. Tissue samples for histological and molecular studies were collected (see Table 2).

2.3. Inoculum preparation

Bradyzoites were obtained from a bull with chronic besnoitiosis. The animal was *B. besnoiti*-seropositive (García-Lunar et al., 2017) and showed characteristic clinical signs of the disease, including hyperkeratosis in the neck and scrotum, alopecia in the carpal and tarsal areas, testicular atrophy and tissue cysts in the ocular conjunctiva. This animal was seronegative for *N. caninum* and *T. gondii* as well as BVD and infectious bovine rhinotracheitis (IBR). The bull was sacrificed in the slaughter house and tissue cysts were also found in the upper respiratory tract. Then, tracheal rings were collected and maintained in phosphate buffer solution (PBS) with 2% penicillin-streptomycin-amphotericin B (Lonza, Walkersville, MD, USA). Afterwards, tracheal mucosa was separated, crosscut and grounded in a Potter-Elvehjem tissue grinder. The mass obtained was homogenized and centrifuged at 1,350 x *g* for 15 min at 4ºC. Released bradyzoites were resuspended in PBS with antibiotics (2%) until inoculation (Fernández-García et al., 2009b; Frey et al., 2013). The absence of bacteria and *Mycoplasma* spp. in the inoculum were checked by culturing an aliquot in blood agar and by using a specific PCR technique, respectively (Venor™ GeM Mycoplasma Detection Kit; Minerva Biolabs, Berlin, Germany). Parasite viability was determined as described by Diezma Díaz et al. (2018), and the bradyzoites were inoculated in cell culture to obtain a new *B. besnoiti* isolate named as Bb-Spain 4. Finally, the absence of BVDV and *Mycoplasma* spp. infections were monitored by qPCR once the new isolate was obtained (Diezma-Díaz et al., 2017). Both Bb-Spain 4 bradyzoites and tachyzoites were genotyped using microsatellite markers (see section 2.9).

2.4. *Besnoitia besnoiti* cell culture and antigen purification

Tachyzoites of *B. besnoiti* (Bb-Spain 1 isolate) (Fernández-García et al., 2009b) were grown in a MARC-145 cell monolayer and maintained with Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Madrid, Spain) supplemented with 5% foetal calf serum (Gibco, Paisley, UK) and 1% penicillin-streptomycin-amphotericin B (Lonza, Walkersville, MD, USA). After several passages, the cells were scraped and the tachyzoites were purified in cold sterile PBS at a pH 7.2 using disposable PD-10 desalting columns (Sephadex G-25; GE Healthcare, Illinois, USA). Pellets were obtained by centrifugation at 1,350 x g for 10 min at 4ºC and stored in vials at -80ºC until use (Fernández-García et al., 2009b).

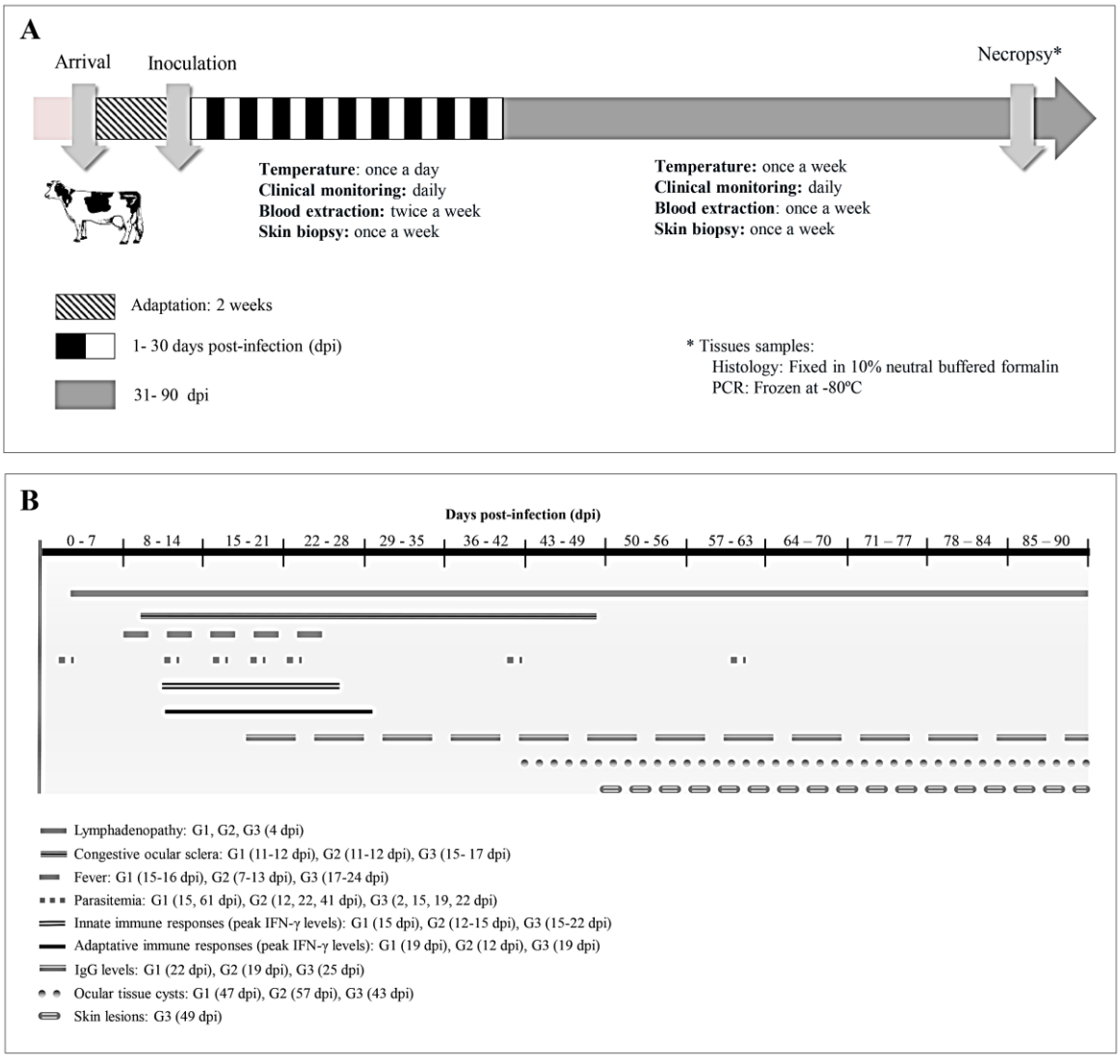
2.5. Innate IFN-γ responses

IFN-γ levels were measured in serum samples from 5 days prior to infection (-5 days pi) until the end of the collection period, with a Bovine IFN-γ ELISA development kit (Mabtech AB, Sweden) according to the manufacturer´s instructions (Arranz-Solís et al., 2016).

2.6. *Besnoitia besnoiti* specific (adaptive) IFN-γ responses

Protein soluble extracts of *B. besnoiti* were obtained as follows for PBMC stimulation: Bb-Spain 1 tachyzoites were maintained *in vitro* as previously described in section 2.4. Purified tachyzoites (2×10^9) were suspended in 1 ml 10 mM Tris–HCl containing 2 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, Madrid, Spain), disrupted by ultrasound treatment (Branson Sonifier 450, Branson Ultrasonic Co., Danbury, USA) in an ice bath and then centrifuged at 10,000 x g for 20 min at 4ºC. The protein content of the supernatant was determined using Quick Start Bradford Protein Assay (Bio-Rad ®, California, USA) and then aliquoted and cryopreserved at −80 °C until use as soluble extracts for PBMC stimulation.

Figure 1: A: Timeline, clinical monitoring and sample collection in experimentally infected calves. **B:** Sequence of post-inoculation events. Clinical and laboratory parameter results.



The PBMCs stimulation protocol was described by Sánchez-Sánchez et al. (2018). Samples were obtained twice a week during the first two weeks of the experiment and once a week thereafter. IFN-γ levels were measured in supernatants by the Bovine IFN-γ ELISA development kit (Mabtech AB, Nacka Strand, Sweden), as previously described (section 2.5.).

2.7. Serological analysis: IgG responses

Besnoitia besnoiti-specific IgGs were determined by ELISA using lyophilized tachyzoites of *B. besnoiti* as antigen (García-Lunar et al., 2017). A RIPC value ≥17.34 indicates a positive result. IgG1/IgG2 ELISA were essentially carried out as described by Diezma-Díaz et al. (2018).

2.8. Histopathology

Tissue samples and biopsies were fixed in 10% neutral buffered formalin. After seven days of fixation, the tissue samples were dehydrated using a graded series of alcohol and were embedded in paraffin used an automatic tissue processor (TP1020, Leica Microsystems, Wetzlar, Germany). Tissue sections of 4-7 μm were cut from each sample with a motorized rotary microtome (RM2255, Leica Microsystems, Wetzlar, Germany) and stained with haematoxylin and eosin (H/E) and a linear staining system (4040, Leica Microsystems, Wetzlar, Germany) for histopathological evaluation. Samples were observed with an optical microscope (BX50, Olympus, Tokyo, Japan). Photographs were taken with a digital camera (DP27, Olympus, Tokyo, Japan) and were analysed with CellsSens Entry 1.15 imaging software (Olympus, Tokyo, Japan).

2.9. DNA extraction/ PCR determinations and microsatellite (MS) genotyping

Genomic DNA was extracted from the PBMCs, skin biopsies, tissue samples collected during necropsy and samples used for genotyping following a previously described procedure (Diezma-Díaz et al., 2017). ITS1-PCR and quantitative PCR (qPCR) assay for the detection of *Besnoitia* spp. DNA from the ungulates were performed according to Frey et al. (2016).

Microsatellite (MS) genotyping was performed as described by Gutiérrez-Expósito et al. (2016). The six MS markers evaluated (Bt-5, Bt-6, Bt-9 and Bt-7, Bt-20, and Bt-21) were used to genotype bradyzoites and tachyzoites from Bb-Spain 4. Moreover, tachyzoites and bradyzoites from Bb-Spain 3, the isolate inoculated in the previous experimental infections in calves (Diezma-Díaz et al., 2018; Diezma-Díaz et al., 2019a) were also genotyped (Gutiérrez-Expósito et al., 2016; Diezma-Díaz et al., 2017). The Bb-Spain 1 isolate was employed as the control. All samples were analysed by fragment analysis and sequenced to confirm the number of repeat motifs (Gutiérrez-Expósito et al., 2016).

2.10. Statistical analysis

Rectal temperatures, numbers of tissue cysts in the scleral conjunctiva and variations in IFN-γ levels and IgG/IgG1-IgG2 were analysed by repeated measures two-way ANOVA tests and Bonferroni post hoc tests. Differences among clinical scores, average diameters and parasite burdens from samples belonging to the same system (reproductive, respiratory, lymphatic, skin and other organs) between infected groups were assessed by non-parametric Kruskal-Wallis tests followed by Dunn's multiple range tests for all pairwise comparisons. Statistical significance for all analysis was established with $P < 0.05$. Finally, a chi-square test was employed to address possible differences among the positive samples by

histology and PCR between groups and systems. All statistical analyses were carried out using GraphPad Prism 6.01 software (San Diego, CA, USA).

3. Results

3.1. Microsatellite analysis

Genotyping results are shown in Supplementary Table 1. MS analysis of the tachyzoite and bradyzoite stages from the Bb-Spain 3 and Bb-Spain 4 isolates and tachyzoites from Bb-Spain1 showed identical genotypes (Gutiérrez-Expósito et al., 2016).

3.2. Clinical signs and clinical score

All animals from G1, G2 and G3 developed lymphadenopathy approximately four days pi; this occurrence was detected in the prescapular and the precrural lymph nodes. The size decreased after one month pi. However, lymphadenopathy extended to the end of the whole experiment in all infected animals (Figure 1B).

A significant increase was observed in the mean temperatures from the different inoculated groups (see Figure 2A) (two-way ANOVA test, $P < 0.001$). The onset of fever (rectal temperature ≥39.5ºC) was as follows: i) the intravenously inoculated calves (G1) developed a sporadic febrile response (one day) at approximately the second week pi; ii) the subcutaneously inoculated calves (G2) developed fever from the first week pi until the end of second week pi (seven days), significant differences were observed between eight to 11 days pi compared to the G1 and G3 ($P < 0.001$, Bonferroni post-test); and iii) the intradermally infected group (G3) showed fever from 17 days pi and it remained high until 24 days pi (eight days) ($P < 0.001$, Bonferroni post-test). The highest temperature values were observed in one animal from G2 (Calf 6; C6) at 11 and 13 days pi (41 ºC and 40.6ºC, respectively). Apart from this animal, only calves from the G3 developed temperatures above 40ºC. Fever was not detected in the control group (G4) throughout the experiment.

Congestive ocular sclera were observed in all infected animals (Figure 1B and 2B) from 11-12 days pi in G1 and G2 and later in G3 at 15-17 days pi until seven weeks pi in all infected calves.

Clinical scores recorded for the acute stage of the disease are shown in Table 1A. Group 1 showed significantly higher scores from 15 to 18 days pi and from 26 to 28 days pi compared with the uninfected group (G4) ($P < 0.05$; Kruskal-Wallis test, Dunn's post-test). Clinical scores in calves from G2 and G3 showed significant differences when compared with G4 at five days pi onwards until 14 days pi and from 19 to 28 days pi, respectively ($P < 0.05$). Lymphadenopathy was less pronounced in G1, but statistically significant differences between infected groups were not observed.

We considered that the chronic stage of the disease started on the first day that pathognomonic tissue cysts were observed in the scleral conjunctivae (Bigalke, 1968) at 43 days pi in one animal from G3 (C9) (Figure 1B). Four days later, visible conjunctival cysts were also observed in G3C7 and G1C1. At 54 days pi, two calves from G1 (C2, C3) and G3C8 also showed ocular tissue cysts, and three days later, all calves from G2 had also developed scleral cysts. G3C9 and G3C7 showed the highest number of tissue cysts (category 3), followed by G1C1 and G3C8 (category 2), whereas the remaining two calves from G1 developed a few tissue cysts (category 1). Regarding G2, a few conjunctival cysts were detected in all animals (category 1). Generally, all three infected groups showed a significant increase in the number of

tissue cysts approximately 60 days pi (Figure 2C). The number of them remained constant in G1 and G2 until the end of the experiment. However, G3 displayed a second significant increase from 81 days until 90 days pi (two-way ANOVA test, $P < 0.001$). G3 showed significant differences compared with G1 (from 81 days pi onwards) and G2 (from 74 days pi onwards) ($P < 0.01$, Bonferroni post-test).

Other clinical signs characteristic of the chronic stage, such as skin lesions, were not observed apart from a few areas of alopecia developed by G3C9 in the hind legs and the muzzle (Figure 2D). These lesions were detected from six and seven weeks pi, respectively, until the end of the experiment.

Clinical scores recorded for the chronic stage of the disease are shown in Table 2B. All infected groups showed significant differences with respect to the uninfected group (G4) ($P < 0.01$) and showed similar clinical scores until 57-60 days pi. Afterwards, the scores increased and reached the highest category in G3 ($P < 0.05$), mainly influenced by the increase of the number of scleral tissue cysts.

3.3. IFN-γ responses

3.3.1. Innate immune responses

In all infected groups the IFN-γ levels were significantly higher than in the uninfected group from 12 days pi until 15 days in G2; 20 days in G1 and 22 days pi in G3 ($P < 0.05$; two-way ANOVA). IFN-γ levels peaked twice in G1, first at two days pi (statistically significant compared with the control group; $P < 0.05$) and again at 15 days pi ($P < 0.01$). Group 2 presented the highest levels of IFN-γ from 12 ($P < 0.001$) to 15 days pi ($P < 0.05$; two-way ANOVA). Finally, G3 showed higher IFN-γ levels from 15 to 22 days pi, with a maximum at 19 days pi ($P < 0.001$). These levels decreased to basal values in all three infected groups from day 25 pi and remained low thereafter (Figure 4A).

3.3.2. Adaptive immune responses

When the PBMCs were stimulated with a *B. besnoiti*-soluble extract, a significant increase of IFN-γ levels in the culture's supernatants was observed in G1 and G3 at 19 days pi and in G2 at 12 days pi ($P < 0.001$; two-way ANOVA test). The kinetics of IFN-γ levels within each group also showed that maximum IFN-γ levels were reached at 19 days pi for all infected groups. However, the levels reached by G3 were significantly lower compared to G1 and G2 ($P < 0.01$; Bonferroni post-test). In addition, average levels of IFN-γ were significantly higher in G1 and G2 compared to G3 at 26 days pi, and in G1 compared to G3 at 33 days pi ($P < 0.01$; Bonferroni post-test). At 33 days pi, the levels started to decrease and remained higher in G1 and G2 compared to G3 (Figure 4C). IFN-γ levels recorded prior to inoculation did not vary throughout the experimental study in G4.

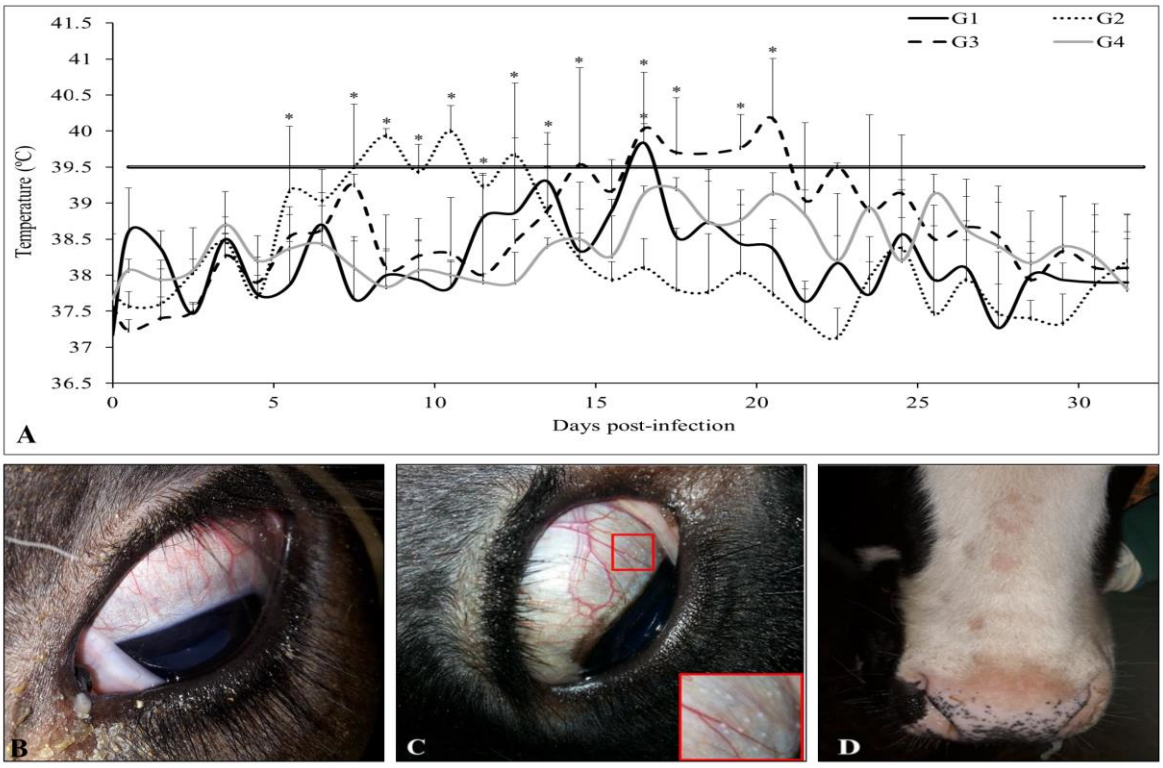
Table 1: Clinical score; A: established for the acute stage of the infection (1 to 42 days pi); **B**: established for the chronic stage of the infection (43 to 90 days pi).

A	Days post-infection											
	1-4	5-7	8-11	12-14	15-18	19-21	22-25	26-28	29-32	33-35	36-39	40-42
G1C1	0	1	1	2	2	2	2	2	2	2	2	2
G1C2	0	1	1	1	2	1	1	2	1	1	1	1
G1C3	0	1	1	1	2	2	2	2	1	2	2	2
G2C4	1	2	2	2	1	1	2	2	2	1	1	1
G2C5	1	2	2	2	2	2	2	2	1	1	1	2
G2C6	1	2	2	2	2	2	1	1	1	1	1	2
G3C7	0	1	1	1	1	2	2	2	1	1	1	2
G3C8	1	2	2	2	2	2	2	2	2	2	2	1
G3C9	1	1	1	1	2	2	2	2	2	2	2	2
G4C10	0	0	0	0	0	0	0	0	0	0	0	0
G4C11	0	0	0	0	0	0	0	0	0	0	0	0
G4C12	0	0	0	0	0	0	0	0	0	0	0	0

B	Days post-infection												
	43-46	47-49	50-53	54-56	57-60	61-63	64-67	68-70	71-73	74-76	77-80	81-83	84-90
G1C1	1	1	1	1	2	2	2	2	2	3	2	2	2
G1C2	1	1	1	1	1	1	1	1	1	1	1	1	1
G1C3	1	1	1	1	1	1	1	1	1	2	2	1	1
G2C4	1	1	1	1	1	2	2	1	1	1	1	2	2
G2C5	1	1	1	1	1	2	2	2	2	2	2	1	1
G2C6	1	1	1	1	1	1	1	1	1	1	1	1	1
G3C7	1	1	1	1	1	3	3	3	3	3	3	3	3
G3C8	1	1	1	2	2	2	2	2	2	2	2	2	2
G3C9	1	1	2	2	2	3	3	3	3	3	3	3	3
G4C10	0	0	0	0	0	0	0	0	0	0	0	0	0
G4C11	0	0	0	0	0	0	0	0	0	0	0	0	0
G4C12	0	0	0	0	0	0	0	0	0	0	0	0	0

G: Group; **C:** Calf.
Clinical score: (Diezma-Díaz et al., 2018).
Acute stage: 0, None (absence of infection); **1**, Mild (local lymphadenopathy); **2**, Moderate (fever, systemic lymphadenopathy, cough/nasal secretion and/or congestive conjunctiva)
Chronic stage: 0, None; **1**; Mild (systemic lymphadenopathy and/or congestive conjunctiva and/or conjunctival cysts of category 1 and/or ocular secretion); **2**, Moderate (systemic lymphadenopathy and conjunctival cysts of category 2 and/or ocular secretion); **3**, Severe (systemic lymphadenopathy and conjunctival cysts of category 3 and/or ocular secretion and/or skin lesión).

Figure 2: Clinical signs developed during acute (A, B) and chronic besnoitiosis (C, D). **A:** Mean rectal temperatures (+SD) recorded during the first month post-infection. Fever was established with values ≥39.5° C. **B:** Congestive ocular sclera and ocular discharge. **C:** *B. besnoiti* tissue cysts developed in conjunctiva (G3C9). **D:** Alopecia in the muzzle (G3C9).



3.4. Humoral immune responses (IgG levels)

B. besnoiti-specific antibody responses throughout the experimental study are shown in Figure 3C. The first group that seroconverted was G2 at 19 days pi, followed by G1 at 22 days pi and G3 at 25 days pi. Differences among all infected groups and the uninfected group appeared from 19 days pi onwards ($P < 0.01$). Antibody levels significantly increased, reaching a maximum at 61 to 68 days pi ($P < 0.001$; two-way ANOVA test).

Non-significant differences were observed between infected groups. However, G3 showed an increase in IgG response at the end of the experiment (87 days pi). In contrast, antibody levels decreased in G1 and G2 ($P < 0.05$, Bonferroni post-test).

Higher IgG1 levels were observed in the infected groups compared to G4 from 19 days pi for G2/G3 ($P < 0.05$; two-way ANOVA) and from 22 days pi for G1 ($P < 0.01$) (Figure 4D). At 33 days pi, significant lower IgG1 levels were found in G2 with respect to G1 and G3, whereas G3 showed higher IgG1 levels than G1 and G2 ($P < 0.05$). Non-significant differences were found among infected groups until 82-90 days pi, when IgG1 levels were significantly higher in G1 than in G2 and G3 ($P < 0.05$). IgG2 antibody levels were higher in all infected groups than in non-infected animals from 33 days pi onwards (G1: $P < 0.01$; G2-G3: $P < 0.05$; two-way ANOVA) (Figure 4E). No significant differences were observed in the IgG2 levels between infected groups, except for 90 days pi when the IgG2 levels were significantly higher in G1 than in G2 ($P < 0.05$).

Figure 3: Macroscopic lesions detected at necropsy (G3C9): Tissue cysts in subcutaneous tissue **(A)** and nasal turbinate **(B)**. *Besnoitia besnoiti* tissue cysts by histology (haematoxylin and eosin). Viable tissue cyst surrounded by pericystic inflammatory infiltrate from skin sample **(C)** and from vas deferens **(D)** (G3C9) (magnification, 10x).

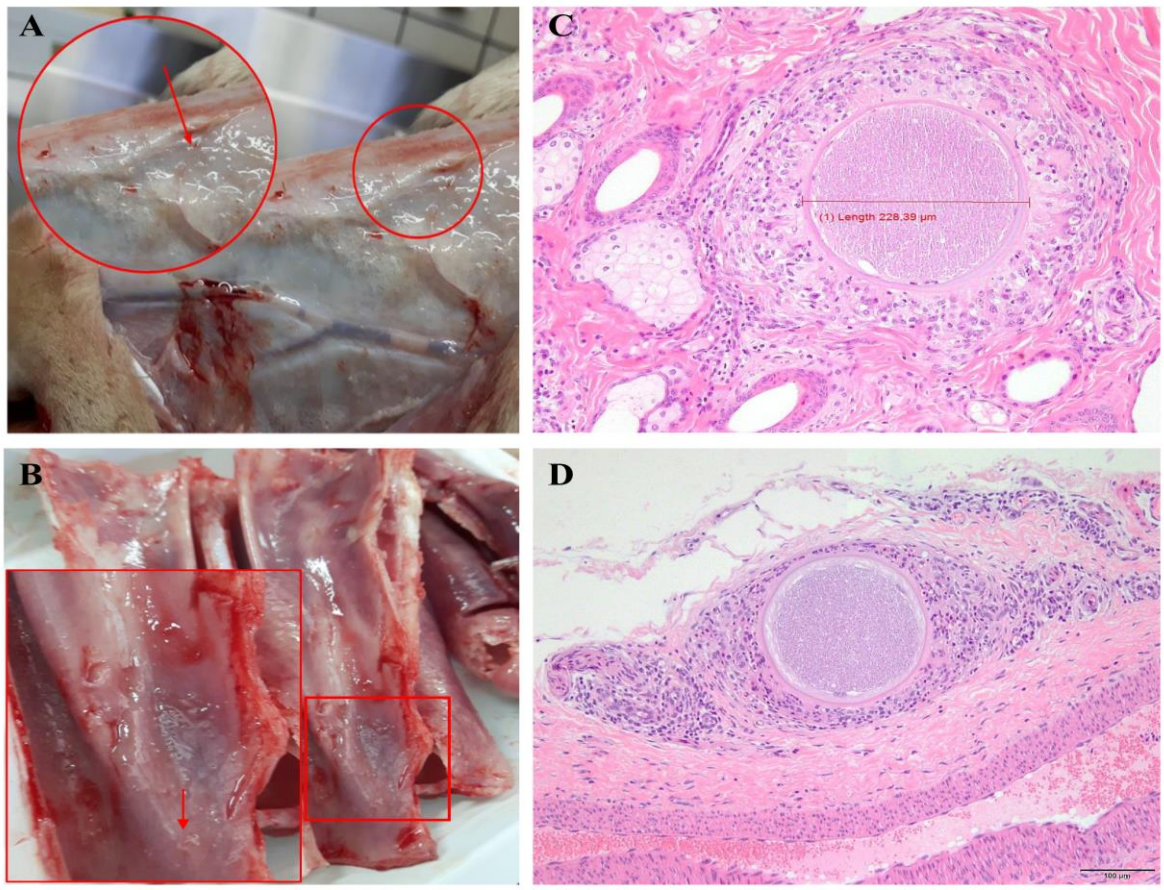
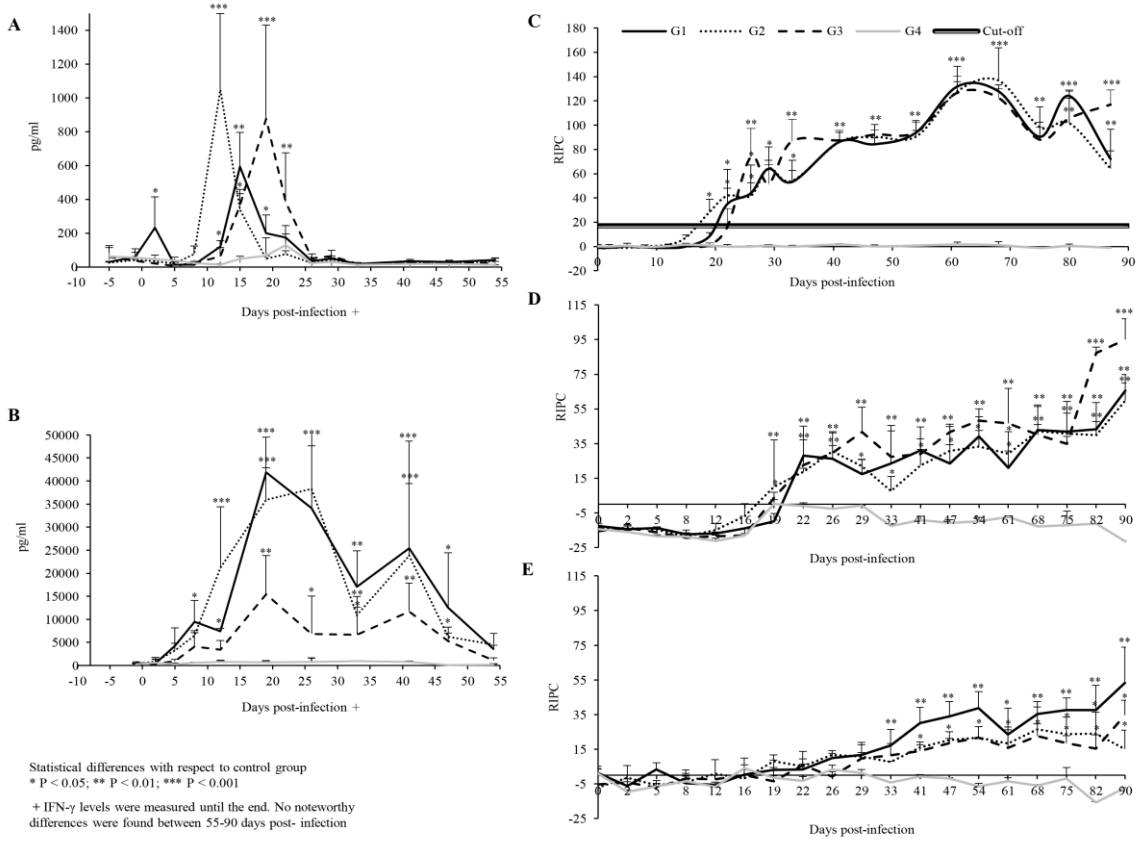


Figure 4: Cellular **(A, B, E)** and humoral **(C, D)** immune responses developed by inoculated calves. **A:** innate IFN-γ responses measured in serum samples; **B:** *B. besnoiti* –specific IFN-γ response measured after stimulation of PBMCs; **C:** *B. besnoiti* –specific IgG responses; **D:** Mean Relative Index (+SD) IgG1 antibody levels; **E:** Mean Relative Index (+SD) IgG2 antibody levels.



3.5. Macroscopic lesions

At the time of necropsy, ascites was present in one calf from G1 (C1) and two animals from G3 (C7 and C9). Macroscopic cysts in ocular conjunctiva were visible in all infected animals and were more numerous in calves from G3. One animal from G3 (C9) also showed skin lesions in facial and carpal areas, multifocal cysts in subcutaneous tissue and respiratory mucous membranes, mainly, in the nasal turbinates and tracheal mucosa (Figure 3A and 3B).

3.6. Detection of tissue cysts

As shown in Table 2, *B. besnoiti* tissue cysts were detected by histology in 49 samples. Most positive samples belonged to animals from G3 (n=41), followed by G1 (n=7) and finally by G2 (n=1). The number of positive skin samples (40.8% of 49 positive samples) was statistically higher than for respiratory (26.5%) and reproductive (18.36%) tract samples, as well as for other organs and lymphatic system (14.3%) ($P < 0.01$, chi-square test). Regarding the tissue cyst size, the average diameter was 181.2 µm (± 60.4 µm) (Figure 3D), and differences between tissue samples and infected groups were not significant.

3.7. Detection of *B. besnoiti* DNA in blood and tissues by ITS1-PCR/qPCR

Parasite DNA was not detected in the skin biopsies. Parasitaemia was detected sporadically in calves of G1 at 15 and 61 days pi (n=1); G2 at 12, 22 and 41 days (n=1); and G3 at 2 (n=1), 15 (n=1), 19 (n=3), and 22 (n=2) days pi.

A total of 624 tissues samples collected at necropsy were analysed by ITS1-PCR. Four hundred sixty-eight samples belonged to infected animals and 156 to control animals, which were PCR negative. As shown in Table 2, *Besnoitia* DNA was detected in 82 samples (17.5% of 468). Statistically, the calves from G3 showed the highest number of PCR-positive tissues (n= 57; 69.5%) compared to G1 (n= 13; 15.8%) and G2 (n= 12; 14.6%) ($P < 0.01$). In G3, 40 positive samples out of 57 positives (70.2%) came from one calf, C9, which had also developed skin lesions (Table 2, Supplementary Table 2). Parasite DNA was mostly detected in the respiratory (35.4%) and reproductive (30.5%) tracts, followed by other organs (15.8%), mainly the ocular sclera, tendon and hoof corium as well as skin (14.6%) and lymphatic system (3.7%). Moreover, parasite DNA was detected in samples where no tissue cysts were found by histological examination, for instance, in brain samples (Table 2).

The amount of parasite/ng bovine DNA in the ITS1-PCR-positive samples are shown in Supplementary Table 2. Significant differences were observed among the infected groups. The parasite load in G3 was higher than in calves inoculated by intravenous (G1) or subcutaneous (G2) routes ($P < 0.0001$; Kruskal-Wallis, Test Dunn). Moreover, samples from the upper respiratory tract and skin from G3 showed the highest parasite load with respect to the other systems ($P < 0.001$).

4. Discussion

We have successfully developed a model for chronic bovine besnoitiosis in calves, in which all infected animals developed macroscopic tissue cysts. This is the first time that bradyzoites have been inoculated by three different routes (intravenous, subcutaneous and intradermal routes) in cattle under the same experimental conditions. Remarkably, more severe clinical signs were detected in calves inoculated by the intradermal route.

Two recent experimental infections carried out in calves formed the basis for the present work, as identical experimental designs were followed. When tachyzoites were inoculated by intravenous (10^6, 10^7 and 10^8 tachyzoites) (Diezma-Díaz et al., 2018), subcutaneous and intradermal routes (10^6 tachyzoites) (Diezma-Díaz et al., 2019a), mild to moderate acute besnoitiosis was evident, followed by the absence of the clinical signs and macroscopic lesions characteristic of the chronic phase and scarce intra-organic distribution of the parasite. The absence of the clinical signs that are characteristic of chronic besnoitiosis hampers the employment of these experimental models based on the inoculation of the tachyzoite stage, regardless of the inoculation route in future drug efficacy trials. Accordingly, the other parasite stage described in the intermediate host, the bradyzoite, was inoculated in the present work. Prior to the experimental inoculations, good health status of the calves and appropriate quality controls of the inoculum were monitored, in contrast to the only two previous experimental infections carried out in the past with this parasite stage in immunologically depressed animals (Bigalke, 1968; Diesing et al., 1988). Moreover, experimental designs followed in the past differed notably in the number of animals, inoculation routes and immunosuppression therapies employed. We again studied three possible routes of inoculation (intravenous, subcutaneous and intradermal route), which could happen in nature since parasite can be transmitted by blood sucking arthropods or through lacerations or wounds during direct contact between infected and non-infected cattle (Álvarez-García et al., 2013).

Table 2: Parasite intra-organic distribution by PCR and histopathology (HP).

Tissue [A]	Group 1		2		3	
	PCR [a]	HP [a]	PCR [a]	HP [a]	PCR [a]	HP [a]
Reproductive System						
[L] Testicle	-	-	-	-	2/3	-
[R] Testicle	-	-	-	-	2/3	1/3
[L] Head Epididymis	1/3	-	-	-	2/3	-
[R] Head Epididymis	1/3	-	-	-	1/3	1/3
[L] Body Epididymis	-	-	-	-	1/3	-
[R] Body Epididymis	-	-	-	-	1/3	-
[L] Tail Epididymis	1/3	-	-	-	2/3	-
[R] Tail Epididymis	2/3	-	-	-	1/3	1/3
[L] Prox vas def.*	-	-	-	-	2/3	3/3
[R] Prox vas def.*	-	-	-	-	1/3	1/3
[L] Dist vas def +Pp**	-	1/3	-	-	1/3	-
[R] Dist vas def+ Pp**	1/3	1/3	-	-	-	-
Bulbourethral gland	-	-	-	-	1/3	-
Seminal vesicles	-	-	-	-	1/3	-
Penis	-	-	-	-	1/3	-
Respiratory System						
[L] Nostril	-	-	1/3	-	2/3	1/3
[R] Nostril	-	-	1/3	-	1/3	2/3
Tongue	1/3	-	1/3	-	-	-
Nasal turbinates	2/3	-	3/3	-	-	2/3
Epiglottis	2/3	-	1/3	-	2/3	1/3
Pharynx	-	-	1/3	-	1/3	-
Larynx	1/3	2/3	-	-	3/3	3/3
Trachea	-	-	-	-	1/3	2/3
[L] Lung	-	-	-	-	1/3	-
[R] Lung	-	-	-	-	1/3	-
Lymphatic System						
Submandibular (LN)	-	-	-	-	1/3	-
[L] Cervical (LN)	-	-	-	-	1/3	1/3
[L] Prescapular (LN)	-	-	-	-	1/3	-
Skin						
Facial	-	-	-	-	1/3	1/3
[L] Neck	-	-	-	-	1/3	2/3
[L] Upper-eyelid	-	-	-	-	1/3	3/3
[L] Ear pinna	-	-	-	-	1/3	-
[L] Carpal	-	1/3	1/3	-	1/3	2/3
[L] Tarsal	-	-	-	-	1/3	3/3
[L] Elbow	-	-	1/3	1/3	1/3	2/3
[L] Thigh	-	-	-	-	1/3	2/3
[L] Perineal	-	-	-	-	1/3	-
Scrotum	-	1/3	-	-	1/3	2/3
Other Organs						
[L] Ocular Conjunctiva	-	-	-	-	1/3	-
[L] Ocular Sclera	-	-	-	-	1/3	1/3
Brain	1/3	-	-	-	3/3	-
Cerebellum	-	-	-	-	1/3	-
[R] Tendon+fascia	-	1/3	2/3	-	2/3	2/3
[R] Hoof Corium	-	-	-	-	1/3	2/3

* Proximal vas deferens
** Distal vas deferens+ pampiniform plexus
[L] Left / [R] Right
(LN): Lymph node
(A) Parasite was not detected either by HP or PCR in bronchus, thymus, spleen, [R] tonsils, bronchial (LN), medíastinum (LN), [L] ventriculum, [L] atrium from infected animals and in all tissues from control group
[a] Number (nº) of positive samples/ total nº of samples

The dose of 10^6 bradyzoites was selected according to previous studies done with tachyzoites to compare results obtained with the same parasite dose (Diezma-Díaz et al., 2018; 2019).

Herein, the acute phase of the infection was classified as mild to moderate, as no severe clinical signs such as oedema or orchitis were detected. The outcome of the acute infection was similar in all infected groups. Lymphadenopathy preceded fever

(Figure 1B), began at approximately four days pi and was maintained until the end of the trial. However, lymphadenopathy was only evident until the first month pi in tachyzoite-inoculated calves, which was probably associated with a lower stimulation of the immune system (Diezma-Díaz et al., 2018; 2019a). Lymphadenopathy was followed by fever, which is a clinical sign associated with Sarcocystidae parasite infections and first replication cycles of the parasite (Benavides et al., 2014). Remarkably, all infected animals showed a febrile response after a prolonged incubation period compared with previous works (Diezma-Díaz et al., 2018; 2019a). Fever appeared in the subcutaneous group at 7 days pi, in the intravenous group at 15 days and finally, in the intradermal group at 17 days pi. In contrast, calves inoculated with tachyzoites by the intravenous route showed a 12-hour incubation period (Diezma-Díaz et al., 2018). Indeed, parasitaemia was more frequently detected during the febrile stage (Bigalke, 1968; Diezma-Díaz et al., 2018) (Figure 1B). In the present study, parasitaemia preceded fever and was more frequently detected in G2 and G3, which could explain why the fever lasted for one week in G2 and G3 in contrast to calves inoculated with tachyzoites by the same inoculation routes, where fever was scarcely detected (Diezma-Díaz et al., 2018; 2019). Based on the present findings and previous assays, the acute phase of bovine besnoitiosis in experimental infections seems to be easily induced regardless of the variables studied (Bigalke, 1968; Basson et al., 1970; Janitschke et al., 1984; Shkap, 1986; Diesing et al., 1988). However, the parasite stage, route of inoculation and immunosuppressive treatments seem to influence on the severity and duration of the clinical signs (Diesing et al., 1988; Diezma-Díaz et al., 2018; 2019).

The chronic phase of the disease was successfully reproduced and varied from mild to moderate clinical scores in the intravenously and subcutaneously inoculated groups to a moderate to severe score in the intradermally inoculated group. Thus, the most successful host-parasite dependent variable combination in inducing the chronic stage of the disease was the bradyzoite stage inoculated by the intradermal route as with the acute stage of the disease. Previous attempts with inoculated tachyzoites failed to induce macroscopic lesions and microscopic tissue cysts were only detected in intravenously inoculated calves (Diezma-Díaz et al., 2018; 2019). Herein, the first macroscopic conjunctival tissue cysts were observed at 43 days pi (18 days post-seroconversion) in intradermal inoculated animals and, subsequently, at 49, 54 and 57 days pi in the remaining infected groups (Figure 1B). Moreover, only skin lesions were observed in one intradermally inoculated calf at approximately 49 days pi (24 days post-seroconversion). The chronobiology of the chronic phase agrees with the observations reported in natural infections during a cohabitation trial in adult cattle (Langenmayer et al., 2015c), where the first macroscopic parasitic cysts were detected in the sclera of infected animals between 19 and 23 days post-seroconversion. Next, skin lesions were found in the proximal part of the hind legs at approximately 25 days post-seroconversion in one cow, which showed clinical signs characteristic of the acute besnoitiosis. These skin alterations were clearly visible within 166 days after seroconversion (Gollnick et al., 2015).

Herein, parasite detection was correlated with the severity of the clinical signs observed. Accordingly, the highest number of samples with tissue cysts and the highest parasite burden corresponded to animals from G3 inoculated by the intradermal route, in particular, the calf with skin lesions developed the highest number of scleral

tissue cysts. Our findings agree with the observations made in natural infections, where the highest number of microscopic tissue cysts has been largely reported in chronically infected animals which showed severe clinical signs and lesions characteristic of chronic besnoitiosis (Pols, 1960; McCully et al., 1966). As expected, the parasite showed tropism for skin, eyes and mucous membranes of the upper respiratory and genital tracks, in agreement with previous reports in naturally (Nobel et al., 1981; Manuali et al., 2011; Gentile et al., 2012; Frey et al., 2013; Diezma-Díaz et al., 2017) and experimentally infected cattle (Diezma-Díaz et al., 2018; 2019a). The cyst morphology and size were normal and compatible with that described for mature viable cysts (Frey et al., 2013; Langenmayer et al., 2015b). The tissue cyst rupture may be observed from 30 days pi onwards (Basson et al., 1970) and could explain the sporadic parasitaemia observed at later stages of the disease (41 and 61 days pi) (Figure 1). Whether the presence of *Besnoitia* in the testes, which was detected in both tissue cysts and parasite DNA, could affect the reproductive function, as stated for naturally infected bulls, remains to be elucidated (Pols, 1960; Kumi-Diaka et al., 1981; Nieto-Rodríguez et al., 2016). However, the low parasite load detected is unlikely to cause sterility, as naturally infected breeding bulls with impaired reproductive function normally present a high number of mature tissue cysts (Kumi-Diaka et al., 1981; Esteban-Gil et al., 2016).

The immune response kinetics were similar in all infected groups and comparable to previous experimental infections in calves (Diezma-Díaz et al., 2018; 2019). After the calves were infected, an innate IFN-γ response was developed, followed by an adaptive IFN-γ response and seroconversion. The most evident differences were observed when we employed tachyzoites or bradyzoites as inoculum. A delay in the innate IFN-γ response was observed in bradyzoites with respect to tachyzoite inoculation (12-20 vs. 4-7 days pi), which could also be associated with a delay in the detection of specific antibody (17 vs. 19-25 days pi). Again, the antibody titres were similar between infected groups, except for calves inoculated by intradermal route, with an increase of antibody levels observed at the end of the trial, possibly due to tissue cysts rupture that may have favoured the re-exposure of parasite antigens to the immune system (Frey et al., 2013). Differences between inoculation routes were confirmed again (Diezma-Díaz et al., 2019a). The parasite exposure to the immune system by subcutaneous or intravenous routes led to an earlier innate IFN-γ response and higher adaptive IFN-γ response, which might have contributed to a better control of the infection compared to the intradermal inoculation. Cellular immune responses are thought to play a key role in the control of the infection regardless of the parasite stage inoculated (Álvarez-García et al., 2014b; Diezma-Díaz et al., 2018). Indeed, in previous experimental infections accomplished with bradyzoites, the best results were obtained under immunosuppressive therapies. Bigalke (1968) inoculated bradyzoites in two immunocompetent bulls by the intranasal route and reproduced the acute and chronic phases of the disease with scant detection of cysts. Later, Diesing et al. (1988) inoculated bradyzoites in seven animals by different routes. Five splenectomised animals either died or were euthanized. Two calves immunosuppressed by cortisone administration and inoculation by intraperitoneal route and by subcutaneous and intravenous route showed severe acute and chronic clinical signs. However, the immunosuppression therapies are not ideal for use in developing a reproducible experimental model of infection, since animals are then more vulnerable to other transmissible diseases.

Based on our results, the bradyzoite stage appears to be a principal key variable in determining the outcome of the chronic infection since all inoculated animals developed tissue cysts regardless of the inoculation route. In Sarcocystidae parasites, the bradyzoite differentiation is an immune evasion mechanism that allow the parasite to persist in the host for a long life (Hemphill and Gottstein, 2006; Jeffers et al., 2018). Indeed, *B. besnoiti* tachyzoites display different antigenic patterns and protein compositions compared to bradyzoites (Fernández-García et al., 2013; García-Lunar et al., 2013a). Moreover, in the closely related *N. caninum*, bradyzoite antigens are less immunogenic compared to tachyzoite antigens (Jiménez-Ruiz et al., 2013). Taking into account the chronobiology of acute besnoitiosis and the finding of proliferative zoites during the acute infection, the rapidly dividing tachyzoite state is assumed to be responsible for the acute phase (Álvarez-García et al., 2013). Consequently, in the present experimental model, bradyzoites should initially switch into tachyzoites that could explain the prolonged incubation period and the delay of the onset of fever compared to these aspects in previous experimental trials (Diezma-Díaz et al., 2018; 2019a). Reactivation of chronic besnoitiosis has been experimentally demonstrated in immunosuppressed hamsters infected with *Besnoitia jellisoni* (Frenkel and Wilson, 1972). Moreover, in *T. gondii*, an immunosuppressive environment (e.g., lack of IFN-γ) eases the bradyzoite to tachyzoite differentiation (Lyons et al., 2002), which agrees with the lowest IFN-γ response and the most frequent parasitaemia and severe clinical signs observed in this study.

The inoculation route could also be important to facilitate the establishment of a chronic infection since a higher intra-organic dissemination of the parasite was detected in calves inoculated by the intradermal route. The increase in the severity by this route is probably attributed to natural route of transmission, as also happens in others bovine pathogens such as bluetongue (Umeshappa et al., 2011). The differences observed among the different inoculation routes could be related to the different effector mechanisms of the host early innate immune response that the bradyzoites would initially face and to the vascularization that facilitates that parasite dissemination and induces an early immune response. Thus, a more rapid and efficient immune response is expected to occur against bradyzoites inoculated by the intravenous route, followed by the subcutaneous route and intradermal route in agreement with our results. Extracellular parasites in blood face a full-blown systemic response. As reported by Rojo-Montejo et al. (2012) in *N. caninum*, while many parasites remain extracellular, these may elicit an enhanced humoral immune response, unlike that associated with a higher cellular internalization of the parasite. Although parasites in subcutaneous tissue are rapidly absorbed through blood vessels, they might be driven by macrophages to draining lymph nodes to be processed by T-cells (Hunsaker and Perino, 2001). Finally, bradyzoites in the dermis are first exposed to a complex immune cell population in the skin, to smaller calibre blood vessels that may make parasite dissemination difficult, with the parasites finally being processed in the draining lymph node facilitated by the enrichment of dendritic cells (Romani et al., 2010; Kabashima et al., 2019). In addition, dendritic cells are major effector innate cells in the dermis that could display a 'Trojan horse' mechanism to facilitate parasite dissemination since is a conserved strategy among Toxoplasmatinae parasites (Lambert et al., 2006; Collantes-Fernández et al., 2012).

Two critical issues arise concerning the employment of bradyzoites as innocula that may limit the reproducibility of this experimental model: i) health status of the donor animals and ii) isolate variability since *N. caninum* (Regidor-Cerrillo et al., 2010; 2011) and *T. gondii* (Verma et al., 2015) isolates display clear differences in virulence. Since bradyzoites cannot be maintained *in vitro*, they must be isolated from infected animals under sterile conditions and immediately inoculated. Thus, the health status of the donor animals is crucial since common pathogens such as BVD is widespread in cattle and could contaminate the inocula. While the health status of the donor is crucial, isolate variability appears to be of minor importance based on the low parasite intraspecific variability reported in *B. besnoiti*. Microsatellite genotyping performed in this work (Supplementary Table 1) is in agreement with previous studies (Gutiérrez-Expósito et al., 2016; Diezma-Díaz et al., 2017; 2018), thereby proving a genetic homogeneity of all *B. besnoiti* isolates.

In conclusion, we have developed a model for bovine besnoitiosis where the chronic stage of the disease was successfully reproduced. The parasite stage (bradyzoite) and the inoculation route (intradermal) were crucial parasite- and host-dependent variables, respectively, that influenced the outcome of the infection. In future research, tachyzoite-bradyzoite switching in *in vitro* models should be investigated to obtain a parasite inoculum that could facilitate a reproducible experimental model.

Acknowledgements

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Appendix A

Supplementary Table 1: Genotyping of Bb-Spain 3 and Bb-Spain 4 isolates (tachyzoites/ bradyzoites) according to the number of repeat motifs in 6 microsatellite loci.

Samples		Specie	Country	Microsatellite Genotype[a]						Reference
				MS- Bt-5	MS- Bt-6	MS- Bt-7	MS- Bt-9	MS- Bt-20	MS- Bt-21	
Tachyzoites propagated in cell culture	Bb-Spain 1*	Cattle	Spain	10	12	8	10	8	13	Gutiérrez- Expósito et al 2016
	Bb-Spain 3	Cattle	Spain	10	12	8	10	8	13	This study
	Bb-Spain 4	Cattle	Spain	10	12	8	10	8	13	This study
Bradyzoites	Bb-Spain 3	Cattle	Spain	10	12	8	10	8	13	Gutiérrez- Expósito et al 2016
	Bb-Spain 4	Cattle	Spain	10	12	8	10	8	13	This study

[a] Microsatellite alleles analysed by sequencing ; * Bb-Spain 1 was employed as control

Supplementary Table 2: Amount of parasite/ng bovine DNA by quantitative real-time PCR detailed by calf.

Group	1			2			3		
Calf	1	2	3	4	5	6	7	8	9
Tissue [A]									
Reproductive									
[L] Testicle	-	-	-	-	-	-	41.5	-	822.3
[R] Testicle	-	-	-	-	-	-	ND	-	1908.2
[L] Head Epid.[a]	-	-	-	-	-	-	-	-	405.6
[R] Head Epid. [a]	591.4	527.4	-	-	-	-	-	-	587.3
[L] Body Epid. [a]	-	-	-	-	-	-	443.6	-	402.7
[R] Body Epid. [a]	-	-	-	-	-	-	-	-	211.4
[L] Tail Epid. [a]	-	-	-	-	-	--	441.6	-	104.8
[R] Tail Epid. [a]	604.1	501.6	-	97	-	-	-	-	111.3
[L] Prox. vas def.[b]	-	-	-	-	-	-	7.22	-	2554.7
[R] Prox vas def.[b]	-	-	-	-	-	-	-	-	199.3
[L] D. vas def+ Pp [c]	-	-	-	-	-	--	-	-	203.6
[R] D. vas def+Pp [c]	-	-	112.3	745.6	-	-	-	-	114.8
Bulbourethral gland	-	-	-	-	-	-	-	-	ND
Seminal vesicles	-	-	-	-	-	-	-	-	ND
Penis	-	-	-	-	-	-	-	-	234.5
Respiratory									
[L] Nostril	-	-	-	-	-	-	-	-	-
[R] Nostril	-	-	-	-	90.2	-	2156.8	-	ND
Tongue	166.3	-	-	-	-	-	-	-	-
Nasal turbinates	203.6	490	-	456.3	104.3	324.1	4789.1	142.3	5489.3
Epiglottis	1198.2	-	ND	ND	211.6	-	203.6	-	5924.3
Pharynx	-	-	-	-	321.4	-	-	-	1804.6
Larynx	3201.4	-	-	-	-	-	2369.7	ND	444.9
Trachea	-	-	-	-	-	-	1303	-	-
[L] Lung	-	-	-	-	-	-	1256.4	-	-
[R] Lung	-	-	-	-	-	-	-	-	675.3
Lymphatic									
Submandibular	-	-	-	-	-	-	-	-	1589
[L] Cervical [d]	-	-	-	-	-	-	-	-	2015.3
[L] Precrural [d]	-	-	-	-	-	-	-	-	1463.6
Skin	-	-	-	-	-	-	-	-	-
Facial	-	-	-	-	-	-	-	-	ND
[L]Neck	-	-	-	-	-	-	-	-	3122.8
[L]Upper-eyelid	-	-	-	-	-	-	-	-	3332.4
[L]Ear pinna	-	-	-	-	-	-	-	-	ND
[L]Carpal	-	-	-	-	-	-	-	-	343.9
[L]Tarsal	-	-	--	569.3	-	-	-	-	208.7
[L]Elbow	-	-	-	589.6	-	-	-	-	1166.5
[L]Thigh	-	-	-		-	-	-	-	388.6
[L]Perineal	-	-	-	-	-	-	-	-	97.1
Scrotum	-	-	-	-	-	-	-	-	1985.6
Other Organs									
[L]O.[e] Conjunc	-	-	-	-	-	-	-	-	ND
[L]O.[e] Sclera	-	-	-	-	-	-	-	-	1157.9
Brain	813.2	-	-	-	-	-	745.3	ND	ND
Cerebellum	-	-	-	-	-	-	-	-	ND
[R] Tendon+fascia	-	-	-	231.3	-	131.3	ND	-	ND
[R] Hoof Corium	-	-	-	-	-	-	-	-	ND
	-	-	-	-	-	-	-	-	-

[a]: Epid.: Epididymis; [b]: Proximal vas deferens; [c]: Distal vas deferens+ pampiniform plexus; [d]: Lymph node; [e]: Ocular; [L] Left / [R] Right

(A) Parasite was not detected either by HP or PCR in bronchus, thymus, spleen, [R] tonsils, bronchial (LN), medíastinum (LN), [L] ventriculum, [L] atrium from infected animals and in all tissues from control group.

Histological findings in experimentally infected male calves with chronic besnoitiosis.

Diezma-Díaz, C.[1], Tabanera, E [2]., Ferre, I.[1], Pizarro-Díaz, M.[2], González-Huecas M.[2], Blanco-Murcia, J.[1, 2], Ortega-Mora, L.M.[1], Álvarez-García, G.[1*]

[1] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

[2] Animal Medicine and Surgery Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

* Corresponding author: Gema Álvarez García. Tel.: +34-913944095. E-mail address: gemaga@vet.ucm.es

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Abstract

Bovine besnoitiosis, caused by the apicomplexan parasite *Besnoitia besnoiti*, is an emerging chronic and debilitating disease in Europe. Bull sterility is a major concern for affected herds, and there are few studies regarding lesions associated with clinical signs of bovine besnoitiosis. Herein, the histological findings associated with *B. besnoiti* infection were exhaustively studied for the first time in target tissues from experimentally and chronically infected calves. Recently, an experimental bovine model of chronic besnoitiosis was developed in which calves were inoculated with 10^6 bradyzoites via intravenous (G1), subcutaneous (G2) and intradermal routes (G3). Pathognomonic sclera cysts were observed in all infected animals. At 90 days post-infection (pi), the animals were euthanized, and tissues from different locations were collected and fixed in neutral buffered formalin for histological examination.

Tissue cysts were more abundant, and lesions were more frequent in G3 calves that previously showed more visible tissue cysts. The most parasitized tissues harbouring tissue cysts were skin, including scrotum (40.8% of positive samples), nostril and nasal turbinate. Tissue cysts were already fully developed, with an average tissue cyst diameter of 181.2 µm. However, tissue cysts did not reach the maximum size reported, and lesions were mild, possibly due to control by the host immune response. Microscopic lesions were detected mainly in skin samples, followed by reproductive and upper respiratory tracts, and were not necessarily associated with the presence of tissue cysts. Lesions compatible with both acute (thrombus, oedema and inflammation) and chronic besnoitiosis (skin lesions, hyperkeratosis and dilated sweat glands) coexisted. Vascular damage and inflammation were more frequently observed in skin (including scrotum), followed by testicular parenchyma, epididymis and pampiniform plexus. Herein, histological findings indicated subclinical chronic besnoitiosis and were not expected to impair fertility during adult life since the seminiferous epithelium of the testes did not show alterations. Future studies should clarify the chronobiology and relevance of the different pathogenic events in the sterility of naturally infected breeding bulls.

Keywords*:* Chronic besnoitiosis, lesions, tissue cysts, skin, testicles.

❖ Objetivo 3: Desarrollo y utilidad de nuevas técnicas serológicas para el diagnóstico temprano de la infección por *Besnoitia besnoiti*.

El diagnóstico temprano de la besnoitiosis bovina es esencial para el desarrollo de programas de control adecuados. Esto es particularmente importante en animales con infección aguda. Sin embargo, las técnicas serológicas actuales, basadas en la detección de inmunoglobulinas específicas del isotipo G (IgG), presentan limitaciones ya que la seroconversión de los animales infectados no se produce hasta la tercera semana pi. En este objetivo, se ha desarrollado una nueva técnica ELISA para detectar inmunoglobulinas del isotipo M (IgM) específicas de la infección por *B. besnoiti*. A continuación, se estudió la utilidad de esta herramienta y la de una técnica ELISA de avidez en un amplio panel de sueros bien caracterizados, procedentes de los animales infectados experimentalmente (objetivos 1 y 2) y de ganado infectado de forma natural. En primer lugar, se determinó la cinética sérica de las IgM específicas en terneros infectados experimentalmente durante la infección aguda y crónica de la enfermedad. Posteriormente, se determinaron las IgM séricas en el ganado infectado con besnoitiosis aguda y crónica de manera natural. Finalmente, se midió el índice de avidez de las IgG en todos los sueros.

Se detectó IgM antes de detectarse IgG específicas (7-19 vs. 17-26 días pi). Una respuesta temprana de IgM séricas se asoció con el inicio de la fiebre en los terneros infectados experimentalmente. Los animales infectados naturalmente y en la fase aguda de la enfermedad con signos clínicos característicos (fiebre, orquitis) mostraron resultados positivos a la detección de IgM y negativos a la de IgG, seguidos de la seroconversión de IgG 2-3 semanas después. El ganado infectado crónicamente mostró resultados positivos de IgM e IgG séricas. Además, se observó un aumento progresivo del índice de avidez en todos los terneros infectados experimentalmente con el progreso de la infección. Sin embargo, valores de baja avidez coexistieron con la detección de quistes oculares macroscópicos en los terneros hasta los 90 días pi. En el ganado infectado de manera natural y en la fase aguda, se detectaron valores de baja avidez cuando los animales seroconvirtieron, a diferencia de los de alta avidez detectados en el ganado infectado crónicamente.

En resumen, las pruebas ELISA de IgM y de avidez mejoraron el diagnóstico temprano *in vivo* de la besnoitiosis bovina. Los resultados positivos de IgM asociados a valores negativos de IgG fueron indicativos de la infección aguda, mientras que los resultados positivos de IgG asociados a una baja avidez confirmaron una infección reciente. Se necesitaría realizar más estudios longitudinales en rebaños infectados para definir la utilidad de la avidez de las IgG en el diseño de programas de control de la enfermedad.

Progress on the diagnosis of bovine besnoitiosis: Added value of IgM detection and low avidity index as indicators of acute disease.

Diezma-Díaz, C.[1], Ferre, I.[1], Saldías, B.[1], Blanco-Murcia, J.[1,2], Ortega-Mora, L.M.[1], Álvarez-García, G.[1*]

[1] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

[2] Animal Medicine and Surgery Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

* Corresponding author: Gema Álvarez García. Tel.: +34-913944095; E-mail address: gemaga@vet.ucm.es

Submitted: *Veterinary Parasitology* (Vetpar-D-19-13610).

Abstract

Early *in vivo* diagnosis of bovine besnoitiosis is crucial for the success of control programmes. Diagnosis is particularly important in acutely infected animals but it is hindered by the low sensitivity available serological tools.

A novel ELISA to detect specific anti-*Besnoitia besnoiti* IgM antibodies was developed. Next, the usefulness of this tool and avidity ELISA were studied with a well-coded sera panel from experimentally and naturally infected cattle. First, the kinetics of specific IgM levels were determined in experimentally infected calves during the acute and chronic infection. Next, IgM levels were determined in naturally infected cattle with either acute or chronic infection. Finally, the IgG avidity index was monitored in both experimentally and naturally infected cattle.

Specific IgMs were detected prior to specific IgGs (7-19 days vs. 17-26 days post-infection). A prompt IgM response was associated with an onset of fever in experimentally infected calves. Naturally and acutely infected animals with clinical signs showed IgM-positive and IgG-negative results, followed by IgG seroconversion 2-3 weeks later. Chronically infected cattle showed IgM- and IgG-positive results. Moreover, a progressive increase in the avidity index (AI) was observed in all experimentally infected calves during the course of the experimental trials. However, low avidity values coexisted with visible tissue cysts until 90 days pi. In naturally and acutely infected cattle, low AI were detected when animals seroconverted, in contrast to a high AI detected in chronically infected cattle.

In summary, IgM and avidity ELISAs improved the early *in vivo* diagnosis of bovine besnoitiosis. IgM-positive and IgG-negative results were indicative of an acute infection, whereas IgG positive results accompanied by low avidity values confirmed a recent infection. Further longitudinal studies are needed in infected herds to define IgG avidity usefulness in the design of control programmes.

Keywords*: Besnoitia besnoiti*, tachyzoites, subcutaneous route, intradermal route, calves.

CAPÍTULO V

GENERAL DISCUSSION

In the present Doctoral Thesis, two experimental models of *B. besnoiti* infection have been developed in calves that mimicked both subclinical besnoitiosis (with inoculated tachyzoites) and chronic besnoitoisis with macroscopic tissue cysts (with inoculated bradyzoites). Moreover, we have improved the diagnosis of acute besnoitiosis with IgM and avidity ELISAs.

Herein, all experimental infections in cattle were performed under the same standardized experimental desing to obtain comparable results. These trials were accompanied by histological analyses performed in target tissues and the study of immune responses. Two key variables were controlled to avoid the limitations of previos studies carried out several decades ago: inoculum quality and previous health status of inoculated animals. The tachyzoite stage arises as the most appropriate inoculum for the experimental infections as it offers several advantages vs. the bradyzoite stage: i) greater availability (tachyzoites can be maintained in cell cultures, whilst bradyzoites can be only isolated from chronically infected cattle; ii) easier quality controls (tachyzoites are easily propagated under controlled conditions and inoculated immediately after cell culture purification, whereas bradyzoite isolation requires prolongued time and must be carefully done under sterile conditions during field sampling and laboratory manipulation); iii) isolate variability is inherent for the bradyzoite based inoculum. Accordingly, we used tachyzoites from a well characterized field isolate with a low passage number in cell culture free of *Mycoplasma* spp. and bovine viral diarrhea virus (BVDV). The bradyzoites used in the last experimental trial were isolated from a *B. besnoiti* infected animal free of BVDV. The inoculum viability and sterility were carefully checked. In adition, a previous good health status was also tested in all animals. Males were selected since they may show severe acute clinical signs compared to females in natural infections. Moreover, testicles are target organs of this parasite during both acute and chronic infection (Gutiérrez-Expósito et al., 2017c).

First, a new isolate capable of producing disease in young animals was obtained from a chronically infected 6-month-old calf (Sub-objective 1.1) and was later used in the experimental inoculations. The new isolate named as Bb-Spain 3 was characterized in an *in vitro* culture system as a low invader and low prolific isolate and behaved similarly to other Spanish isolates (Bb-Spain 1, Bb-Spain 2) (Frey et al., 2016). It is in accordance with the microsatellite analyses performed herein and in previous works that did not reveal intra-specific differences among Spanish isolates (Gutiérrez-Expósito et al., 2016; Nieto-Rodríguez et al., 2016). Moreover, this case report was the first exhaustive description of an unusual case report of chronic bovine besnoitiosis in a young animal. Chronic besnoitiosis in the calf was similar to previous descriptions in adult animals based on parasite intra-organic distribution and tissue cyst type. We found three different type of cysts (viable without inflammation, with marked pericystic inflammation or degenerated). The most parasitized tissues were the predilection locations for the parasite (skin, respiratory and reproductive tracts) (Álvarez-García et al., 2013; Frey et al., 2013). *Besnoitia besnoiti* DNA was also detected in the lower respiratory tract (bronchi and lungs),

reproductive tract (cervix, uterus, oviducts and ovaries), circulatory system (heart and aorta), tonsils, the bronchial lymph nodes and brain.

Next, different tachyzoite doses (10^8, 10^7 and 10^6) from Bb-Spain 3 isolate were inoculated intravenously in calves. Similar clinical signs were obtained regardless the dose employed. However, a higher number of PCR positive tissues were detected in calves inoculated with 10^6 tachyzoites. Thus, this dose was chosen to be inoculated in bulls. When we compared the results obtained in calves and bulls, age did not seem to be a key host factor since macroscopic clinical signs and lesions characteristic of chronic besnoitiosis were absent (Sub-objective 1.2). Thus, calves were used again in the next trials as they are easily handled. Similar results were obtained with 10^6 *B. besnoiti* tachyzoites inoculated by subcutaneous and intradermal route (Sub-objective 1.3) so that last trial included the inoculation of 10^6 bradyzoites by intravenous, subcutaneous and intradermal routes (Sub-objective 2.1).

The acute phase of bovine besnoitiosis was easily induced with fever and lymphadenopathy regardless the parasite and host dependant variables studied. Following a previously stablished score, the acute phase was classified as mild-moderate as no severe clinical signs such as oedema or orchitis were detected. However, the parasite stage and inoculation route seem to influence on the severity, but for a few differences observed regarding the onset and duration of clinical signs observed. After intravenous inoculation of *B. besnoiti* tachyzoites, all infected animals (calves and bulls) developed fever around the first week pi. Higher temperature values were early detected in calves inoculated with the highest doses from 12 hours pi. It has been suggested that tachyzoite dose plays an important role in determining a severe vs. mild infection (Álvarez-García et al., 2014b). However, a high number of zoites are not likely to be trasmitted under natural conditions, as horizontal transmission seems to be the main route of parasite transmission either by cyst rupture during direct contact or by blood suckling arthropods bites (Álvarez-García et al., 2013). Subcutaneously infected calves compared with animals infected with the same tachyzoite dose by the intravenous route developed a lower febrile response that was absent in the intradermally infected group. Although fever might not be a necessary requisite for a successful infection, it is likely associated with the first replication cycles of the parasite (Benavides et al., 2014) and might be indicative of disease severity since severely affected animals during the acute stage of the infection may develop high fever for a few days (Álvarez-García et al., 2014b). This is in accordance with long lasting fever and lymphadenopathy observed in calves inoculated with bradyzoites. Remarkably, intradermally inoculated animals developed fever for a prolongued period of time that corresponded with a more severe chronic phase. Lymphadenopathy was maintained until the end of the trial in contrast with previous inoculations where lymphadenopathy dissapereared after the first month pi.

Next, the chronic phase was monitored and parasite DNA as well as seroconversion were detected in most animals. However, apart from a microscopic tissue cyst detected no macroscopic tissue cysts

and skin lesions characteristic of chronic besnoitiosis were developed in calves and bulls inoculated with the tachyzoite stage. These animals mimicked a subclinical besnoitiosis frequently found under field conditions (Frey et al., 2013; Gollnick et al., 2015). The low parasitie load detected by PCR could explain the absence of tissue cysts and differences among infected groups were hardly found. Notably, a higher number of PCR positive samples belonged to calves inoculated with the lowest dose (10^6 tachyzoites). Rojo-Montejo et al. (2012) suggested in *N. caninum* infection that when a high parasite dose was administered a large number of tachyzoites might remain extracellular and stimulate the immune response more efficiently, whereas a lower parasite dose might facilitate the evasion of the immune response. Fortunately, when bradyzoites were employed as inoculum, macroscopic clinical signs characteristic of chronic besnoitiosis were developed. Ocular tissue cysts were evidenced from 43 days pi onwards in intravenously, subcutaneously and intradermally inoculated groups, with the highest number of ocular sclera cysts and skin lesions detected in calves inoculated via intradermal route (49 days pi). Likewise, our histological and molecular findings confirmed a higher parasite load (number of tissue cysts and parasite DNA) and severity of lesions in intradermally inoculated calves (Sub-objective 2.2.). As expected parasite showed tropism for skin, eyes and mucous membranes of the upper respiratory and genital tracks, in agreement with previous reports in naturally infected animals (Nobel et al., 1981; Manuali et al., 2011; Gentile et al., 2012; Frey et al., 2013). Herein, tissue cysts were already fully developed, with an average tissue cyst diameter of 181.2 µm. Basson et al. (1970) observed that tissue cysts measuring more than 100 µm were fully developed and mature cysts measured up to 330 µm at 71-77 days pi in skin samples from experimentally infected cattle. However, in calves inoculated with bradyzoites, the existence of smaller cysts at 90 days pi could be explained by a prolongued incubation period that should be considered for the tissue cysts development (Bigalke, 1968), together with the induction of peripheral and local host immune responses that could have limited the growth of the tissue cysts. The predominant lesions were inflammatory infiltrates (perivascular, perifollicular, in the lamina propria or submucosa layer) and vascular damage (oedema, thrombus, hyaline degeneration of the muscular middle layer in vessels). These lesions were more frequently found in skin samples, followed by reproductive and upper respiratory tracts, in many cases, associated with detection of *B. besnoiti* DNA. In skin, vascular lesions also coexisted with lesions characteristics of the chronic phase, such as hyperkeratosis and dilated sweat glands, which were associated with a higher number of tissue cysts in the skin. Accordingly, lesions compatible with both acute and chronic besnoitiosis seem to coexist in both experimental and naturally infected animals (Dubey et al., 2013). Lesions in the respiratory tract were scarce and mild. *Besnoitia besnoiti* predilection for the upper respiratory tract has been frequently reported (Gentile et al., 2012; Álvarez-García et al., 2013; Frey et al., 2013) and did not alter respiratory function because lesions were absent in the lower respiratory tract. In reproductive tract, lesions were studied in detail due to impact of this parasitic infection in bulls sterility (Gutiérrez-Expósito et al., 2017c). However, seminiferous epithelium structure was not altered and corresponded

to normal six-month calf testis (Rawlings et al., 2008). Moreover, slight fibrosis is unlike to alter heat exchange in the testicles (Esteban-Gil et al., 2016).

The immune response kinetics was similar in all experimentally infected animals. After inoculation, an innate IFN-γ response was developed, followed by an adaptive IFN-γ response and seroconversion. Similarly, this kinetics has been also reported in experimentally infected cattle with closely related apicomplexan parasites such as *N. caninum* (Regidor-Cerrillo et al., 2014). However, differences in cellular immune responses were observed among infected groups. A delayed innate IFN-γ response was observed when bradyzoites vs. tachyzoites were inoculated (12-20 vs. 4-7 days pi), which was also associated with a later IgG seroconversion (17 vs. 19-25 days pi). The longer incubation period (later appearance of fever) and delayed immune responses could be due to the bradyzoite-tachyzoite switch as tachyzoites are the fast replicating parasite stage responsible for the acute phase (Lyons et al., 2002). The inoculation route could also affect the parasite exposure to the immune system. In general, subcutaneous or intravenous routes resulted in an earlier innate IFN-γ response and higher adaptive IFN-γ response that might have contributed to a better control of the infection compared to the intradermal inoculation. These findings could be explained by differences in vascularization that may influence on parasite dissemination and effector mechanisms of the host early innate immune response against *B. besnoiti* in the different tissues (Muñoz-Caro et al., 2014). After intravenous inoculation, 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). 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 is rapidly absorbed through blood vessels, this bypasses the skin immune cells, and the migration into lymph nodes is more efficient, leading to a more rapid immune response (Escobar-Chávez, 2010). We corroborated that high antibody levels are neither predictive of the outcome of the infection nor protective against the infection. However, the cellular immune response seemed to play a key role in the control of the infection. Indeed, in previous experimental infections, the best results were obtained under immunosuppressive therapies. Diesing et al. (1988) inoculated tachyzoites from peritoneal fluid or bradyzoites in animals by different routes. Five splenectomised animals either died or were euthanized. Two calves immunosuppressed by cortisone administration and inoculated with bradyzoites showed severe acute and chronic clinical signs. However, the immunosuppression therapies may compromise the reproducibility of these trials since animals are then more vulnerable to environmental infections.

Based on all the results obtained the bradyzoite stage and the intradermal route appear to be key factors in determining the outcome of the acute and chronic infection. In Sarcocystidae parasites, the bradyzoite differentiation is an immune evasion mechanism that allow the parasite to persist in the host

for a long life (Hemphill and Gottstein, 2006; Jeffers et al., 2018). *Besnoitia besnoiti* bradyzoites display a different antigenic pattern and protein composition compared with the tachyzoite stage that might explain the delayed immune response and prolongued incubation period (Fernández-García et al., 2013; García-Lunar, 2014). Moreover, the intradermal route may better mimick natural parasite transmission as it also happens in other vector-borne bovine pathogens (Umeshappa et al., 2011). However, a further refinement of this experimental model is necessary to overcome bradyzoite inoculum limitations. It is important to note that the sporozoite infective stage present in *Besnoitia* oocysts has not been tested as the definitive host remains unknown (Álvarez-García et al., 2014b). Parallel to this Doctoral Thesis, a failed experimental infection was carried out in young domestic cats (*Felis silvestris catus*) that were fed with a high dose of bradyzoites in order to obtain oocysts in their feces. Although seroconversion was detected at 22 days pi in infected kittens, oocysts were not detected in their feces for 30 days pi. This period time was selected since in other *Besnoitia* spp., which have the cat as definitive host, the minimum prepatency period was 9 days pi (*B. oryctofelisi*) (Dubey et al., 2003b) and the maximum period of oocyst elimination (patency) was until 30 days in *B. neotomofelis* (Dubey and Yabsley, 2010). Despite the considerations with respect to age and dose, findings agreed with previously described results reported by Diesing et al. (1988), Basso et al. (2011) and Marcén-Seral (2011). Only, Peteshev et al. (1974) reported shedding of oocysts in cats feces after ingestion of cattle tissues parasitized with *B. besnoiti* that were able to later induce bovine besnoitiosis in calves. However, other autors have not been able to reproduce these results, as well as the lack of serological and molecular tests make them not comparable (Basso et al., 2011) (see Annex I).

Finally, experimental infections in calves provided us with a well-coded sera panel that was employed to improve the early serological diagnosis of bovine besnoitiosis (Objective 3). First, an IgM ELISA test was developed and standardized. Next, specific IgM levels and IgG avidity kinetics were determined in sera from experimentally and naturally infected cattle. In *B. besnoiti* infection, specific IgM kinetics has not been previously measured and IgG based serological tests lack a diagnostic window during the acute phase comprising the appearance of initial clinical signs until IgG seroconversion at 3 weeks pi or even later (García-Lunar et al., 2013b; Schares et al., 2013). IgMs were mostly detected earlier than IgGs in all experimentally infected calves. An earlier IgM response corresponded to calves inoculated with the tachyzoite stage (vs. the bradyzoite stage) inoculated by subcutaneous and intravenous route vs. intradermal route, which was correlated with a shorter incubation period and an earlier IgG response mentioned above. A relevant finding was that first IgMs appeared when fever was also detected. Fever is likely associated with the first replication cycles of the parasite (Benavides et al., 2014), that would lead to an early stimulation of the immune system. The diagnosis of acute infection caused by the closely related parasite *T. gondii* in humans relies on the detection of specific IgMs since IgMs can be detected two weeks prior to IgG seroconversion (Dard et al., 2016). Herein, results obtained from acutely infected animals confirmed an earlier IgM response compared with IgG seroconversion

and revealed that *B. besnoiti* specific IgM detection (IgM+) in combination with negative IgG values (IgG-) and compatible clinical signs would confirm an acute besnoitiosis. IgM+ and IgG+ simultaneous detection is also a usual diagnostic finding in human toxoplasmosis since IgMs may remain detectable in serum from infected patients for months or even years after the primo infection (Dard et al., 2016). Persistence of IgMs in chronic besnoitiosis was also confirmed so that avidity ELISA test usefulness was studied since in human toxoplasmosis low avidity is indicative of a recent infection and high avidity is associated with an acquired toxoplasmosis at least four months before sampling (Villard et al., 2015).

When tachyzoites were inoculated in calves, low avidity values switched to high avidity values from 47 to 56 days pi onwards. In contrast, when bradyzoites were administered, despite avidity values progressively increased, low avidity was maintained until 90 days pi. In fact, low avidity coexisted with visible tissue cysts from 49 days pi onwards. As Schares et al. (2013) previously reported, the avidity maturation of IgG is slower than cyst development. These authors found wide differences to give rise high avidity in IgG antibodies from 32 days pi to 5 months pi in a cohabitation trial and associated the rapid maturation of the immune response with the unapparent course of the acute infection. This hypothesis would agree with our observations since prolongued fever and macroscopic tissue cysts were detected in calves with low avidity antibodies. Acutely and naturally infected cattle showed low avidity values once IgG levels were detectable after seroconversion. High avidity values were found in chronically infected animals that were IgG seropositive for at least two years. Based on our results and a previous study (Schares et al., 2013), low IgG avidity would be useful as an indicator of recent infection and high avidity as an indicator of ancient chronic infection. Further studies to monitor IgG avidity kinetics at individual (longitudinal studies) and at herd level (outbreaks vs. herds with endemic besnoitiosis) should be performed in order to determine the usefulness of the avidity ELISA in control programmes (Gutiérrez-Expósito et al., 2017c).

In summary, two experimental models of *B. besnoiti* infection in calves were developed representative of either a subclinical infection or a chronic infection with macroscopic clinical signs and lesions when tachyzoites and bradyzoites were inoculated, respectively. This later model could be used to test potencial therapeutic or vaccine candidates and bradyzoite stage and intradermal route were key factors. Furthermore, IgM/IgG pair-wise comparison serology allowed us to come with a diagnosis of acute besnoitiosis until two-three weeks earlier than IgG detection as well as to discard a recent infection when high IgG avidity values were measured.

CAPÍTULO VI

CONCLUSIONES

CONCLUSIONS

Objetivo 1: Inoculación de taquizoítos de *Besnoitia besnoiti* en bovinos: Influencia de la dosis parasitaria, ruta de inoculación y edad de los animales.

- **Primera:** Tras describir el primer caso clínico de besnoitiosis bovina en un animal menor de seis meses, inusual por la edad a la que se desarrolló la enfermedad, se demuestra que tanto las lesiones como la distribución intraorgánica del parásito son semejantes a lo observado en los casos clínicos de animales adultos. El nuevo aislado obtenido (Bb-Spain 3) mostró un comportamiento *in vitro* y un genotipado por microsatélites similares al de otros aislados españoles y europeos. Por lo tanto, la escasa variablilidad intraespecífica observada en *B. besnoiti* indicaría que la variable aislado no sería un factor determinante para el desarrollo de la enfermedad.
- **Segunda:** La inoculación de taquizoítos de *B. besnoiti* (aislado Bb-Spain 3) por diferentes vías de administración (intravenosa, subcutánea e intradérmica) causa una besnoitiosis subclínica en los bovinos infectados (terneros y novillos). Las variables estudiadas, dosis parasitaria (10^8, 10^7 y 10^6 taquizoítos), ruta de inoculación y edad del hospedador, no fueron determinantes en el éxito de la infección. Todos los animales infectados desarrollaron signos clínicos compatibles con una besnoitiosis aguda clasificada como leve-moderada y, en algunos casos, se detectaron lesiones microscópicas características de la fase crónica, en ausencia de signos clínicos patognomónicos de esta enfermedad.

Objetivo 2: Inoculación de bradizoítos de *Besnoitia besnoiti* en terneros: Factores clave en la aparición de los signos clínicos característicos de la besnoitiosis crónica y descripción de los hallazgos histológicos en los tejidos diana.

- **Primera:** Se ha desarrollado un modelo experimental de besnoitiosis crónica en terneros, en el que el estadio parasitario (bradizoíto) y la ruta de inoculación (intradérmica) son variables determinantes. Los animales infectados presentaron quistes patognomónicos de la enfermedad en la conjuntiva ocular, así como una marcada distribución intraorgánica del párasito en los diferentes tejidos diana, principalmente en el grupo inoculado por vía intradérmica. El empleo de bradizoítos como inóculo muestra limitaciones en cuanto a su obtención y a los rigurosos controles de calidad que deben realizarse. Estas limitaciones deberán ser consideradas para un futuro refinamiento del modelo.
- **Segunda:** La vía de administración de los bradizoítos influye en el desarrollo de la respuesta inmune en los animales inoculados, lo que podría deberse a los diferentes mecanismos efectores de la inmunidad innata en los puntos de inoculación. Se observó un retraso en el desarrollo de la respuesta inmune, tanto innata como adaptativa, asociado a la ruta de inoculación intradérmica.Sin embargo, la respuesta inmune desarrollada pudo haber limitado la progresión

de la enfermedad, ya que los quistes tisulares no alcanzaron el tamaño máximo y el infiltrado inflamatorio granulomatoso fue la lesión predominante.

- **Tercera:** El modelo de besnoitiosis crónica se caracterizó por la coexistencia de lesiones inflamatorias y vasculares (características de la fase aguda) junto con la presencia de quistes tisulares (característicos de la fase crónica). Estos quistes se localizaron principalmente en la piel, seguido del tracto reproductivo y el respiratorio, tal y como se ha descrito en infecciones naturales. En relación con el aparato reproductor, las lesiones vasculares fueron más frecuentes en la piel del escroto que en el plexo pampiniforme y el parénquima, aunque, probablemente sin repercusión en la fertilidad, ya que estas lesiones no alteraron ni la estructura de los túbulos seminíferos ni las células de la línea germinal.

Objetivo 3: Desarrollo y utilidad de nuevas técnicas serológicas para el diagnóstico temprano de la infección por *Besnoitia besnoiti*.

- **Primera:** Se ha desarrollado una nueva prueba ELISA basada en la detección de inmunoglobulinas del isotipo M (IgM) específicas para *B. besnoiti*, la cual se puede emplear en el diagnóstico serológico de la besnoitoisis bovina al mostrar unos valores elevados de sensibilidad. En este sentido, se recomienda la detección pareada de IgM e inmunoglobulinas del isotipo G (IgG) para el diagnóstico de la besnoitiosis aguda, ya que los signos clínicos característicos de esta fase coinciden con valores altos de IgM en ausencia de IgG.
- **Segunda:** Por el contrario, la detección simultánea de ambas inmunoglobulinas no confirma una infección reciente, ya que los valores elevados de IgM persisten durante varios años en los animales con besnoitiosis crónica. En estos casos, se recomienda el empleo *a posteriori* de una prueba ELISA de avidez, puesto que los valores de avidez baja se detectan tras la seroconversión de IgG. En fases tempranas de la infección, la aparición de quistes tisulares en la conjuntiva ocular no coincide con el cambio de los valores de baja a alta avidez, por lo que una avidez baja pueden mantenerse en los animales infectados durante varios meses.

Objective 1: Inoculation of *Besnoitia besnoiti* tachyzoites in cattle: Influence of parasite dose, administration route and animal age.

- **First:** The description of the first and unusual case report of bovine besnoitiosis in a calf less than 6 months shows that both lesions and parasite intraorganic distribution are similar to previous reports in adult cattle. The new *B. besnoiti* isolate (Bb-Spain 3) yielded similar *in vitro* behaviour and molecular genotyping by microsatellite analyses to others Spanish and European isolates. Therefore, the low intra-specific variability observed in *B. besnoiti* lead us to hypothesize that the variable isolate might not be a key factor for the outcome of the disease.
- **Second:** The inoculation of *B. besnoiti* tachyzoites (Bb-Spain 3) by different administration routes (intravenous, subcutaneous and intradermal route) induces subclinical besnoitiosis in infected cattle (calves and young bulls). Parasite dose (10^8, 10^7 and 10^6 tachyzoites), inoculation route and host age are not crucial for the outcome of the infection. All infected animals developed clinical signs compatible with mild-moderate acute besnoitiosis. Microscopic lesions characteristic of the chronic phase were detected in a few animals but pathognomic clinical signs of this disease were absent.

Objective 2: Inoculation of *Besnoitia besnoiti* bradyzoites in calves: Key factors in the appearance of clinical signs characteristic of chronic besnoitiosis and description of histological findings in target tissues.

- **First:** An experimental model for chronic besnoitiosis has been developed in calves. Herein, the parasite stage (bradyzoite) and the inoculation route (intradermal) are crucial variables that influence the clinical outcome of the chronic infection. Infected animals showed pathognomonic tissue cysts in ocular sclera, as well as a noticeable intraorganic distribution of the parasite in target tissues, being more evident in calves inoculated via intradermal route. The bradyzoite stage employed as inoculum shows limitations due to the inherent time consuming isolation procedure and the exhaustive quality controls needed that should be considered for future refinement of the experimental model.
- **Second:** The inoculation route of bradyzoites influences the development of the immune response in infected animals. This finding could be explained by the different effector mechanisms of the host early innate immunity developed at the inoculation points. A delay in both innate and adaptive immune responses was associated with the intradermal inoculation route. However, the immune response developed may have controlled the disease progression since tissue cysts did not reach the maximun size and the granulomatous inflammatory infiltrate was the predominant lesion.
- **Third:** The experimental model of chronic besnoitiosis was characterized by the coexistence of inflammatory and vascular lesions (characteristics of the acute phase), as well as tissue cysts

(characteristic of the chronic phase). Tissue cysts were found mainly in skin, reproductive and respiratory tracts, as described in naturally infected cattle. In reproductive tract, the vascular lesions were more frequently observed in the scrotal skin vs. the pampiniform plexus and the parenchyma. The negative impact of the infection on the fertility is unlikely to occur since no changes in the normal architecture of the seminiferous tubulus and germ line cells were detected.

Objective 3: Development and usefulness of novel serological tools for the early diagnosis of *Besnoitia besnoiti* infection.

- **First:** A novel ELISA test based on the detection of *B. besnoiti*-specific immunoglobulins isotype M (IgM) has been developed and standardized for the serological diagnosis of bovine besnoitoisis that showed high sensitivity. It is recommended the IgM/IgG pair-wise comparison for the diagnosis of acute besnoitiosis, since clinical signs characteristic of the acute phase are in accordance with high IgM values in the absence of IgG.
- **Second:** Conversely, simultaneous IgM and IgG detection does not confirm a recent infection, since high IgM levels persist for years in chronically infected animals. In this case, an avidity ELISA should be used *a posteriori* based on the detection of low avidity values detected after IgG seroconversion. In early infection stage, the appearance of visible pathognomonic ocular tissue cysts does not match with high avidity values and low avidity values persist in infected animals for several months.

CAPÍTULO VII

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ANEXO I

ANNEX I

Failed *Besnoitia besnoiti* oocyst shedding in kittens (*Felix silvestris catus)* experimentally infected with a high dose of bradyzoites.

Diezma-Díaz, C.[1], Ferre, I.[1], Ortega-Mora, L.M.[1], Álvarez-García, G.[1*]

[1] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

* Corresponding author: Gema Álvarez García. Tel.: +34-913944095; E-mail address: gemaga@vet.ucm.es

Draft version of the manuscript

Abstract

Besnoitia besnoiti (Protozoa, Apicomplexa) is the causal agent of bovine besnoitiosis, a chronic and debilitating cattle disease. It has been hypothesized that the life cycle of *B. besnoiti* is heteroxenous as in other Sarcocystidae members. However, the definitive host has not been identified yet. The aim of this work was to clarify the role of the domestic cat as a possible definitive host of *B. besnoiti*. Thus, an experimental infection was performed in three 3-month-old kittens that were fed with a high dose of tissue cysts (290,000 cysts/animal) isolated from a breeding bull with chronic besnoitiosis. During the first month post-infection (pi), daily faecal examination by a flotation technique with sucrose was performed in order to detect oocysts. In addition, blood samples were weekly collected until the eighth week pi to detect the presence of specific antibodies by Western blot. The three infected animals seroconverted at 22 days pi and remained seropositive until the end of the experiment (55 days pi). However, oocyst-like forms were not observed in the faecal samples. The cat is unlikely to be a definitive host of *B. besnoiti*.

Keywords: *Besnoitia besnoiti*, *Felix silvestris catus*, definitive host, oocysts, antibodies.

1. Introduction

Besnoitia besnoiti (Protozoa, Apicomplexa) is the causal agent of bovine besnoitiosis, a chronic and debilitating cattle disease responsible for considerable economic losses due to skin lesions and impairment of fertility (Guitiérrez-Expósito et al., 2017b). The trade of animals with unknown health status and the absence of commercial drugs and vaccines have favoured its dissemination (EFSA, 2010). However, undiscovered epidemiology aspects of the parasite such as the complete life cycle and the unknown definive host could have also contributed.

The life cycle of *B. besnoiti* is considered to be heteroxenous, where animals of subfamilies Bovinae and Antilopinae are the intermediate hosts (Álvarez-García et al., 2013). The domestic cat (*Felix silvestris catus*) has been considered a potential definitive host similar to other *Besnoitia* species (*B. darlingi*, *B. wallacei*, *B. oryctofelisi* and *B. neotomofelis*) (Jellison, 1956; Wallace and Frenkel, 1975; Dubey et al., 2002; Dubey and Lindsay, 2003; Dubey et al., 2003b; 2003c), Peteshev et al. (1974) reported *B. besnoiti* oocysts in domestic cats and in one wild cat fed with tissues from a naturally infected cow. In contrast, similar studies carried out later could not confirm these findings (Diesing et al., 1988; Basso et al., 2011; Marcén-Seral, 2011). First, Diesing et al. (1988) fed four domestic cats of unknown age with *B. besnoiti* tissue cysts from a cow. Later, Basso et al. (2011) performed an experimental infection in adult cats where two animals were infected with tachyzoites (Bb-GER 1 isolate) and three animals with bradyzoites from a naturally infected cow. In both experiments no oocysts were observed in faeces. Moreover, Marcén-Seral (2011) reported oocyst-like forms in faeces from two kittens (2-3 months of age) fed with *B. besnoiti* tissue cysts at 26 and 27 days post-infection (pi) without parasite identity confirmation by PCR.

Herein, we considered the age and parasite doses as key varibles. In *Toxoplasma gondii*, age has been described as a risk factor since kittens are more susceptible and they can shed a larger amount of cysts (Dubey, 2001; Fritz et al., 2012), Thus, the aim of the present work was to clarify the potential role of domestic cat (*Felix silvestris catus*) as definitive host in *B. besnoiti* life cycle. Herein, 3-month-old kittens were fed with a high dose of *B. besnoiti* tissue cysts (290,000 cysts per animal) isolated from a naturally infected bull.

2. Materials and methods

2.1. Ethics statement

All experimental procedures were approved by the Animal Welfare Committee of the Community of Madrid, Spain, following proceedings described in Spanish and EU legislation (PROEX 163/15 Law 32/2007, R.D. 53/2013) and in EU Council Directive 2010/63/EU. Animals remained in the Animal Health Department of the Faculty of Veterinary Medicine at Complutense University of Madrid (registration number: ES280790000155). Cats were housed in individual cages (floor: 1.5 m^2; shelves: 0.5 m^2; height: 2 m). All animals had their own clinical history from birth until the end of the trial and were provided with an enrichment environment, adequate nutrition, water "*ad libitum*" and appropriate care for their health and well-being.

At the end of the study, all the cats were given in adoption with perfect health status.

2.2. Experimental design

Three 12-week-old kittens from an authorized commercial hatchery (Isoquimen S.L., Barcelona, Spain) were employed. Kittens were vaccinated with a polyvalent vaccine (Feligen CRP, Virbac ®, Carros, France) against feline Panleukopenia, Rhinotracheitits and Calicivirus viruses. Moreover, they were free of ectoparasites and endoparasites (helminths, *Cystoisospora* spp., *Sarcocystis* spp., *Haemobartonella felis* and *T. gondii*). Upon arrival, a quarantine adaptation period of two weeks was established.

To emulate the potential natural infection route, animals were fed with scrotum skin (see section 2.3) obtained from a naturally infected breeding bull with lesions characteristic of chronic besnoitiosis (thickened scrotal skin and ocular tissue cysts). During the trial (55 days pi), daily clinical monitoring (general health, loss of appetite and frequency and consistency of faeces) was carried out. Faeces were daily collected and examined for oocysts detection during 30 days pi (see section 2.4). This period was selected based on previous reports in other *Besnoitia* spp., where cats act as definitive hosts. The minimum prepatency period was 9 days pi in *B. oryctofelisi* (Dubey, et al., 2003b) and the maximum period of oocyst elimination (patency) was 30 days in *B. neotomofelis* (Dubey and Yabsley, 2010). Each sample was processed in duplicate; one sample for microscopic observation and another one was kept at -20ºC for the extraction of DNA to confirm oocysts identity by PCR. In addition, blood samples were taken from infected animals with the following frequency: pre-inoculation (-7 days pi), infection day (0 days pi) and onwards weekly until 8 weeks pi. After centrifugation (1500 x *g* for 10 min) serum was obtained for the detection of specific anti-*B. besnoiti* antibodies by Western blot (WB) (see section 2.5).

2.3. Inoculum preparation

Scrotum from a breeding bull with clinical signs characteristic of chronic besnoitiosis and specific anti-*B. besnoiti* antibodies (García-Lunar et al., 2017) was employed as inoculum. This animal was seronegative for *N. caninum* and *T. gondii* as well as bovine diarrhoea and infectious bovine rhinotracheitis viruses. In a previous work, bradyzoites from this bull were isolated in cell culture to obtain a new *B. besnoiti* isolate named as Bb-Spain 4. Microsatellite genotyping was performed in the new isolate that showed identical microsatellite pattern to other Spanish isolates and was subsequently employed in experimental infections in calves. These animals developed clinical signs compatible with acute and chronic besnoitiosis (Diezma-Díaz et al., 2019b).

Herein, large number of tissue cysts in scrotal skin from naturally infected breeding bull were previously visualized by compression of small fragments under glass plates and subsequent visualization under a microscope (BX50, Olympus, Tokyo, Japan). Cysts count was performed in 0.3 g of skin and 1,000 cysts per gram was quantified. Scrotum homogenisation mixed with their usual food was voluntarily ingested by the cats for three consecutive days: 60 g for the first day, 90 g for the second and 140 g for the third day (parasite dose consumed: 290,000 per cat).

2.4. Faecal examination

Faeces from each cat were daily examined between 1 and 30 days pi and were processed by a combined sedimentation-flotation technique using sucrose solution (Panreac AppliChem ITW Reagents ®, Barcelona, Spain), previously described for *N. caninum* (Schares et al., 2005) and subsequently employed in the experimental infections performed by Basso et al. (2011).

2.5. Western blot (WB)

Western blot was performed in a 15% polyacrylamide gel following the protocol previously described by García-Lunar et al. (2013b) with the following modifications. Cat sera were analysed at 1/20 dilution and two secondary antibodies were added. First, a FITC-labelled anti-cat IgG produced in goat (Sigma-Aldrich ®, St. Louis, Missouri, United States) (dilution 1/200) and later, a monoclonal anti-goat IgG labelled with peroxidase (Sigma-Aldrich ®, St. Louis, Missouri, United States) (dilution 1/1000). In the absence of positive control cat sera at the beginning of the study, bovine sera were used as positive and negative controls that were incubated with an anti-bovine monoclonal IgG conjugated with peroxidase (ThermoFisher Scientific®, Waltham, Massachusetts, USA) (dilution 1/1000). Positivity criteria was also established by García-Lunar et al. (2013b) (Figure 1).

3. Results and discussion

Three 3-month kittens were fed with a high dose of tissue cysts in order to clarify the role of *Felix silvestris catus* as a definitive host of *B. besnoiti*. Although, young cats have been reported to be more susceptible to *T. gondii*-infection, in previous studies carried out in *B. besnoiti*, age was not taken into account (Diesing et al., 1988) or adult cats (19-40 months of age) were only used (Basso et al., 2011). Thus, Marcén-Seral (2011) employed kittens (3-4 months of age) that were inoculated with 3 x 10^8 bradyzoites. In our work, a higher dose has been also considered. As far as we are aware, 5.8 x 10^{10} bradyzoites (estimated quantity with 200.000 zoites per cyst) is the highest inoculation dose employed until now.

Herein, none of the infected animals showed loss of appetite or any other compatible clinical sign with the infection throughout the study period. Stool showed normal consistency in infected animals. Seroconversion was detected by WB in all infected animals at 22 days pi orwards. Sera showed previously reported antigen recognition pattern from 22 days until 55 days pi (Basso et al., 2011; García-Lunar et al., 2013b) (Figure 1). Previously, Basso et al. (2011) had reported seroconversion at week 5 pi in two of the three cats fed with 2 x 10^7 *B. besnoiti* bradyzoites by means of an immunofluorescence antibody test (IFAT). However, reactions against a specific 27kDa band were already observed from three weeks pi by WB. The weak antigen recognition could be due to a lower bradyzoites dose ingested by the cats. Ours results agree with the serological findings reported by Marcén-Seral (2011), who reported seroconversion by IFAT in three of the 11 infected kittens at 23 days pi, that evidenced an exposition to the parasite.

Oocyst-like structures compatible with *B. besnoiti* oocysts were not detected in faeces of any cat during the whole observation period (30 days pi) in agreement with Diesing et al. (1988), Basso et al. (2011) and Marcén-Seral (2011). Diesing et al. (1988) fed various species of reptiles, birds and mammals, including four cats with tissues containing *B. besnoiti* cysts. Only, a leopard (*Panthera pardus*) excreted a few unsporulated *Cystoisospora*-like oocysts without infective capacity in calves. Basso et al. (2011) inoculated five cats with *B. besnoiti* tachyzoites (2 cats) and bradyzoites (3 cats) without detection of oocysts in faecal samples for 20 weeks pi. On the contrary, Peteshev et al. (1974) reported faecal elimination of oocysts in cats after ingestion of tissue parasitized with *B. besnoiti*. Rommel (1975) reported unsporulated oocysts in six out of 14 cats inoculated with skin containing cysts at 6-23 days pi (above mentioned by Marcén-Seral, 2011). However, the lack of data on their feeding and breeding conditions in these studies, as well as the lack of serological and molecular tests make their results not comparable

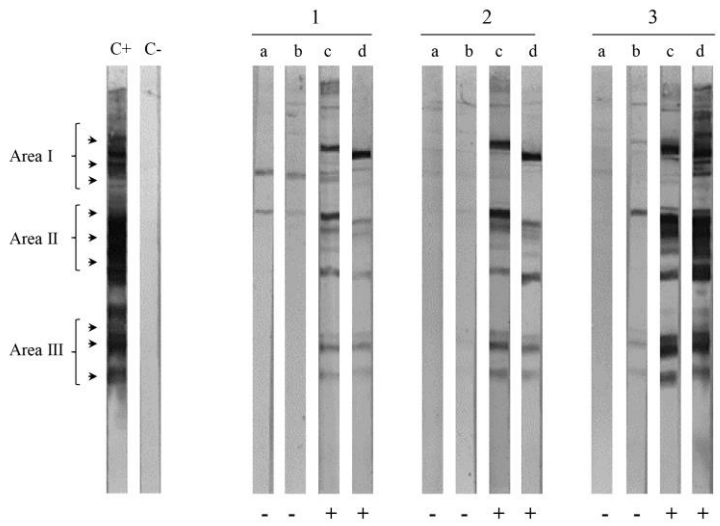

Figure 1: Detection of serum anti-*Besnoitia besnoiti* antibodies by Western blot in experimentally infected cats (1, 2 and 3) at 0 (a); 13 (b); 22 (c) and 55 (d) days post-infection respectively (notice seroconversion in all animals from 22 days pi onwards. Bovine positive (C+) and negative (C-) controls.
+: Positive result
-: Negative result

Seropositivity criteria: The presence of at least three antigenic bands in at least two of the described areas was considered IgG-positive (García-Lunar et al., 2013b).

(Basso et al., 2011). In other *Besnoitia* spp. where cats are the definitive host (*B. wallacei*, *B. darlingi*, *B. oryctofelisi* and *B. neotomofelis*) a low number of sporulated oocysts were excreted in faeces (Jellison, 1956; Wallace and Frenkel, 1975; Dubey et al., 2002; Dubey and Lindsay, 2003; Dubey et al., 2003b; 2003c). Curiously, in the above mentioned *Besnoitia* spp. where the intermediate hosts are rodents or rabbits (*B. oryctofelisi*) that could be depredated by cats under natural conditions. However, in *Besnoitia* spp. infecting ungulates: bovines and antelopes (*B. besnoiti*), donkeys and zebras (*B. benneti*), caribous and deers (*B. tarandi*), as well as goats (*B. caprae*), the role of cat as predator is questioned.

It is hypothesized that, at least in Europe, the disease is mainly transmited through direct contact between infected and non-infected animals or haematophagous vectors without the participation of a definitive host. This hypothesis is supported by the low intra-species diversity found among *B. besnoiti* isolates (Arnal et al., 2016; Diezma-Díaz et al., 2019b) that contrast to the variability reported in *T. gondii* where sexual recombination occurs frequently in the definitive hosts. Futher studies should include African wildlife (carnivores and scavengers) since antelopes can act as intermediate hosts and bovine besnoitiosis is endemically present in many sub-Saharan African countries (Hofmeyr, 1945; Bigalke, 1968).

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ANEXO II

(Artículos publicados)

ANNEX II

(Peer-reviewed papers)

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Research paper

Bovine chronic besnoitiosis in a calf: Characterization of a novel *B. besnoiti* isolate from an unusual case report

C. Diezma-Díaz[a], A. Jiménez-Meléndez[a], M. Fernández[b], D. Gutiérrez-Expósito[a], P. García-Lunar[a], L.M. Ortega-Mora[a], J.A. Pérez-Salas[c], J. Blanco-Murcia[c], I. Ferre[a], G. Álvarez-García[a,*]

[a] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040, Madrid, Spain
[b] Instituto de Ganadería de Montaña (CSIC-ULE), 24346, León, Spain
[c] Animal Medicine and Surgery, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

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Case report

ABSTRACT

Bovine besnoitiosis, caused by the apicomplexan *Besnoitia besnoiti*, is a chronic and debilitating disease characterized by cutaneous and systemic manifestations that primarily affects adult beef cattle. Previous studies have reported that clinical besnoitiosisis is rare in calves. However, we isolated *B. besnoiti* from a chronically infected calf for the first time. The identity of the *Besnoitia* species was determined after parasite isolation and molecular genotyping. According to the results obtained *in vitro* the new isolate, named as Bb-Spain3, was characterized in a reproducible *in vitro* model and was categorized as a low invader and low prolific isolate with a slower lytic cycle compared to Bb-Spain 1 isolate. Specific traits that differentiate isolates obtained from adult animals from those infecting calves were not found. Next, we described the first case report of chronic besnoitiosis in a female calf less than 6 months-old with a low body condition. The disease was confirmed by the presence of specific anti-*B. besnoiti* antibodies and parasite detection in the skin. At post-mortem examination, tissue samples were collected for histological, immunohistochemical and molecular analyses. DNA-parasite was detected in 31 different calf's tissues, being the most highly parasitized tissues the skin and the respiratory and reproductive tracts. In addition, the parasite was also present in heart, eyes, lymph nodes and brain. The high parasite load, a wide intra-organic parasite distribution and the presence of both viable and degenerated cysts, were indicative of a rapid progression of the disease. This case report underlines the need to include the inspection of young animals in besnoitiosis control.

1. Introduction

Bovine besnoitiosis, caused by the cyst forming apicomplexan parasite *Besnoitia besnoiti* (Besnoit and Robin, 1912), is a chronic and a debilitating disease that primarily affects beef cattle and has a negative impact on productive and reproductive parameters, welfare and causes occasional deaths. The European Food Safety Authority in 2010 (European Food Safety Authority, 2010) was alerted on the re-emergence of this disease in Europe from areas where the disease was traditionally endemic (French Pyrenees, the Alentejo region in Portugal and the northeastern part of Spain). At present, the disease has already reached Ireland and Eastern countries (Beck et al., 2013; Hornok et al., 2014; Vanhoudt et al., 2015; Ryan et al., 2016). Unfortunately, there are still gaps in the epidemiology of the disease, and this lack of knowledge hampers its control, which solely relies on diagnosis and management measures in the absence of either drugs or vaccines. In particular, the definitive host and transmission routes remain to be elucidated (Álvarez-García et al., 2013).

It has been postulated that *B. besnoiti* could have a heteroxenous life cycle, where cattle, antelopes, red deer and roe deer act as intermediate hosts (Arnal et al., 2017; Gutiérrez-Expósito et al., 2016). In the intermediate host, two different asexual parasitic stages develop. First, the tachyzoites invade the vascular endothelium of blood vessels and are responsible for the acute stage of the disease. During the acute stage of the disease, the infected animals develop non-specific clinical signs that may go unnoticed, such as fever, nasal and ocular discharge, depression, lameness, orchitis and subcutaneous oedema. Next, tachyzoites switch into bradyzoites as a mechanism of immune response evasion, which pack inside thick-walled cysts mostly found in subcutaneous tissues and mucous membranes during the chronic stage of the disease. Consequently, chronically infected cattle develop hyperkeratosis, alopecia, atrophy of the testes, as well as pathognomonic tissue cysts in the sclera conjunctiva or the *vestibulum vaginae* (Álvarez-García et al., 2014b; Gollnick et al., 2015).

* Corresponding author.
E-mail addresses: gemaga@vet.ucm.es, gemalgar@yahoo.es (G. Álvarez-García).

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Parasite and host dependent factors may determine the outcome of the infection (Álvarez-García et al., 2014b), such as the isolate. Recently, Frey et al. (2016) has studied and compared seven *B. besnoiti* isolates, from six countries and two continents, and reported different *in vitro* characteristics (invasion and proliferation) but no obvious difference between them with regard to virulence. Gutiérrez-Expósito et al. (2016) using microsatellite analysis, could only find variation in a unique MS marker in one isolate (Bb- Italy2) that differed from 9 other, homogeneus, *B. besnoiti* isolates. This may be due to the fact that all compared isolates had been obtained from adult cattle clinically affected. Until now, *B. besnoiti* isolates have not been obtained from affected young animals, which may allow us to address if there are specific traits that differentiate isolates obtained from adult animals from those infecting younger animals.

Whether animal age has influence in the infection remains to be clarified. There are only two recent reports of the disease in calves. Hornok et al. (2014) reported the first case of besnoitiosis in Hungary and found three seropositive calves with respiratory disorders. More recently, Ryan et al. (2016) detected scleral tissue cysts in 41.9% of the calves present in an affected dairy herd in Ireland. The calves tested by ELISA proved to be seronegative. In the past, several authors (Bigalke, 1968; Alzieu, 2007) have concluded that the clinical incidence of besnoitiosis is very low in animals under one year of age. Indeed, clinical signs are more often observed among 2–4-year-old adults and rarely occur in calves under 6 months of age (Janitschke et al., 1984); this finding is also supported by recent reports demonstrating an increase of prevalence rates with age (Fernández-García et al., 2010; Gutiérrez-Expósito et al., 2014).

In the present study, firstly we obtained and characterized the first *B. besnoiti* isolate from a calf origin in an *in vitro* system. Next, we described the first case report of chronic bovine besnoitiosis in a calf younger than 6 months of age. After the post-mortem examination of the animal, an exhaustive tissue collection was carried out, and data on lesions and intra-organic parasite distribution were obtained by means of histopathological and PCR techniques.

2. Material and methods

2.1. Case report and herd samplings

In August 2013, local veterinarians reported three suspicious clinical cases of chronic bovine besnoitiosis located in Central Spain (northeastern part of Toledo province), where the presence of the disease had not previously been confirmed. The affected animals were an adult Limousin cow and a calf from a closed beef cattle herd. This herd was composed of 176 animals raised under extensive conditions where natural mating was the rule. In addition, a Charolais cow in a nearby farm that practised similar management measures was also affected.

The adult animals showed compatible clinical signs of chronic besnoitiosis, such as hyperkeratosis, skin folding in the neck and limbs, nodules and cracks in the udders and tissue cysts that could be seen by the naked eye in the sclera conjunctivae (Supplementary figure). The 4-month-old Limousin calf also showed signs of alopecia in the periorbital region and muzzle (Fig. 1). Two months before, the animal had displayed non-specific clinical signs compatible with the acute stage of bovine besnoitiosis, such as fever, depression and anorexia.

Sera from these animals were collected and examined for specific antibodies by ELISA. Biopsies from the skin in the tarsal region in the calf and from the *vestibulum vaginae* in the adult cow were collected and squashed between trichinelloscopy plates to visualize tissue cysts by direct microscopic examination. The serological analysis was repeated in the calf at 7 and 8 months of age. Subsequently, it was donated to the Veterinary Medical Teaching Hospital (Complutense University of Madrid) for further examination.

Due to poor body condition, the calf was sacrificed at 8 months of age. The animal was sedated with xylazine hydrochloride (Rompun®; Bayer) and immediately euthanized by an intravenously overdose of embutramide and mebezonium iodide (T61®; Intervet) in the ruminant medical facilities at the Veterinary Medical Teaching Hospital. All experimental procedures complied with current EU legislation (Directive 2003/65/CE and 2010/63/EU).

A complete and systematic post-mortem study was carried on the carcass, and tissue samples from 35 different locations were taken (Table 1). Tissues were fixed in neutral buffered 10% formalin solution for histopathology and immunohistochemistry studies and frozen at −80ºC for PCR analyses. In addition, an epiglottis sample was also collected for parasite isolation in cell culture.

2.2. Parasite isolation and in vitro assays

The presence of *B. besnoiti* tissue cysts in the calf was confirmed in samples from the epiglottis after tissue compression on a trichinelloscopy plate and visualization under the microscope (Nikon eclipse 50*i*). The tissue was homogenized using a Potter Elvehjem homogenizer (Sigma-Aldrich®) in a solution consisting of PBS (phosphate buffer saline) with 2% antibiotic (Penicillin/Streptomycin + Amphotericin B (Lonza®)). Bradyzoites released from the tissue cysts were observed at 40× in a light microscope, and aliquots of 4×10^7 bradyzoites were inoculated onto fresh monolayers of Marc-145 cells in T75 flasks (Nunc®; Thermofisher Scientific). All isolates were maintained on this type of cell, employing the same procedure described by Fernández-García et al. (2009). Invaded bradyzoites were observed as soon as 48 h post infection (hpi). Tachyzoites were maintained and passaged every 3–5 days in confluent cell cultures. This newly obtained isolate was named Bb-Spain 3. Tachyzoites from Bb-Spain 1 and the newly obtained Bb-Spain 3 isolates were grown in Marc-145 cells and purified as described by Frey et al. (2016). In order to avoid adaptation of the parasites to the cell line employed, isolates with a low passage number were included in the experiments (Bb-Spain 1 from 10 to 16 and Bb-Spain 3 from 6 to 10 passage number). Bb-Spain 1 was included as an internal control of reproducibility in invasion and proliferation assays. All isolates used for *in vitro* assays tested negative to *Mycoplasma* spp. infection by PCR (Venor™GeM Mycoplasma Detection Kit; Minerva Biolabs) and bovine viral diarrhea virus (BVDV) by quantitative real-time PCR (qPCR) (Hoffmann et al., 2006). Foetal calf serum used in all the experiments was previously checked for the absence of either specific IgG against *B. besnoiti*, *Neospora caninum* and *Toxoplasma gondii* by IFAT or BVDV RNA by qPCR (Fernández-García et al., 2009).

2.2.1. Parasite invasion rate

For invasion assays, 10^5 Marc-145 cells/well were grown to confluence in P24 cell culture plates (Nunc®; Thermofisher Scientific) incubated at 37 °C with 5% CO2. The next day, 10^3 tachyzoites of each isolate (infection rate 1:100) were added to the cell cultures. Four washes with PBS were performed at 4, 6, 8 and 24 hpi to discard non-invaded tachyzoites. One ml of fresh culture medium was added, and the plates were incubated at 37 °C with 5% CO2. In addition, non-washed plates were kept until fixation. After 72 hpi, IFAT (see Section 2.6.) was carried out in order to count the total number of invasion events (parasitophorous vacuoles and/or lysis plaques) per well according to the procedure established by Frey et al. (2016). Three replicates of invasion assays were repeated in three independent experiments for each isolate.

2.2.2. Proliferation kinetics, tachyzoite yield and doubling time determination

For the proliferation assay, P24 cell culture plates with confluent Marc-145 cells (10^5 cells/well), maintained with DMEM 10% FCS (HyClone®; Thermofisher Scientific), were used. The monolayers were infected with 10^6 purified tachyzoites/well (infection rate 10:1) suspended in 1 ml DMEM 5% FCS. After 4 h, the wells were washed three times with DMEM and 5% FCS, and the infected monolayers were

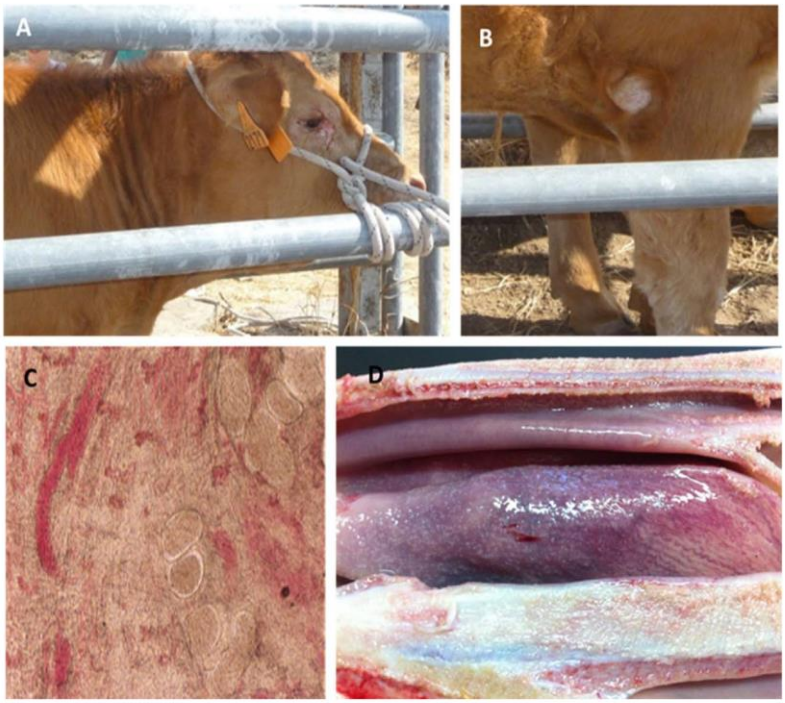

Fig. 1. Clinical signs, gross lesions and parasitological findings in the calf infected with *B. besnoiti*. A and B: Clinical signs compatible with chronic besnoitiosis (alopecic in the periorbital region and limbs). **C**: Microscopic tissue cysts visualized in a skin biopsy. **D**: Macroscopic tissue cysts in the tongue and nasal turbinates.

further cultured at 37 °C and 5% CO2. At 4, 8, 24, 48 and 72 hpi, the supernatants were discarded, and the parasites were harvested following the manufacturer's instructions included in the DNeasy blood and tissue Kit (Qiagen®). DNA was extracted and stored at −20 °C until further qPCR analysis (see Section 2.5.). Proliferation assays were carried out in triplicates and were repeated in three independent experiments for each isolate. To account for variations in the DNA content of the samples, the number of tachyzoites per µl was normalized using the DNA concentration per µl determined using a Biotek multiplate reader (Biotek ®), and the results were finally expressed as tachyzoites per nanogram of DNA (tachyzoite yields, TY).

In parallel, to study the proliferation kinetics of both isolates, replicates of cell cultures in coverslips were infected as described above and labelled using a double immunostaining. Three coverslips were photographed for each condition using an inverted fluorescence microscope (Nikon Eclipse TE 200, Chiyoda, TYO, JP).

The doubling time (Td) was defined as the period of time required for a tachyzoite to duplicate during the exponential multiplication period, excluding the lag phase (when there is not parasite multiplication) and the egression phase.

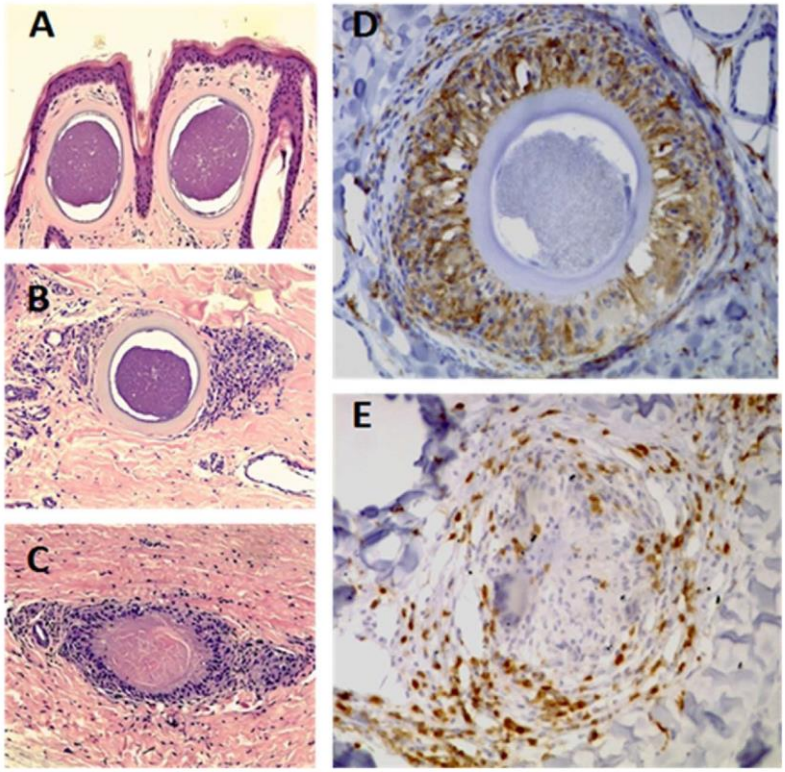

Fig. 2. *Besnoitia besnoiti* tissue cysts in the skin sections. HE; **A**: Viable tissue cyst with no surrounding inflammatory cells. **B**: Viable tissue cyst surrounded by pericystic inflammatory infiltrate. **C**: Lysed tissue cyst, with necrotic debris inside the capsule, in the lamina propria with pericystic inflammatory infiltrate. Inflammatory infiltrate. IHC; **D**: Recent recruited macrophages (positive labelling for myeloid/histiocyte-antigen. Clone MAC387 antibody) close to the hyaline capsule. **E**: T lymphocytes within the infiltrate (rabbit anti-human CD3 polyclonal antibody).

Table 1
Parasite intra-organic distribution by histopathology (HP) and PCR.

	PCR			HP		
Tissue[a]	ITS-1rDNA	qRT[b]	Results	Viable cysts (VC)	VC+ Imflammation	Degenerated cysts
Circulatory system						
Aorta	Positive	ND[d]	Negative			
Ventricle	Positive	ND[d]	Negative			
Atrium	Positive	ND[d]	Negative			
Respiratory system						
Tongue	Positive	2.8	Positive	20	2	
Muzzle	Positive	0.2	Positive	> 50	> 50	> 50
Nasal turbinates	Positive	5.6	Positive	> 50	> 50	> 50
Nasal sinus	Positive	241.8	Positive	5		
Pharynx	Positive	ND[d]	Positive	1	1	2
Epiglottis	Positive	6.3	Negative			
Larynx	Negative		Negative			
Trachea	Positive	0.2	Negative			
Bronchi	Positive	0.3	Negative			
Lungs	Positive	0.2	Negative			
Reproductive system						
Vulva	Negative		Positive		2	5
Vagina	Negative		Negative			
Cervix	Positive	0.1	Negative			
Uterus	Positive	ND[d]	Negative			
Uterine horns	Positive	ND[d]	Negative			
Oviducts	Positive	0.1	Negative			
Ovaries	Positive	98.7	Negative			
Skin						
Neck	Negative		Positive	5	20	20
Upper-eyelid	Positive	234.6	Positive	> 50	> 50	> 50
Carpal	Positive	1558.7	Positive	> 30	> 50	> 50
Tarsal	Positive	4210.2	Positive	> 50	> 50	> 20
Ear pinna	Positive	0.2	Positive	9		
Thigh	Positive	1224.4	Positive	5	20	20
Udder	Positive	ND[d]	Negative			
Nipple	Positive	0.1	Positive	1	2	10
Perineal	Positive	109.2	Positive	1	8	9
Lymphatic system						
Bronquial lymph node	Positive	ND[d]	Negative			
Tonsils	Positive	ND[d]	Negative			
CNS						
Brain	Positive	ND[d]	Negative			
Other organs						
Kidney	Negative		Negative			
Liver	Negative		Negative			
Ocular conjunctiva	Negative	ND[d]	Positive	15	2	
Ocular sclera	Positive	ND[d]	NA[c]			
Auditory canal	Positive	ND[d]	NA[c]			

[a] Tissues that tested negative: Lymphatic system (Retropharyngeal, Axillary, Mandibular, Subscapularis, Mesenteric, Traqueal, Iliac, Popliteal, Portal, Inguinal, Ileocaecal lymph nodes and Parotid, Thymus and Spleen) and Digestive system (Esophagus, Abomasum, Reticulum, Duodenum, Jejunum, ileum, caecum and rectum).
[b] N° tachyzoites/ng cow DNA by quantitative real-time PCR (qPCR).
[c] NA: not available.
[d] ND: not detected by qPCR

2.3. ELISAs

All sera were analysed by SALUVET in-house ELISA based in lyophilized tachyzoites of *B. besnoiti* as antigen (García-Lunar et al., 2017) to discriminate between seropositive and seronegative animals. The optical density was converted into the RIPC (Relative Index Percent) using the formula described by Fernández-García et al. (2010). An animal with an RIPC ≥ 17.34 was considered positive.

To discriminate between acute and chronic infection, the calf's sera collected at 4, 5, 7 and 8 months of age were also tested by an avidity SALUVET in-house ELISA. The test was essentially carried out as described above but included one additional incubation step with 6 M urea in the tested samples and PBS-Tween in the controls after the incubation step with sera (Schares et al., 2013). The cut-off to discern between low and high avidity values (acute and chronic stages, respectively) was estimated using a panel of 11 sera from naturally infected and seropositive cattle based on the results obtained by SALUVET in-house ELISA. Four sera samples came from animals that showed clinical signs compatible with the acute stage of the disease, and 7 sera samples came from seropositive chronically infected animals with tissue cysts. Serial dilutions of the sera were performed starting from 1:100 to 1:102,400, and the avidity indexes were calculated, and the cut-off was established at 50.8, according to Schares et al. (2013).

2.4. Histopathology (HP) and immunohistochemistry (IHC)

After five days of fixation, tissue samples were dehydrated using a graded series of alcohols and were embedded in paraffin. Tissue sections of 5 µm were cut from each sample and stained with haematoxylin and eosin (H/E) for the histopathological evaluation. The

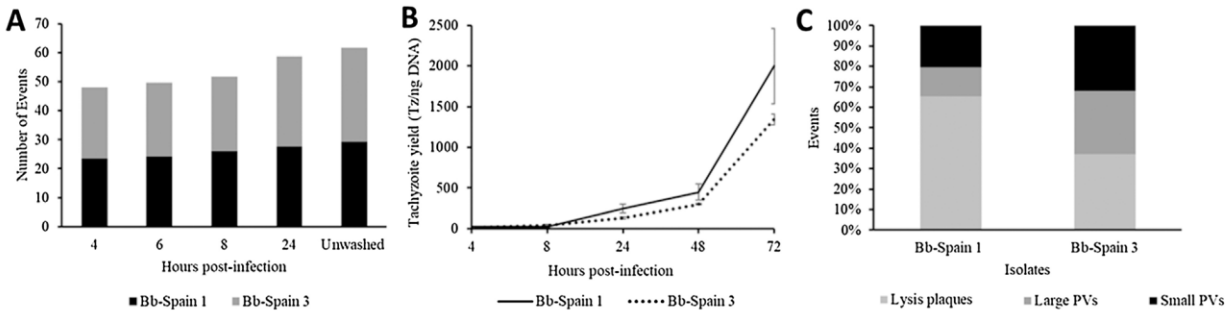

Fig. 3. *In vitro* behaviour of Bb-Spain 1 and Bb-Spain 3 isolates. **A:** Number of invasion events at different time points post-infection; **B**: Growth measured as tachyzoite yield as a function of time in a proliferation assay; **C**: Proportions of invasion outcomes counted per isolate.

immunohistochemical labelling of the parasites was performed using deparaffinised sections from the collected tissues. Primary in-house rabbit polyclonal antibodies against *B. besnoiti* tachyzoites were used at a 1:3000 dilution following the same protocol described by Frey et al. (2013). Parasite cysts found in the histological sections were counted when there were less than 30 or were subjectively classified as between 30 and 50 or more than 50.

On selected sections, the inflammatory cells infiltrating the area adjacent to the tissue cysts were characterized by immunohistochemical labelling. Primary antibodies specific for T-lymphocytes (rabbit anti-human CD3 polyclonal antibody) and monocytes/macrophages (mouse anti-human CD68 monoclonal antibody and mouse anti-human myeloid/histiocyte-antigen monoclonal antibody; clone MAC387) were used following the procedure described by Frey et al. (2013).

2.5. DNA extraction, ITS1-PCR and qPCR

The DNA extraction of the tissue samples were completed using Maxwell® 16 Instrument (Promega) with the Maxwell® 16 Tissue DNA Purification Kit (Promega) (Frey et al., 2013). The DNA content from each sample was adjusted to 40 ng/µl, and it was measured with a NanoPhotometer® (Implen).

The products of the DNA extraction process were tested for the specific detection of *Besnoitia* spp. by ITS-1 rDNA PCR (Cortes et al., 2007). The forward primer ITS1F (50-TGACATTTAATAACAATCAACCCTT-30) and the reverse primer ITS1R (50-GGTTTGTATTAACCAATCCGTGA-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 were visualized after electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The positive control was DNA extracted from *in vitro* cultured tachyzoites of *B. besnoiti,* and PCR grade water was used as the negative control.

The qPCR assay for the detection of *Besnoitia* spp. DNA from the ungulates was performed according to Frey et al. (2016). In each PCR, 10-fold serial dilutions of genomic DNA corresponding to 0.1-10,000 Bb-Spain 1 (Fernández-García et al., 2009) tachyzoites were included. To quantify the amount of DNA, dilutions of DNA extracted from the liver of a cow corresponding to 100, 20, 4, and 1 ng/µl were included. The cycling conditions were 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence emissions were measured during the 60 °C step. A dissociation stage was added.

2.6. Indirect fluorescent antibody test (IFAT)

IFAT was carried out on infected MARC-145 monolayers at 72 hpi. The protocol used was the same as that previously described by Frey et al. (2016). The total number of invasion events per well (IR; invasion rate) was counted using a fluorescence microscope (Nikon eclipse TE200) at 200X magnification. Three categories of invasion outcomes were distinguished: small parasitophorous vacuoles (PVs), large PVs, and lysis plaques. Vacuoles filled with tachyzoites forming a rosette and with individually distinguishable tachyzoites were regarded as small PVs. When the PV was packed with tachyzoites that were not individually discernible, a large PV was recorded. A lysis plaque was identified as soon as the host cell lysed, manifesting in an accumulation of multiple infected cells with just a few tachyzoites infecting each cell (with the infected cells typically being located around a central space with no cells at all and with extracellular tachyzoites) (Frey et al., 2016).

2.7. Data analysis

To assess differences in time points in invasion and proliferation assays for each isolate, and between isolates (Bb-Spain 1/Bb-Spain 3), ANOVA and Mann-Whitney *U* test were performed, respectively. We represented dT for each isolate as the average value obtained from all of the determinations that revealed a linear regression, $R^2 \geq 0.95$ (Jiménez-Pelayo et al., unpublished results). Doubling time was determined by applying non-linear regression analysis and an exponential growth equation as described by Regidor-Cerrillo et al. (2011). Finally, a chi-square test was employed to address possible differences regarding the different invasion events between both isolates.

Statistical analysis was performed with GraphPad statistic software (San Diego, CA, USA).

3. Results

3.1. Bb-Spain 3 in vitro behaviour

At 24 hpi, the maximal IRs of Bb-Spain 1 and Bb-Spain 3 isolate was 3% and 2–3%, respectively ($P > 0.05$) (Fig. 3A). However, there were no statistically significant differences for Bb-Spain 1 and Bb-Spain 3 among the different time points studied (Fig. 3A, ANOVA; $P < 0.05$). Nevertheless, there was an increase in the number of parasites that had invaded longer than 24 hpi (Fig. 3A).

Regarding parasite proliferation results evidenced that there was no multiplication of the parasite until 24 hpi, establishing a lag phase no longer than 24 hpi (Figs. 3 B and 4). Growth curves obtained for both isolates fitted an exponential growth pattern (Fig. 3B, $R^2 > 0.95$) and both isolates showed a significant increase in tachyzoite numbers between 24 and 48 hpi ($P < 0.05$, ANOVA, Mann-Whitney *U* test) (Figs. 3 B and 4). Significant differences were found when mean tachyzoite yields (tachyzoites/ng DNA) for both isolates at 72 hpi were compared ($p < 0.05$, Mann-Whitney test). Doubling times during the exponential growth period were 12.74 ± 4.66 and 10.45 ± 2.37 h for Bb-Spain 1 and Bb-Spain 3 isolates, respectively.

Egress of tachyzoites after lysis of their host cells was observed by light microscopy between 48 and 72 hpi (Fig. 4). After 72 hpi, the infected cultures exhibited small PVs, large PVs and lysis plaques in both isolates (Fig. 3C). These three events were simultaneously present and

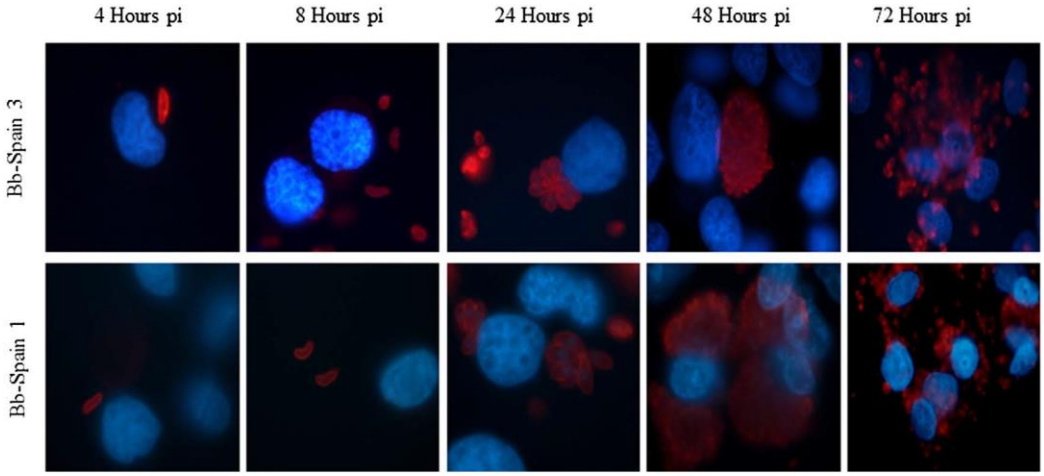

Fig. 4. Microscopic follow up of invasion events over 72 hpi in Marc-145 cell cultures infected with Bb-Spain1 and Bb-Spain3 isolates.

homogeneously distributed over the cell monolayer for Bb-Spain 1 and Bb-Spain 3 isolates. In Bb-Spain 1, the predominant event was the formation of lysis plaques (65.5% of the events) *versus* large and small PVs (14% and 20.4%, respectively). For isolate Bb-Spain 3, the different invasion events were similarly represented (32.1% small PVs, 30.9% large PVs, and 37% lysis plaques) and differed significantly from Bb-Spain 1 isolate ($P < 0.05$, ANOVA) (Fig. 3C).

3.2. Specific anti-B. besnoiti IgG antibodies

Anti-*B. besnoiti* antibodies were detected in the calf at 4 months of age (RIPC = 165.5), in the adult cow of the same farm (RIPC = 27.5), and simultaneously in the Charolais cow from the nearby farm (RIPC = 61.4). Specific antibody levels remained high in the calf at all samplings analysed at 6, 7 and 8 months of age (RIPC = 152.7, 140.5, 166.6, respectively), until it was slaughtered. The avidity indexes tested in those same samples were 48, 73, 74 and 60.

3.3. Lesions, histopathology, parasite distribution and burden in positive samples

3.3.1. Gross lesions

Macroscopic tissue cysts were detected in the ocular conjunctiva, epiglottis and the nasal turbinate of the calf (Fig. 1). Tissue cysts were visualized through the trichinelloscopy plates in the biopsies collected from the tarsal region in the calf and from the *vestibulum vaginae* in the adult cow (Fig. 1).

3.3.2. Microscopic lesions

Microscopic lesions were mostly related to the presence of tissue cysts and were found in the skin and oral mucosa. They were characterized by a multifocal infiltration of lymphocytes, macrophages, plasma cells and neutrophils in the epithelium, where there was also hydropic degeneration of the keratinocytes and lamina propria-submucosa of the tongue. A similar infiltration was found in the dermis of the skin from the tarsus, nipple and the perineal area. In the vagina, there were a few small foci of mononuclear inflammatory cells in the propria-submucosa in the absence of tissue cysts.

3.3.3. HP and IHC

Parasite tissue cysts were detected in 15 different locations. Cysts were observed in the tissue sections from skin samples (neck region, upper-eyelid, carpal, tarsal, ear pinna, thigh region, nipple and perineal area), the respiratory system (tongue, muzzle, nasal turbinate, nasal sinus and pharynx), the reproductive tract (vulva) and the ocular conjunctiva (Table 1). The skin and the upper respiratory track (muzzle and nasal turbinates) were the most parasitized locations.

Parasite tissue cysts were classified into three categories, according to Langenmayer et al. (2015b): a) developed tissue cyst, b) developed tissue cyst with pericystic inflammation, and c) lysed cyst with necrotic debris and pericystic inflammation (Table 1 and Fig. 2).

A subjective evaluation of the immunohistochemical labelling of cell populations in the inflammatory lesions showed that the infiltrate was mainly formed by CD3+ lymphocytes. Macrophages were mostly positive to the labelling for myeloid/histiocyte-antigen (MAC387 clone) and negative for CD68 expression (Fig. 2). The host cells of the parasite cysts were negative to all antibodies employed.

3.3.4. PCR detection and quantification

As shown in Table 1, 30 tissues samples were positive by PCR. From the 16 locations where parasite cysts were found in the histological examination, 13 of them were also positive by PCR analysis, whilst no *Besnoitia* spp. DNA was detected in the vulva, the skin from the neck, and the ocular conjunctiva, tissues where parasite tissue cysts were evidenced by HP.

On the other hand, *Besnoitia* spp.DNA was amplified in samples where no tissue cysts were found by histological examination (atrium, ventricle and aorta, as well as trachea, bronchi and lungs). The reproductive tract from cervix to ovaries and skin samples from the different areas were also PCR positive (Table 1).

Regarding the lymphatic system, only the bronchial lymph node and tonsils yielded positive results. Remarkably, parasite DNA was detected in the brain. Other assessed tissues, such as the ocular sclera and the auditory canal, were also positive. Finally, the parasite was not detected in the kidney, liver and the digestive track.

The amount of tachyzoites/ng cow DNA in the ITS1PCR positive samples are shown in Table 1. The highest parasite loads corresponded to skin samples from the tarsal, carpal and thigh regions and the upper-eyelid, followed by the perineal skin. Low parasite loads were detected in the respiratory and reproductive tracks, but for the ovaries, the load was relatively higher.

4. Discussion

Bovine besnoitiosis is rare in calves and cases of chronic disease have not been described up to date. Thus, the control of this re-emergent disease focus solely on adult cattle. In the present study, we have obtained and characterized the first isolate from calf origin and we have described this unusual case report in a calf younger than 6 months. The identity of *Besnoitia* species present in the calf was determined after parasite isolation (named as Bb-Spain 3 isolate) and molecular genotyping. The newly obtained Bb-Spain 3 isolate had been already

genotyped by Gutiérrez-Expósito et al. (2016) (named as skin biopsy 3). Six microsatellite sequence loci were sequenced and they showed identical microsatellite patterns compared with other *B. besnoiti* isolates from different countries. Herein *in vitro* behaviour of the new isolate was compared with Bb-Spain 1 reference isolate. We have evidenced again that *B. besnoiti* shows limited invasion capacity (low invasion rates), requires prolonged time to invade host cells and shows high extracellular survival periods that may extend up to 24 hpi regardless the isolate, in contrast to *N. caninum* or *T. gondii* (Naguleswaran et al., 2003; Dellarupe et al., 2014). Moreover, Bb-Spain 1 isolate showed similar results as those obtained by Frey et al. (2016), where Bb-Spain 1 isolate was classified as a low invader (3.2% IR) and low prolific isolate. Accordingly, this *in vitro* culture system represents a useful and reproducible tool for further assays including the study of *B. besnoiti* intraspecies variability or parasite drug screenings.

Bb-Spain 3 behaved similarly to Bb-Spain 1 and was classified in the same category like other European isolates (Bb- Spain 2 and Bb- Italy 2) with IR around 3%. Both isolates showed a growth curve that fitted an exponential growth up to 72 hpi, a lag phase and doubling times in accordance to other Spanish isolates (Frey et al., 2016), despite some inherent variability. However, the significantly different TYs suggest that Bb-Spain 3 has a slower lytic cycle and is less prolific than Bb-Spain 1, which was corroborated by immunofluorescence findings being lysis plaques less frequent. Similar to other *B. besnoiti* isolates, the new isolate showed non-synchronic growth pattern at 72 hpi, since PVs and lysis plaques coexisted. However, different percentages of invasion outcomes were found. Bb-Spain 3 isolate produced more PVs, whereas Bb-Spain 1 isolate showed more lysis plaques. In agreement, Frey et al. (2016) reported that lysis plaques represented up to 80% of the invasion outcomes at 72 hpi for Bb-Spain 1 isolate.

Biological diversity is another remarkable difference between the closely related *N. caninum* or *T. gondii* and *B. besnoiti,* that relies on extended genetic variability (Regidor-Cerrillo et al., 2013; Verma et al., 2015) and a correlation between *in vitro* phenotypic traits and *in vivo* virulence (Jungersen et al., 2002; Regidor-Cerrillo et al., 2011, 2014). In contrast, genetic homogeneity is characteristic of all *B. besnoiti* isolates and no *in vitro* parameter was found indicative of the parasite virulence in cattle as previously reported by Frey et al. (2016).

The present study also represents the first thorough description of a case report of chronic bovine besnoitiosis in a calf less than 6 months of age. It has already been demonstrated that clinical cases in young cattle rarely occur in animals under 1 year of age (Pols, 1960). These findings might be explained by i) the lower risk of young animals being exposed to the parasite by the horizontal transmission route, ii) a lower receptivity of a young calf due to passive immunity acquired from the dam, and iii) innate protective immunity as it apparently occurs in *Babesia* spp. infections in young cattle (Bock et al., 2004; Zintl et al., 2005). However, these clinical cases might be more frequent that originally thought. Hornok et al. (2014) reported three seropositive calves under 6 months of age showing respiratory signs. In addition, Ryan et al. (2016) mentioned that exposure to besnoitiosis occurs in animals before six months of age, which was evidenced by the detection of 41.9% of calves with scleral tissue cysts. More recently, a systemic chronic besnoitiosis in a juvenile roe deer was reported, and molecular findings verified *B. besnoiti* identity (Arnal et al., 2017). However, up to now, all reported severely chronically infected animals with skin lesions and wide parasite dissemination corresponded to adult animals (Álvarez-García et al., 2014a).

Herein, the clinical signs compatible with the disease (alopecia, folding and thickening of the skin), tissue cysts detection, specific anti-*Besnoitia* spp. antibodies (also corroborated by western blot; data not shown), as well as the avidity maturation of the IgGs, led us to confirm chronic besnoitiosis in a calf, similar to previous descriptions in adult animals (Álvarez-García et al., 2014a; Gollnick et al., 2015). This severe case of chronic besnoitiosis was characterized not only by macroscopic lesions but also by wide intra-organic parasite distribution evidenced by HP and PCR techniques. Skin samples at different locations showed a high parasite load, followed by the upper respiratory tract, vulva and ocular conjunctiva. These locations are predilection sites for *B. besnoiti* that show tropism for the connective tissue of the superficial skin layers, scleral, conjunctiva and mucous membranes of the upper respiratory and distal genital tracts (Pols, 1960; Nobel et al., 1981; Manuali et al., 2011; Gentile et al., 2012; Frey et al., 2013). Skin lesions, along with the high parasite load detected, evidenced a dermatotropic tropism of the parasite. Remarkably, *B. besnoiti*-DNA was also detected in the lower respiratory tract (bronchi and lungs), reproductive system (cervix, uterus, both oviducts and ovaries), circulatory system (heart and aorta), tonsils, the bronchial lymph nodes and brain. Similarly, Basso et al. (2013) and Langenmayer et al. (2015b) found *B. besnoiti* in these locations in adult cattle. In particular, we corroborated for the second time that *B. besnoiti* may be present in the CNS in cattle. The parasite stage present remains to be elucidated. Interestingly, Arnal et al. (2017) recently detected tissue cysts in the brain of a roe deer, which had affected the grey and white matter. The cyst size was smaller compared to those located in the target locations. The authors suggested that an immune response could have limited cyst growth, which was supported by the inflammatory response found in the brain cortex, considering that tissue cyst development is rather synchronous. The parasite load was low in the remaining PCR positive tissues. It is possible that either intracellular tachyzoites or tissue cysts were present in these highly vascularised tissues since endothelial cells are the primary target cells for initial parasite replication (Basson et al., 1970). Moreover, previous studies showed that these tissues do not harbour any or very seldom contain singular cysts (McCully et al., 1966; Langenmayer et al., 2015b). The low parasite load detected in these locations may explain the discordant results obtained between the histologic and more sensitive PCR techniques (Schares et al., 2011).

Another distinct feature of chronic bovine besnoitiosis was the detection of three different type of cysts found herein, and reported by other authors in chronically and sub clinically infected adult cattle (Frey et al., 2013; Langenmayer et al., 2015a). Viable cysts without any signs of pericystic infiltrates, cysts with marked pericystic inflammation and degenerated cysts were found. Previous studies in *Besnoitia* spp. infections agreed that viable tissue cysts might show variable host immune reactions. These reactions vary from an absent inflammatory response to an intense granulomatous response (Nobel et al., 1981). Langenmayer et al. (2015b) reported apparently degenerated cysts, which appeared from 30 days post-infection onwards (Basson et al., 1970), surrounded by inflammatory cells. According to this author, the lysis of degenerated cysts could occur when pericystic inflammation breaks the hyaline layer. Frey et al. (2013) suggested that in these cases, bradyzoites could reach the bloodstream, leading to parasite exposure to the immune system and the development of high levels of specific antibodies as detected herein. Several authors have recently characterized the inflammatory infiltrate surrounding the tissue cysts in chronically infected cows; it was composed of numerous T-lymphocytes, recently recruited macrophages and fewer eosinophils, which agree with the present findings (Frey et al., 2013; Langenmayer et al., 2015b).

High avidity antibodies were also indicative of the establishment of a chronic infection, despite having an avidity index lower than the established cut off in the first sampling. Schares et al. (2013) claimed that the avidity maturation of IgG is slower than cyst development.

Our hypothesis is that this calf might have been infected through horizontal transmission, as it usually occurs in adult cattle, either by direct contact with the infected adult cow or by hematophagous arthropod bites. The serological analyses in the calf ruled out the vertical transmission since colostral antibodies decrease after 2 months post-infection (Shkap et al., 1994) and the calf's mother was seronegative (data not shown). We ignore whether any immunosuppressive factor could have favoured the outcome of the infection in the calf. An association between BVDV infection and other protozoan infections (e.g., *N.*

caninum) have been reported in cattle (Waldner 2005; Vanleeuwen et al., 2009). In the present study, BVDV infection was discarded in an ear notch sample of the calf (data not shown). However, other immunosuppressive factors were not excluded.

5. Conclusion

In summary, we have isolated *B. besnoiti* from a calf for the first time. Genotyping or *in vitro* behaviour differences of the new isolate were not found with respect other Spanish isolates obtained from adult cattle. Moreover, this is the first exhaustive description of a severe clinical case of chronic bovine besnoitiosis reported in a calf less than 6 months of age. Lesions, clinical signs, parasite load and intra-organic distribution were characteristic of a severe chronic besnoitiosis and demonstrated the rapid progression of the disease (Nobel et al., 1981; Frey et al., 2013; Gollnick et al., 2015). Further studies should elucidate the molecular mechanisms that may underlay the disease progression. This case report shows the need to include the inspection of young animals in the control of the disease.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2017.09.017 .

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ORIGINAL ARTICLE

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Effect of parasite dose and host age on the infection with *Besnoitia besnoiti* tachyzoites in cattle

Carlos Diezma-Díaz[1] | Alejandro Jiménez-Meléndez[1] | Michela Tatiana Re[1,2] | Ignacio Ferre[1] | Maria del Carmen Ferreras[3] | Daniel Gutiérrez-Expósito[1,3] | Silvia Rojo-Montejo[1,4] | Alicia Román-Trufero[4] | Julio Benavides-Silván[3] | Paula García-Lunar[1] | Lydia Calleja-Bueno[2] | Javier Blanco-Murcia[1,2] | Koldo Osoro[4] | Luis-Miguel Ortega-Mora[1] | Gema Álvarez-García[1]

[1]SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Madrid, Spain

[2]Animal Medicine and Surgery Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Madrid, Spain

[3]Livestock Health and Production Institute (ULE-CSIC), León, Spain

[4]Regional Service for Research and Agri-Food Development (SERIDA), Animal Production Systems, Villaviciosa, Spain

Correspondence
Gema Álvarez García, SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.
Email: gemaga@vet.ucm.es

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Abstract

Bovine besnoitiosis is continuing to spread in Europe. Therefore, the development of ruminant animal models of infection is urgently needed to evaluate therapeutic and prophylactic tools. Herein, we studied the effect of parasite dose and host age on the infection dynamics with *Besnoitia besnoiti* tachyzoites in cattle in two independent experimental infections. In experiment A, twelve 3-month-old male calves were inoculated intravenously with either three different doses of tachyzoites (G1: 10^8; G2: 10^7; G3: 10^6) or with PBS (G4). In experiment B, six 14-month-old bulls were inoculated with 10^6 tachyzoites based on results obtained in experiment A. In both trials, clinical signs compatible with acute and chronic besnoitiosis were monitored daily; blood and skin samples were collected regularly for 70–115 days post-infection (pi). Finally, animals were killed, and tissues were collected for lesion and parasite detections. Infected animals developed mild–moderate signs compatible with acute besnoitiosis. Lymphadenopathy and fever were observed in both calves (from 12 hr until 7 days pi) and bulls (from 6 days until 9 days pi). Seroconversion was detected at 16–19 days pi, and antibody levels remained high. Infected animals did not developed characteristic clinical signs and macroscopic lesions of chronic besnoitiosis. However, successfully, parasite-DNA was detected in a reduced number of target tissues: conjunctiva, ocular sclera, epididymis, skin of the scrotum and carpus in calves (n = 10, 6 of which belonged to G3), and pampiniform plexus and testicular parenchyma in bulls. Remarkably, one tissue cyst and mild microscopic lesions were also detected. In summary, inoculated animals developed the acute besnoitiosis and chronic infection was evidenced by microscopic findings. However, our results suggest that tachyzoite dose and host age are not key variables for inducing clinical signs and macroscopic lesions characteristic of chronic besnoitiosis. Thus, a further refinement of this model should evaluate other parasite- and host-dependent variables.

KEYWORDS

Besnoitia besnoiti, bull, calf, experimental infection, tachyzoites

1 | INTRODUCTION

Besnoitia besnoiti is a cyst-forming apicomplexan parasite responsible for bovine besnoitiosis. At present, the disease is considered to be re-emerging in Europe by the European Food Safety Authority due to an increased number of cases and the geographic expansion of besnoitiosis into cattle herds in several Western and Central European countries (Álvarez-García, 2016; European Food Safety Authority, 2010). The control and prevention of this disease relies only on diagnostic and management measures, as no chemotherapeutics are available and no vaccines are licensed in Europe (Álvarez-García, Frey, Mora, & Schares, 2013). Promising drugs have been tested in vitro (Cortes, Muller, Boykin, Stephens, & Hemphill, 2011; Jiménez-Meléndez et al., 2017). However, an appropriate ruminant experimental model has not been developed yet; this model is a requirement to test the safety and efficacy of these potential prophylactic and therapeutic tools.

In this regard, only *B. besnoiti* tachyzoite and bradyzoite stages have been used so far for the inoculation of animals. *Besnoitia besnoiti*-oocysts are not available as the definitive host remains unknown (Basso, Schares, Gollnick, Rutten, & Deplazes, 2011). Several laboratory rodents (hamster, gerbils and various strains of white mice) have been infected with *B. besnoiti* (Basso et al., 2011; Shkap, Pipano, & Greenblatt, 1987). Although gamma-interferon knockout mice are very susceptible to the acute infection (Schares et al., 2009), they are not recommended as an appropriate laboratory model due to the rapid onset of the acute stage and death. Rabbits showed susceptibility to experimental infection by developing clinical signs of the acute phase of the disease (Lienard et al., 2015), but the results were variable with respect to the chronic stage (Basson, McCully, & Bigalke, 1970; Bigalke, 1968). The disease was successfully replicated in these animals through subcutaneous inoculation of bradyzoites (6×10^6) (Lienard et al., 2015) resulting in mild clinical signs and the formation of cysts in the leg dermis, nasal mucosa, eyelid and penis. However, the authors suggested that the establishment of *B. besnoiti* and its adaptation to a heterologous host was difficult.

Between the 1960s and 1980s, there were a few attempts to establish a reproducible in vivo model for bovine besnoitiosis, but failed due to the difficulty in inducing the characteristic clinical signs (Álvarez-García, García-Lunar, Gutiérrez-Expósito, Shkap, & Ortega-Mora, 2014). Unfortunately, all the inoculations were carried out under different experimental conditions (e.g., different ages of the infected animals, unknown previous health status and use of immunosuppressive therapies). Moreover, the inocula were obtained from different sources, such as blood or tissue cysts from cattle with chronic besnoitiosis (Bigalke, 1967), blood from acutely infected rabbits (Basson et al., 1970) or from a blue wildebeest strain maintained in cell culture (Bigalke, Schoeman, & McCully, 1974). In infected animals, fever and lymphadenopathy have been detected on several occasions (Basson et al., 1970; Bigalke, 1968; Bigalke et al., 1974). However, specific clinical signs of the acute phase, such as oedema and anasarca, and of the chronic phase (tissues cysts) were only reported in infected adult animals (Basson et al., 1970), as well as in immunosuppressed animals that had been infected with bradyzoites by Diesing et al. (1988). This heterogeneity in the experimental assay parameters does not allow for the development of an experimental model of besnoitiosis in cattle. Additionally, most results have been based on clinical inspection and histopathology, as molecular tests were not available and serological tests were rarely employed, which is crucial as immunity to re-infections has been reported (Basson et al., 1970; Bigalke, 1968; Janitschke, De Vos, & Bigalke, 1984; Shkap et al., 1987).

Nevertheless, relevant information concerning pathogenesis has been derived from these trials. It is known that both tachyzoite and bradyzoite stages are infective for cattle (Bigalke, 1967, 1968; Diesing et al., 1988; Pols, 1960). We expect that the parasite dose is important in determining the severity of the infection. Moreover, the incubation period may depend on the route of infection. Information regarding age suggests that the disease is mainly present in older animals due to longer periods of exposure (Álvarez-García et al., 2014). However, the disease was recently confirmed in a 4-month-old calf (Diezma-Díaz et al., 2017), demonstrating that animals younger than 6 months old can also be chronically infected.

The aim of this study was to study the effect of parasite dose and host age on the infection dynamics with *B. besnoiti* tachyzoites in cattle and their impact on both the acute and chronic phases of the disease. Accordingly, two independent experimental infections were carried out in calves and bulls. We addressed the limitations of previous trials by: (a) the employment of a well-characterized *B. besnoiti* isolate; (b) checking the viability and quality of the inoculum and (c) checking the animal health status prior to inoculations. Moreover, exhaustive clinical monitoring and regular samplings were carried out, employing complementary serological, histological and molecular tools.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All our experimental procedures were approved by the Animal Welfare Committee of the Community of Madrid, Spain, following proceedings described in Spanish and EU legislations (PROEX 92/14, Law 32/2007, R.D. 53/2013), and Council Directive 2010/63/EU.

Animals were housed in clinical facilities belonging to the Faculty of Veterinary Sciences of Complutense University of Madrid (Number of register: ES280790000101).

2.2 | Animals, experimental design, clinical monitoring and collection of samples

The experimental design followed in both trials is shown in Figure 1. The same clinical and laboratory parameters were evaluated with slight differences in their periodicity.

2.2.1 | Experiment A

The calves used in this study came from a single Holstein Friesian dairy herd located in Madrid province and were free from relevant infectious (tuberculosis and brucellosis) and parasitic diseases of dairy cattle. Twelve 2-month-old male calves were selected after assessing the absence of specific antibodies against *B. besnoiti* and other closely related sarcocystidae parasites (*Neospora caninum*, *Toxoplasma gondii* and *Sarcocystis* spp.) by Western blot. Prior to the inoculation and to guarantee the health status of the animals, the calves were vaccinated against Bovine Respiratory Syndrome (Cattle Master®). The absence of gastrointestinal parasites was checked by means of coprological analyses. Additionally, haematological and biochemical analyses were also carried out.

Calves were randomly allocated into four different groups of three animals each. Group 1 (G1) was inoculated intravenously with 10^8 tachyzoites, group 2 (G2) with 10^7 tachyzoites and group 3 (G3) with 10^6 tachyzoites by the same route of administration. Group 4 (G4), used as a non-infected control group, was inoculated with PBS and MARC-145 cells, where tachyzoites are routinely propagated (see Section 2.3 for the inoculum). Rectal temperatures and other clinical signs characteristic of either acute or chronic phase infections were monitored periodically along the experimental period (Figure 1). Any temperature above 39.5°C was considered as a fever. A clinical scoring system was established to classify the severity of the outcome of the infection (Table 1). The number of tissue cysts in the ocular conjunctiva was estimated and assigned to the following categories: 1: 1–10 cysts; 2: 11–20 cysts; 3: 21 to >30 cysts in both eyes (Gollnick, Scharr, Schares, & Langenmayer, 2015).

A criterion for the humanitarian slaughtering of the animals consisting of 1 day of total anorexia and prostration was established.

Blood samples were collected regularly until the end of the experiment by jugular venipuncture into 5-ml Vacutainer tubes (Becton Dickinson and Company, Plymouth, UK) with and without ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Haematological and biochemical parameters were assessed in blood samples at 7 days prior to infection and at 4, 7, 14 and 21 days post-infection (pi). For biochemical and serological analyses, the blood samples that were collected in vacutainers without anticoagulant were allowed to clot and were centrifuged (1,200× *g* for 10 min) to obtain serum samples (stored at −80°C until use). For haematological analyses and detection of parasitaemia, the blood samples that were collected in vacutainers containing EDTA were immediately processed to obtain peripheral blood mononuclear cells (PBMC).

Once a week, two skin biopsies from each animal were collected from the inner thigh using 6- to 8-mm-diameter biopsy punches to perform *B. besnoiti* ITS-1 PCR and histopathology. At the end of the

TABLE 1 Clinical score employed and based on the severity of the clinical signs compatible with acute and chronic phases of bovine besnoitiosis monitored throughout the experimental infections

Score	Category	Acute besnoitiosis	Chronic besnoitiosis
0	Absence of infection	None	None
1	Mild	Local lymphadenopathy	Systemic lymphadenopathy and/or congestive conjunctiva and/or conjunctival cysts (cat. 1) and/or ocular secretion[a]
2	Moderate	Fever and/or systemic lymphadenopathy and/or cough/nasal secretion and/or congestive conjunctiva[a]	Systemic lymphadenopathy and conjunctival cysts (cat. 2) and/or ocular secretion
3	Severe	Oedema, orchitis, lameness, anorexia	Systemic lymphadenopathy and conjunctival cysts (cat. 3) and/or ocular secretion and/or skin lesions[a]

[a]At least the detection of two clinical signs.

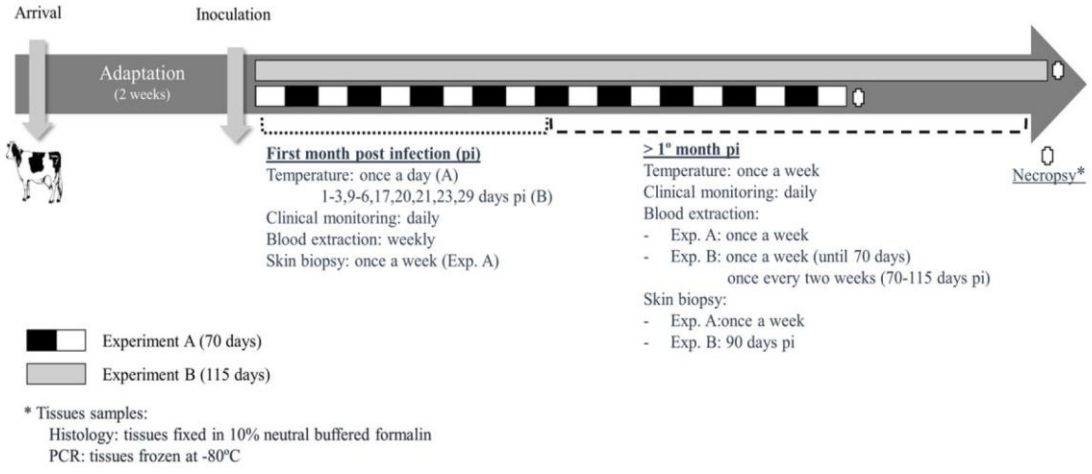

FIGURE 1 Timeline, clinical monitoring and sample collection in experimentally infected calves (a) and bulls (b) [Colour figure can be viewed at wileyonlinelibrary.com]

experiment, the animals were sedated with xylazine hydrochloride (Rompun®; Bayer, Mannheim, Germany) and immediately killed by an intravenously overdose of embutramide and mebezonio iodide (T61®; Intervet, Salamanca, Spain). Post-mortem examination of the calves was carried out immediately after euthanasia and tissue samples were collected for PCR and histopathological analyses. Tissues collected were as follows: reproductive system (testis, epididymis head, body and tail, vasa deferential, bulbourethral gland, prostatic gland, seminal vesicles and penis), respiratory tract (nostrils, nasal turbinates, larynx, pharynx, epiglottis, trachea, bronchi and lungs), digestive tract (tongue), 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) as well as other organs (ventriculum, atrium, ocular conjunctiva, sclerotic, distal fascia-tendon from the rear leg and hoof corium).

2.2.2 | Experiment B

Six 14-month-old young bulls (B1-B6) of the Asturiana de la Montaña breed were included in this trial. Health status monitoring of the animals and laboratory analyses were done as in experiment A. The animals were inoculated with 10^6 tachyzoites through jugular venipuncture (see Section 2.3). The experimental design in experiment B was similar to A. The periodicity of clinical monitoring and skin biopsies and blood samplings are shown in Figure 1.

Bulls were killed at 115 days pi. Tissues from the reproductive tract (testicular parenchyma, epididymis, pampiniform plexus and penis), respiratory tract (nostrils, epiglottis and trachea), skin (scrotum, carpus, tarsus, elbow, perineum and pinna), spleen and ocular conjunctiva were collected for PCR and histopathological analyses.

2.3 | Parasites

Tachyzoites from the Bb-Spain 3 isolate were inoculated in both experiments. This isolate was obtained from a calf with chronic besnoitiosis and showed similar in vitro traits as other Spanish isolates (Diezma-Díaz et al., 2017). Tachyzoites were propagated in a MARC-145 cell monolayer (passage number = 4) according to a previously described protocol (Fernández-García et al., 2009) and were free of bovine viral diarrhoea virus (BVDV) and *Mycoplasma* spp. Tachyzoites were recovered from the cell monolayer when the majority of the parasites were still intracellular. The tachyzoites were then counted using a Neubauer chamber and parasite viability was determined using trypan blue exclusion and a plaque invasion assay by counting lysis plaques and parasitophorous vacuoles (Rojo-Montejo et al., 2009).

To prepare the inocula, tachyzoites were resuspended in phosphate buffer saline (PBS) at the required dose (10^6, 10^7 and 10^8 tachyzoites in a final volume of 2 ml) and were administered to animals within 1 hour of harvesting from cell culture. MARC-145 cells were also counted using a Neubauer chamber to determine the number of cells per dose. These were resuspended in PBS and inoculated in G4.

2.4 | Biochemical and haematological analyses

Biochemical and haematological analyses were performed according to procedures previously described (Langenmayer, Scharr, Sauter-Louis, Schares, & Gollnick, 2015). They were analysed by comparing values from infected animals with bovine reference values, taking into account that values may vary due to age and environmental factors (George, Snipes, & Lane, 2010).

2.5 | IFN-γ innate responses

IFN-γ levels were measured in sera by the Bovine IFN-γ ELISA development kits (Mabtech AB, Stockholm, Sweden), following manufacturer's recommendations. Colour reaction was developed by the addition of 3, 3′, 5, 5′-tetramethylbenzidine substrate (TMB, Sigma–Aldrich, Madrid, Spain) and incubated for 5–10 min in the dark. Reactions were stopped by adding 2N H_2SO_4. Then, plates were read at 450 nm. The cytokine concentrations were calculated by interpolation from a standard curve generated with recombinant cytokines provided with the kits.

2.6 | Humoral immune responses

Besnoitia besnoiti–specific IgG was determined by ELISA using lyophilized *B. besnoiti* tachyzoites as antigen (García-Lunar, Ortega-Mora, Schares, Diezma-Díaz, & Álvarez-García, 2017). Serum samples were analysed and the value of the optical density (OD) was converted into a relative index per cent (RIPC) using the following formula: RIPC = (OD_{405} sample − OD_{405} negative control)/(OD_{405} positive control − OD_{405} negative control) × 100. An RIPC value ≥17.34 indicates a positive result.

Besnoitia besnoiti–specific IgG1 and IgG2 serum isotypes were also determined by ELISA. Briefly, ELISA was performed as described above using a 1:100 dilution of sera samples and anti-bovine IgG1 or IgG2 peroxidase conjugated as secondary antibody (1:1,000; Bio-Rad, Hercules, California, USA). Sera from naturally infected and non-infected cattle were used as positive and negative controls respectively. For each plate, the values of the optical density read at 405-nm wavelength (OD405) were converted into the corresponding RIPC value as described above.

2.7 | DNA extraction and PCR determinations

Whole blood collected in a heparin tube was used to obtain PBMC. An aliquot of 800 μL of blood was gently mixed for 5 min with 960 μl of cold lysing solution (0.83% NH_4Cl + 2.06% Tris) at room temperature. After a centrifugation for 30 s at 12,000× *g*, the supernatant was decanted, and the process was repeated until the liquid was clear red. The pellet was stored at −80°C. Genomic DNA was extracted from PBMC and tissue samples using the Maxwell® 16

MouseTail DNA Purification Kit (Promega, Wisconsin, USA). The DNA content of each sample was measured using a NanoPhotometer® (Implen, Munich, Germany) and adjusted to 40 ng/µl. ITS-1 PCR was performed as described previously (Cortes et al., 2007).

2.8 | Histopathology and immunohistochemistry

Tissue samples and biopsies were fixed in 10% neutral buffered formalin and conventionally processed through a graded alcohol series before being embedded in paraffin wax. Four-µm-thick sections were obtained and stained with haematoxylin and eosin (H/E).

The immunohistochemical labelling was performed on those samples with histological lesions consistent with *B. besnoiti* infection and those where PCR-positive results were obtained. A primary in-house rabbit polyclonal antibody against *B. besnoiti* tachyzoite antigens (Gutiérrez-Expósito et al., 2012) was used for this purpose at 1:4,000 dilution. Briefly, an enzymatic digestion was carried out with trypsin, the primary antibody was incubated overnight at 4°C and, later on, a polymer-based detection system was used (EnVision+ System Labelled Polymer-HRP anti-rabbit; Dako, Glostrup, Denmark). The reaction was developed with 3, 3′-diaminobenzidine tetrahydrochloride (DAB Peroxidase Substrate Kit; Vector Laboratories, California, USA). The sections were counterstained with Mayer's haematoxylin. The specificity of the technique was assessed by omitting the primary antibody and also using pre-immune rabbit serum.

2.9 | Statistical analysis

Rectal temperatures and serology were analysed using a two-way ANOVA repeated measures test followed by a Tukey post-test. Statistical significance for all analyses was assessed with $p < 0.05$. All statistical analyses were carried out using GraphPad Prism 6.01 software (San Diego, CA, USA).

3 | RESULTS

3.1 | Clinical signs and clinical score

From the first day pi onwards, the mean temperatures of the infected calves were significantly higher than the uninfected group ($p < 0.001$) (Figure 2). However, there were no significant differences between infected groups ($p > 0.05$; two-way repeated measures ANOVA, Tukey post-test). All infected animals presented with fever from 1 day pi onwards until the end of the first week. The onset of fever was earlier in the higher dose–infected group (G1). These animals showed temperatures higher than 39.6°C at 12 hr pi. The highest temperature values were observed in one animal from G1 at 1 and 3 days pi (40.8°C and 40.3°C respectively). The remaining calves from G1 also developed high temperatures up to 40.3°C. A febrile response was also observed in all calves from G2 for as long as 7 days pi. The highest values from G2 were observed in one animal at 1 and 3 days pi (40.3°C and 40.7°C respectively). In G3, all animals developed fever. The highest values were observed in two animals at 4 days pi (40.1°C and 40°C). Animals from the uninfected control group (G4) had temperatures below 39.5°C throughout the experimental period.

The only temperatures higher than 39.5°C at 8 days ($n = 3$) and 9 days pi ($n = 1$) were detected in four of six infected bulls (Figure 2).

All calves from G1, G2 and G3 developed lymphadenopathy at 4 days pi in at least two of the three lymph nodes (submandibular, pre-scapular and pre-crural) examined. Lymphadenopathy remained longer in G1, for as long as 34 days pi *versus* 30 days pi in G2 and G3. Respiratory signs were also detected sporadically in calves from infected groups. Most animals coughed and had respiratory distress with an increased expiratory effort, at least once during the first month pi, which is characteristic of diseases that affect the lower respiratory tract.

Other signs characteristic of the acute stage of the disease, such as oedema, orchitis or lameness, did not develop in any of the infected animals. The outcome of the acute infection was classified as a 'mild-moderate infection' according to the clinical score presented in Table 2a, as no severe clinical signs (orchitis, oedemas or lameness) were found. In vivo clinical signs characteristic of the chronic stage, such as skin lesions, were not detected.

All infected bulls developed lymphadenopathy in one or both lymph nodes (only pre-crural or pre-scapular was monitored in bulls). This enlargement was observed at 3 days pi in five of six bulls and after 6 days in all infected animals. Lymphadenopathy was maintained until 55 days pi but was more evident within the first 24 days. Respiratory signs such as cough and nasal discharge were observed mainly between 8 and 20 days pi. Respiratory distress with an increased expiratory effort was observed in all animals between 17 and 20 days pi. Similar to the calves, the bulls did not develop other signs of severe acute stage or chronic phase of the disease, and the outcomes of the infections were classified as mild–moderate based on the clinical score shown in Table 2b.

3.2 | Kinetics of IFN-γ innate responses

Maximum serum IFN-γ levels were observed at 7 days pi in G1 and at 4 days pi in G2 and G3 and were significantly higher compared to the control group ($p < 0.05$; two-way ANOVA). At 4 days pi, IFN-γ levels were significantly higher in G2 and G3 ($p < 0.05$; Tukey post-test) compared to G1 and bulls. IFN-γ levels decreased to basal values in all three infected groups from day 10 pi and remained low onwards (Figure 3a).

In bulls, IFN-γ levels peaked at 8 days pi and were significantly higher compared to calves ($p < 0.05$; Tukey post-test). Levels decreased to basal values at 16 days pi. (Figure 3a).

3.3 | Humoral immune responses

After infection, seroconversion was detected at 16 days in bulls, 17 days in G1 and G2 and at 21 days in G3. The antibody levels were significantly higher in infected calves than in G4 from 17 days

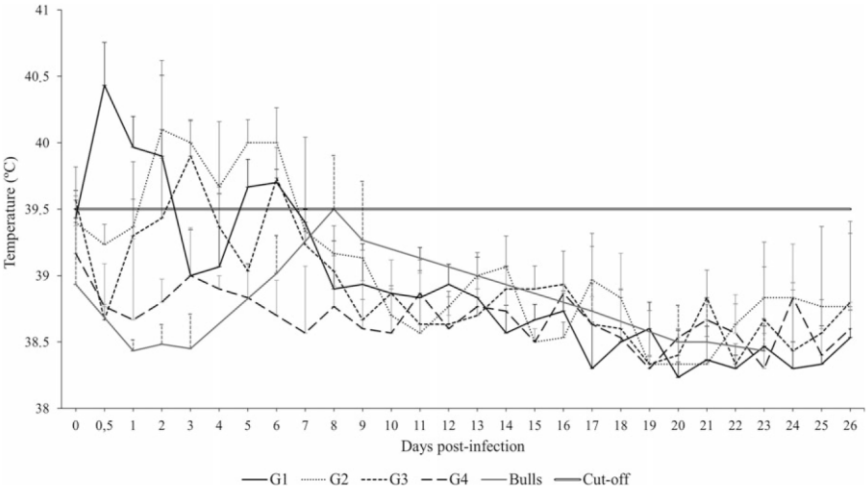

FIGURE 2 Mean rectal temperatures (+*SD*) recorded during the 1st month post-infection. The cut off for fever was ≥39.5°C (see legend)

TABLE 2 Clinical score estimated in calves (A) and bulls (B)

		Days post-infection										
A	**Group**	**0–2**	**3–6**	**7–9**	**10–13**	**14–16**	**17–20**	**21–23**	**24–27**	**28–30**	**31–34**	**34–70**
C1	1	2	2	2	2	2	1	1	1	0	1	0
C2	1	2	2	2	2	2	2	2	1	1	1	0
C3	1	2	2	2	2	2	1	1	1	1	1	0
C4	2	2	2	2	2	2	2	1	1	1	0	0
C5	2	2	2	2	2	1	1	1	1	1	0	0
C6	2	2	2	2	2	1	1	1	0	1	0	0
C7	3	2	2	2	2	2	2	1	0	1	0	0
C8	3	0	2	2	1	2	1	1	1	1	1	0
C9	3	0	2	2	2	2	2	1	0	1	0	0
C10	4	0	0	0	0	0	0	0	0	0	0	0
C11	4	0	0	0	0	0	0	0	0	1	0	0
C12	4	0	1	0	0	0	0	1	1	1	0	0

	Days post-infection																
B	**0**	**1**	**2**	**3**	**6**	**8**	**9**	**17**	**20**	**21**	**23**	**29**	**31**	**35**	**42**	**48**	**55**	**>64**
B1	0	1	1	2	2	2	2	2	2	2	1	1	1	2	1	1	1	0
B2	0	1	1	1	2	2	2	2	2	1	1	1	1	1	1	1	1	0
B3	1	1	1	2	2	2	2	2	2	1	1	1	1	1	1	1	1	0
B4	0	1	1	2	2	2	1	2	2	1	1	1	1	1	1	1	1	0
B5	0	0	0	0	1	1	1	2	1	1	1	1	1	1	1	1	1	0
B6	1	1	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	0

C: Calf (1–12); B: Bulls (1–6).

pi onwards ($p < 0.05$; two-way repeated measures ANOVA, Tukey post-test).

In calves, the higher dose–infected group (G1) showed significantly higher antibody levels compared with the lower dose–infected group (G3) starting at 17 days and remained higher from 24 days pi onwards ($p < 0.05$; two-way repeated measures ANOVA, Tukey test). However, there were no significant differences between G1 and G2 ($p > 0.05$; two-way repeated measures ANOVA, Tukey post-test). The antibodies kinetics were similar in calves and bulls with a few significant differences. The antibody levels in bulls were significantly higher than in the G4 from 16 to 70 days pi ($p < 0.05$; two-way repeated measures ANOVA, Tukey post-test) (see Figure 3b).

Significantly higher levels of IgG1 were detected in the infected groups compared to uninfected animals from 17 days pi ($p < 0.01$; two-way repeated measures ANOVA, Tukey post-test) (Figure 3c). Infected calves, G1 had significantly higher levels than the other

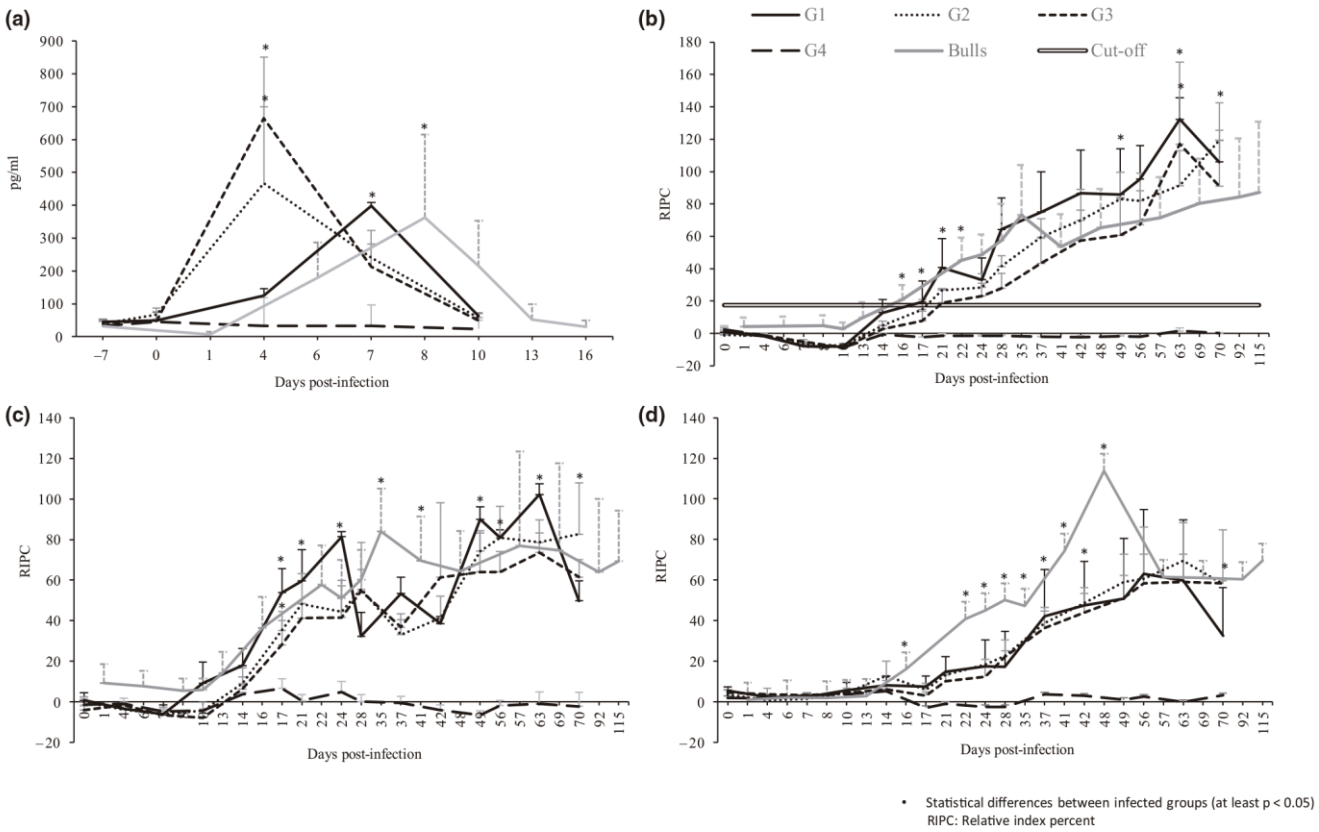

FIGURE 3 Immune responses measured in calves and bulls. (a): Innate IFN-γ responses measured in serum samples; (b): *B. besnoiti*–specific IgG responses; (c): Mean Relative Index (+*SD*) IgG1 antibody levels; (d): Mean Relative Index (+*SD*) IgG2 antibody levels

infected groups between 21 and 24 days pi ($p < 0.01$) and higher than G3 at 56 and 63 days pi ($p < 0.05$). The levels in G2 were significantly higher than G3 at 56 days pi, and higher than the other infected groups at the end of the trial ($p < 0.05$). IgG1 levels in bulls were significantly higher than infected calves between 37 and 41 days pi, ($p < 0.05$).

In calves, IgG1 levels were higher than IgG2 levels throughout the whole trial. Although IgG2 levels were detected approximately 21 days pi, significant differences between infected animals and the negative control group were not detected until 37 days pi (two-way repeated measures ANOVA, Tukey post-test $p < 0.05$). As IgG2 levels decreased in G1, significant differences were only observed between G1 and the other two infected groups in day 70 pi ($p < 0.01$).

An increase in IgG1-IgG2 levels was observed in bulls at 16 days pi and levels remained high until the end of the experiment. IgG2 levels were always lower than IgG1 except for 41 and 48 days pi, when IgG2 levels reached their highest values (Figure 3d). IgG2 levels were higher in bulls than in infected calves between 16 and 48 days pi ($p < 0.001$).

3.4 | Detection of *B. besnoiti* DNA in blood and tissues by PCR

Parasitaemia was detected sporadically in calves from G1 and G2 until 7 days pi. Parasitaemia was observed in two bulls between 10 and 24 days pi. However, the parasite was not detected in skin biopsies in any of the groups (Table 3).

After necropsy, 10 tissue samples from calves were PCR positive with the highest number of PCR-positive tissues (skin, reproductive and eyes samples) in G3. In the experiment B, only two tissue samples (testicular parenchyma and pampiniform plexus) from the same animal were positive by PCR (Table 3).

3.5 | Histopathology

Macroscopic lesions were not detected at necropsy. In contrast, compatible and characteristic microscopic lesions of chronic infection were found. Vasculitis was found in infected calves, particularly, in the skin (neck, tarsus, carpus, pinna and perineum), reproductive tract (testis, epididymis, vasa deferens and bulbourethral gland) and other locations (nostrils, tonsils, pre-scapular lymphnode, lung and hoof corium) (Supporting information Figure S1a). This lesion was characterized by the infiltration of lymphocytes, plasma cells, macrophages and eosinophils within the arteriole and venule walls and hyaline deposits at the arteriolar wall. Granulomatous vasculitis with foreign body giant cells in the testicle and reticular dermis of the perineum was observed in four positive-PCR animals: one from G1 (C3), two from G2 (C5, C6) and one from G3 (C9) (Table 3).

TABLE 3 Detection of *Besnoitia besnoiti* DNA in blood and tissues

Groups		Blood: days post-infection (animals)	Tissues (animals)
Calves	G1	4 (C2, C3); 7 (C1,C2,C3)	Carpal (C2) and scrotum skin (C3)
	G2	7 (C5, C6)	Proximal vas deferens (C5), epididymis body (C5) and scleral (C6)
	G3	Negative	Epididymis tail (C9), ocular conjunctiva (C7, C8), scleral (C8) and carpal skin (C7)
	G4	Negative	Negative
Bulls		10 (B3, B6), 13 (B6), 16 (B6), 24 (B3)	Parenchyma testicular (B2) and pampiniform plexus (B2)

C: Calf (1–12); B: Bulls (1–6).

Focal lymphoplasmacytic inflammatory infiltrates with the presence of eosinophils and macrophages were found in all infected animals. These infiltrates, often perivascular, were more numerous in lamina propria of the respiratory mucosa (larynx and nostrils), in skin dermis (scrotum, tarsus and neck) as well as in the stroma of genital organs (epididymis). Regarding other lesions in the male genital tract, C3 from G1 and C9 from G3 (both positive by PCR) showed testicular degeneration characterized by the formation of multinucleated giant cells in the lumen of seminiferous tubules.

A tissue cyst was only found in one animal (C2) from G1 in carpal skin (Supporting information Figure S1b). This tissue cyst was located in the deep dermis near vessels, surrounded by inflammatory cells (lymphocytes, macrophages and eosinophils) accompanied by fibrinous necrosis of the muscular of the arterioles.

In bulls, mild local inflammation was detected in most of the tissue samples from skin, reproductive and respiratory tract. These foci were formed mainly by lymphocytes and, to a lesser extent, macrophages and plasma cells. They were located at the skin, dermis and lamina propria of the respiratory mucosa. Specifically, these lesions were mainly found at the trachea and scrotum (all animals), epiglottis and skin of the elbow, carpus and thigh (five of six animals) and skin of the tarsus (four of six animals). In addition, the spleen of two bulls showed mild lymphoid hyperplasia. Regarding the genital tract, three animals showed foci on non-purulent inflammatory infiltrate adjacent to vessels at the epididymis and one of them (B2) also at the pampiniform plexus.

All immunohistochemistry labelled samples were negative for *B. besnoitii* antigen but for the carpal skin sample from C2G1.

4 | DISCUSSION

An in vivo ruminant model of besnoitiosis is a major need. Thus, we studied the effect of parasite dose and host age on the infection dynamics with *B. besnoiti* tachyzoites in cattle with the ultimate goal of developing a reproducible experimental model of *B. besnoiti* infection in cattle. We have successfully reproduced the acute phase of the disease and microscopic findings also evidenced the chronic infection. Indeed infected animals without detectable clinical signs and macroscopic lesions characteristic of the chronic phase are more frequently found than clinically affected animals in endemically infected herds (Gollnick et al., 2018). However, to test potential drugs or vaccines, an ideal experimental bovine model should be able to reproduce the acute stage followed by the characteristic clinical signs of the chronic stage of the disease. Fulfilling this requirement is not an easy task based on the variable results obtained by others in the past, where the absence of a well-standardized experimental design hampered the ability to infer conclusions about the key variables and, consequently, to reproduce the results (Álvarez-García et al., 2014).

To address the previous limitations, we carried out two experimental infections where we examined the tachyzoite dose and host age as parasite- and host-dependent variables respectively. Three key elements were carefully controlled to ensure reproducible results: (a) the employment of a well-characterized *B. besnoiti* isolate; (b) the viability and quality of the inocula and (c) the animal health status prior to infections.

We chose the Bb-Spain 3 isolate for several reasons: first, it was obtained from an animal younger than 4 months with marked skin lesions and, second, its recent isolation rules out the possibility of in vitro adaptation that might compromise its ability to form tissue cysts in vivo. Moreover, it has shown similar in vitro behaviour compared to other Spanish isolates (Diezma-Díaz et al., 2017). We also carefully monitored: (a) the inocula to ensure that the tachyzoites were free of BVDV and *Mycoplasma* spp. infections; (b) the animal health status prior to inoculations and (c) the numerous clinical signs and laboratory parameters measured by complementary techniques.

On the other hand, we selected males for these experiments, even though both sexes may be equally infected (Álvarez-García et al., 2014). However, bulls are at higher risk of acquiring and transmitting the infection as they serve a large number of cows and they appear to show more acute signs and a higher mortality rate (Álvarez-García et al., 2014; Gazzonis et al., 2017). Moreover, testicles are target organs for *B. besnoiti* replication and persistence (Kumi-Diaka, Wilson, Sanusi, Njoku, & Osori, 1981; Pols, 1960) and recent studies have provided evidence for the serious consequences of male sterility for herd fertility due to *B. besnoiti* infection (Esteban-Gil et al.,2016; Gazzonis et al., 2017).

Herein, we successfully reproduced the acute stage of the disease. Calves and young bulls developed fever followed by lymphadenopathy and respiratory signs. All infected calves developed fever during the 1st week pi and the incubation period lasted less than 12 hours, which agrees with the less than 2-day incubation period established previously using different intravenous inocula (Bigalke, 1968). However, the incubation period was longer in bulls. Four of six inoculated bulls showed temperatures higher than 39.5°C at 8–9 days pi. Respiratory signs of infection were observed in bulls later and the lymphadenopathy was maintained longer. It is possible

that a longer incubation period leads to a delay in the appearance of acute clinical signs. This delay could also have influenced on parasitaemia, as the parasite was detected in blood until 7 days pi in calves versus 24 days pi in bulls. These findings agree with previous studies that detected proliferative organisms in blood smears from the 3rd to the 12th day after the beginning of the febrile reaction in experimentally infected cattle (Basson et al., 1970). However, this finding was not corroborated in natural infections (Gollnick et al., 2015), where examinations of blood smears from naturally infected cows during the acute phase did not reveal either free or intracellular tachyzoites in peripheral blood. However, the sensitivity of blood smears is lower than PCR, which might explain these negative results. Our analyses of haematological and biochemical parameters showed very little relevant changes, which is in agreement with the mild-to-moderate outcome of the infection in both calves and bulls (unpublished data). We only observed an increase in serum globulin fractions that was correlated with seroconversion in infected animals. There is only one previous study where haematological and biochemical alterations in sera were studied in *B. besnoiti*-seropositive cows during disease progression of naturally acquired bovine besnoitiosis (Langenmayer et al., 2015). Disturbances in Aspartate Aminotransferase or Creatine Kinase activities, and typical alterations of chronic inflammatory diseases such as hyper-(gamma)-globulinemia or reduced erythrocyte were described by these authors.

No relevant differences were observed during the acute stage regarding the parasite dose and host age variables studied. It has been suggested that tachyzoite dose plays an important role in determining a severe versus a mild infection (Álvarez-García et al., 2014). In this study, this variable influenced only the occurrence of fever and the incubation period, but not the clinical score of the disease. Higher temperature values were detected in the group inoculated with the highest tachyzoite dose, similar to previous observations (Basson et al., 1970), but no relevant differences in the severity of the acute phase were observed among groups. It is tempting to hypothesize that doses higher than 10^8 tachyzoites would not significantly exacerbate clinical signs as relevant differences in the outcome of the infection were not observed among the three infected groups. In four experimental infections carried out in the past, tachyzoite doses higher than 10^8 were inoculated in cattle (Álvarez-García et al., 2014; Basson et al., 1970; Bigalke, 1968; Diesing et al., 1988). Acute phase was observed in all inoculated animals with mild–moderate severity, but for one immunosuppressed cow that died during this phase (Basson et al., 1970). However, severe clinical signs of chronic phase were only reported in one bull that had been splenectomiced previously (Basson et al., 1970). We must carefully consider these results as the inoculations were carried out under different experimental conditions. Moreover, a high number of zoites are not likely to be involved in natural infections with *B. besnoiti*, where horizontal transmission seems to be the main route of parasite transmission, either by cyst rupture during direct contact or by blood suckling arthropods bites (Álvarez-García et al., 2013). In fact, in naturally infected herds where animals might be exposed to similar infective doses, only a few animals develop characteristic clinical signs of bovine besnoitiosis, which may be due not only to parasite dose but also to other parasite and host-dependent factors. Regarding host age, according to previous experimental infections (Bigalke, 1970; Bigalke et al., 1974), the incubation period as well as the onset and duration of the febricula were highly variable in adult animals and were probably influenced by the different sources of the inocula. Herein, the inocula employed in both experiments were the same so that the most feasible explanation for the delay in the onset of clinical signs observed in bulls compared to calves might be the maturity of the immune system. The immune system maturation progresses in calves from birth until 6 months, when innate and adaptive immune responses reach values close to the ones developed by adults (Chase, Hurley, & Reber, 2008).

After the acute stage of the disease, the chronic stage of the infection was evidenced by parasite detection and microscopic lesions in target tissues despite no macroscopic lesions characteristic of the chronic stage of the disease, such as hyperqueratosis or alopecia, were detected in vivo. In this study, vasculitis was the most relevant lesion present in skin, genital and respiratory tract in all infected groups. Despite being a non-specific lesion, target locations where vasculitis was found suggested that this might be a consequence of endothelial damage produced by the parasite during the acute phase. Similar lesions have been described under natural infection (Langenmayer et al., 2015). Focal degenerative lesions in testicle of the calves were observed only in relation to vasculitis that probably contributed to mild degenerative changes in the germinal epithelium. However, the lesions observed in the reproductive tract were compatible with normal testicular function. Remarkably, in this study, a tissue cyst was located at the reticular dermis of the carpus region, a less frequent location than the papillary dermis (Langenmayer et al., 2015). As expected, a higher parasite intra-organic distribution was found by PCR compared with histopathological results. PCR-positive tissues were predilection sites for *B. besnoiti* that show tropism for the connective tissue of the superficial skin layers, scleral, conjunctiva and mucous membranes of distal genital tract (Frey et al., 2013; Gentile et al., 2012; Manuali et al., 2011; Nobel, Neumann, Klopper, & Perl, 1981). The DNA detected is expected to belong to the bradyzoite stage according to the infection dynamics as it was detected at 70–110 days pi during the chronic phase of the disease (Álvarez-García et al., 2014). Slight differences were found among infected groups as a higher number of PCR-positive samples (six of the 10) belonged to calves inoculated with 10^6 tachyzoites. It appears that inoculation with a lower dose of the parasite could have caused less stimulus of immune response, facilitating the evasion of the parasite and this could have allowed a higher early intra-organic distribution. This fact could be in agreement with what happens in natural conditions where low parasite doses might be inoculated under natural conditions through either direct contact or via haematophagous vectors as mentioned above (Álvarez-García et al., 2013). Based on the similar results between infected calves, the dose of 10^6 tachyzoites was chosen for the inoculation of bulls, where only two testicular samples were PCR positive. Regarding host age, 3- to 6-month-old Holstein friesian calves were

experimentally infected with *B. besnoiti* bradyzoites in the past and animals only developed tissue cysts and skin lesions under immunosuppressive treatment regardless the route of infection employed (Diesing et al., 1988). When tachyzoites were used as inocula only one of eight infected animals develop a few cysts without skin lesions in agreement with the results obtained in this study.

Herein, efficient immune responses elicited by immune competent animals were likely to have cleared most parasites, which is evidenced by the low parasite load detected by histology and correlated with mild lesions despite the high doses of parasites and the route of inoculation employed and reported by others (Álvarez-García et al., 2014; Bigalke, 1968). Accordingly, in both calves and bulls, the infection was controlled, and the immune response kinetics were similar in all infected groups. After infection, an innate IFN-γ response was developed during the 1st week pi, followed by an acquired immune response evidenced by seroconversion at 2–3 weeks pi and the maintenance of high antibody levels until the end of the experiment. IgG1 levels increased simultaneously with total IgG levels followed by an increase in IgG2 levels a few days later. Similar immune response kinetics have been reported in experimentally infected cattle with closely related apicomplexan parasites such as *Neospora caninum* (Regidor-Cerrillo et al., 2014). The basis of an effective immune response that governs the control of *B. besnoiti* infection remains to be clarified. However, a few remarkable findings were obtained. First, we corroborated that high antibody levels are neither predictive of the outcome of the infection nor protective against the infection, as no relevant differences were observed among groups regardless tachyzoite dose and host age. In contrast, it has been suggested that cell-mediated immune responses may play a key role in the control of the infection (Álvarez-García et al., 2014). Indeed, *B. besnoiti*-induced neutrophil extracellular trap formation was recently demonstrated as an important innate immune response mechanism of PMN acting against *B. besnoiti* (Caro, Hermosilla, Silva, Cortes, & Taubert, 2014) that might be influenced by parasite dose and host age based on the results obtained. It was also reported that *B. besnoiti* infection triggers early innate immune responses in endothelial cells (Maksimov, Hermosilla, Kleinertz, Hirzmann, & Taubert, 2016). Although the information we obtained is limited to IFN-γ and IgG2 responses, the results are in agreement with this hypothesis. The late IFN-γ response displayed by G1 and G2 calves and bulls, together with the higher IgG2 levels observed in bulls might be responsible for a better control of the infection. This mild dose-dependent modulation of the immune response has been previously reported by Rojo-Montejo et al. (2012), who suggested that when a high parasite dose is administered, a large number of tachyzoites may remain extracellular and stimulates the immune response more efficiently, whereas lower parasite doses might facilitate parasite immune evasion. Further works should elucidate the roles played by the immune T-cells repertoire, the Th1/Th2 balance and other cytokines, among others, in the pathogenesis and control of *B. besnoiti* infection.

In summary, infected animals developed clinical signs compatible with the acute stage and microscopic lesions characteristic of the chronic stage of the disease. Neither parasite dose nor host age seem to be relevant parasite- and host-dependent factors when tachyzoites were inoculated, as they did not significantly influenced the outcome of the infection. Moreover, we have set the stage for carrying out further controlled experimental infections in bovines to refine the present experimental model and induce chronic clinical signs. Thus, other variables such as alternative inoculation routes or parasite stages (e.g. bradyzoites) should be investigated in order to be able to reproduce clinical signs and macroscopic lesions characteristic of chronic besnoitiosis.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

ORCID

Gema Álvarez-García http://orcid.org/0000-0002-6627-7577

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SUPPORTING INFORMATION

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Short communication

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

C. Diezma-Díaz[a], I. Ferre[a], M. Re[a,b], A. Jiménez-Meléndez[a], E. Tabanera[b], M. González-Huecas[b], M. Pizarro-Díaz[b], D. Yanguas-Pérez[a], P.L. Brum[c], J. Blanco-Murcia[a,b], L.M. Ortega-Mora[a], G. Álvarez-García[a,*]

[a] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040, Madrid, Spain
[b] Animal Medicine and Surgery Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040, Madrid, Spain
[c] Laboratory of Microbiology and Parasitology, UNIPAMPA, Federal University of Pampa, Dom Pedrito, Brazil

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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

* Corresponding author.
E-mail address: gemaga@vet.ucm.es (G. Álvarez-García).

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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

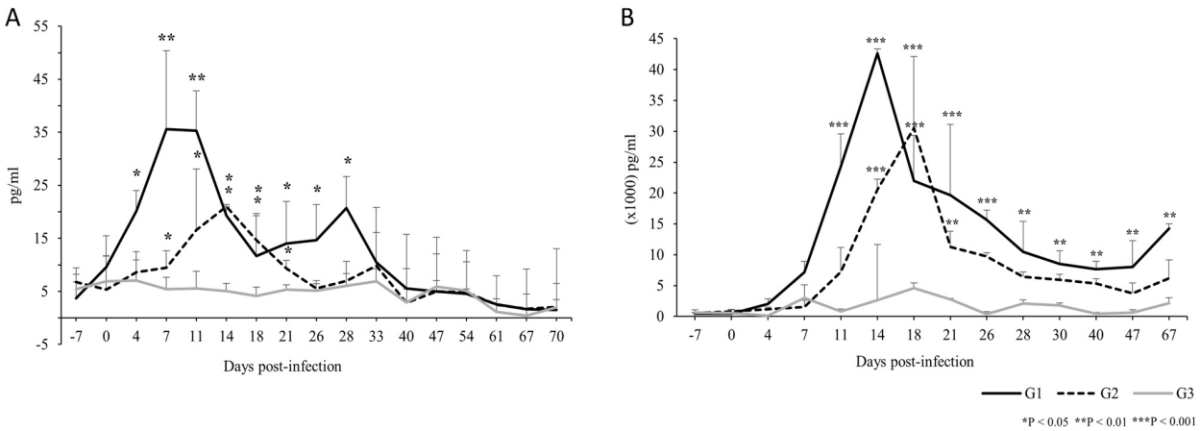

Fig. 1. Cellular immune response: **A**: Innate IFN-γ responses measured in serum samples; **B**: Adaptive IFN-γ responses measured in cell culture supernatants.

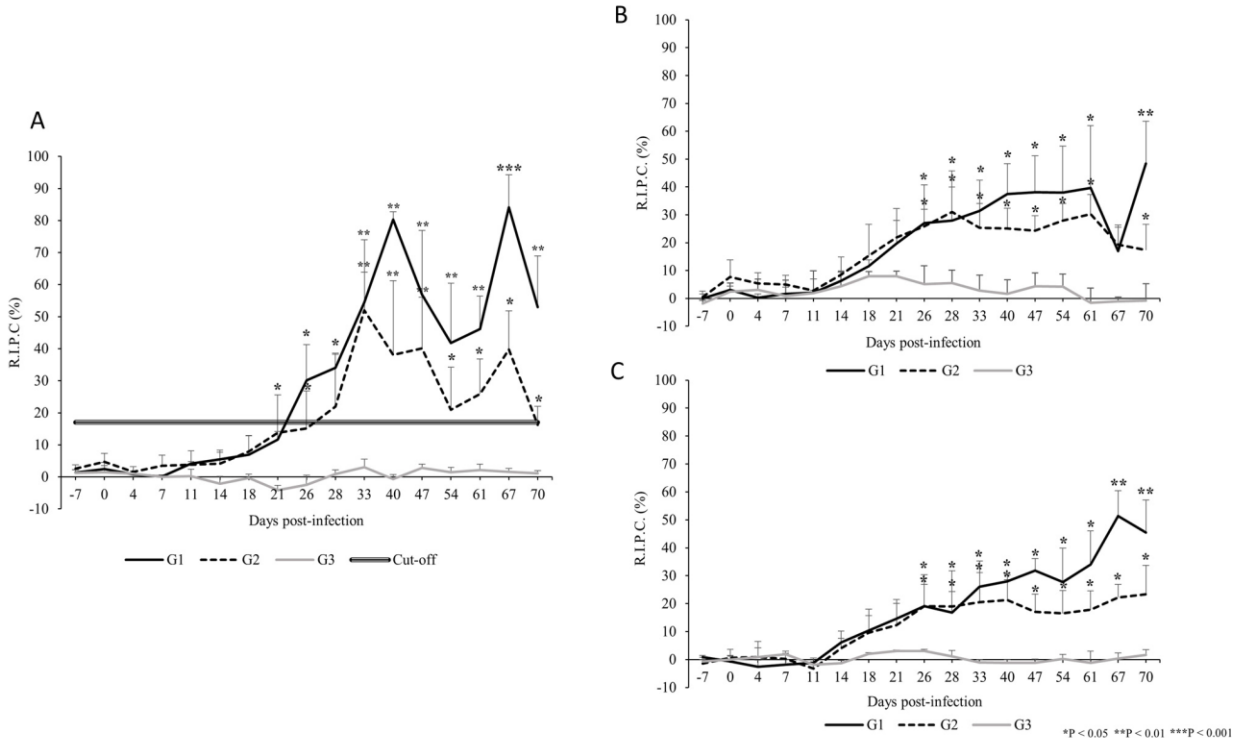

Fig. 2. Humoral immune response: **A**: *Besnoitia besnoiti*–specific IgG responses; **B**: IgG1 antibody levels; **C**: IgG2 antibody levels. Mean Relative Index (+Standard Deviation). (R.I.P.C: Relative index per cent).

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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ORIGINAL ARTICLE

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A model for chronic bovine besnoitiosis: Parasite stage and inoculation route are key factors

Carlos Diezma-Díaz[1] | Ignacio Ferre[1] | Michela Re[1,2] | Alejandro Jiménez-Meléndez[1] | Enrique Tabanera[2] | Manuel Pizarro-Díaz[2] | Marta González-Huecas[2] | María Alcaide-Pardo[1] | Francisco Javier Blanco-Murcia[1,2] | Luis-Miguel Ortega-Mora[1] | Gema Álvarez-García[1]

[1]Animal Health Department, Faculty of Veterinary Sciences, SALUVET, Complutense University of Madrid, Ciudad Universitaria s/n, Madrid, Spain

[2]Animal Medicine and Surgery Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, Madrid, Spain

Correspondence
Gema Álvarez García, Animal Health Department, Faculty of Veterinary Sciences, SALUVET, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain.
Email: gemaga@vet.ucm.es

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Abstract

In this work, an experimental model for chronic besnoitiosis in bovine was developed and characterized. Using a previously established calf model, two new variables (parasite stage and inoculation route) were combined and used. Twelve Holstein Friesian 3-month-old male calves were randomly divided into four groups of three animals each. Bradyzoites were obtained from a chronically infected bull and used for inoculation via three different inoculation routes. Three groups were inoculated with 10^6 bradyzoites by intravenous (G1), subcutaneous (G2) and intradermal (G3) routes, and a non-infected control group (G4) was inoculated with PBS. The trial lasted for 90 days and included daily clinical monitoring as well as weekly skin biopsies and blood sampling. Sera were obtained to analyse both cellular and humoral responses. Once the calves were euthanized, tissues from the skin, eyes, respiratory and reproductive tracts, among others, were collected to study presence of the parasite. Clinically, the infection was classified as mild to moderate for the acute stage since all infected calves showed lymphadenopathy from four days post-infection (pi) and fever from one week pi until 24 days pi. However, the most relevant results were achieved during the chronic stage that was classified as moderate to severe. In fact, pathognomonic conjunctival cysts were observed in all infected calves from 40 days pi onwards and were more abundant in G3. Moreover, one calf from this group developed skin lesions (49 days pi). The microscopic tissue cysts and Besnoitia DNA were detected primarily in skin, reproductive tract and respiratory tissue samples, and parasite load was higher in G3. In conclusion, the parasite stage (bradyzoite) and the inoculation route are key factors that influence the outcome of an infection. In particular, the intradermal route led to more severe clinical signs of the chronic phase in the inoculated calves.

KEYWORDS

Besnoitia besnoiti, bradyzoites, chronic besnoitiosis, in vivo model, inoculation routes

1 | INTRODUCTION

Bovine besnoitiosis, caused by the apicomplexan protozoan *Besnoitia besnoiti*, is a chronic and debilitating disease that causes local and systemic clinical signs (Bigalke, 1968) and is responsible for considerable economic losses, mainly in cattle (Álvarez-García, Frey, Ortega-Mora, & Schares, 2013). Currently, this transboundary disease poses a serious concern to the cattle industry in beef cattle in some EU areas since fertility rates are notably reduced in infected herds (Gazzonis et al., 2017). Moreover, the disease is spreading in several European countries, mainly in association with animal trade (Basso et al., 2013; European Food Safety Authority, 2010; Gentile et al., 2012; Hornok, Fedak, Baska, Hofmann-Lehmann, & Basso, 2014; Ryan et al., 2016; Vanhoudt et al., 2015).

Unfortunately, control of the disease is only possible through management measures coupled to diagnosis since chemotherapeutics are not available and no vaccines are licensed in Europe. Drugs are promising options to fight against the tachyzoite stage responsible for the acute stage. Regarding the chronic stage of the disease, therapeutic options are unlikely to be successful, due to the limited accessibility to the bradyzoites packed inside tissue cysts, which are responsible for the lesions developed during this phase (Gutiérrez-Expósito, Ferre, Ortega-Mora, & Álvarez-García, 2017). Remarkably, in vitro models have provided proof-of-concept of putative effective drugs, such as arylimidamides (Cortes, Muller, Boykin, Stephens, & Hemphill, 2011), bumped kinase inhibitors (BKIs) (Jiménez-Meléndez et al., 2017) and commercially available drugs, such as decoquinate, diclazuril (Jiménez-Meléndez et al., 2018), naphthoquinone buparvaquone (Müller, Manser, & Hemphill, 2018) and curcumin (Cervantes-Valencia et al., 2018).

Currently, in vivo testing is an essential step in drug and vaccine efficacy trials. The availability of well-characterized experimental models is needed to obtain comparable, robust and reliable data (Joachim et al., 2018). Unfortunately, in bovine besnoitiosis, several laboratory animals offer serious limitations for this purpose and preclinical drug screening can only be achieved in in vitro systems. Various rodent species are resistant to the disease (Shkap, Pipano, & Ungar-Waron, 1987), while others, including rabbits, are characterized by a rapid onset of the acute stage of the disease and sudden death and show difficulty in reproducing the chronic phase (Lienard et al., 2015). Ideally, an in vivo experimental model of *B. besnoiti* in bovine should be developed. In the 1960s and 1980s, numerous experimental infections were conducted in cattle. Unfortunately, a reproducible model was not obtained due to variable inoculants and experimental designs (Basson, McCully, & Bigalke, 1970; Bigalke, 1968; Bigalke, Schoeman, & McCully, 1974; Diesing et al., 1988).

Recently, we developed a standardized cattle model of besnoitiosis. Several parameters were evaluated in a bovine model using tachyzoites as the challenge parasite stage. In a first trial, different tachyzoites doses were intravenously inoculated into calves and bulls. Infected animals developed clinical signs compatible with the acute stage and microscopic lesions characteristic of the chronic stage (Diezma-Díaz et al., 2018). In a second trial, 10^6 tachyzoites were inoculated by a subcutaneous and intradermal route to mimic parasite transmission under natural conditions by bloodsucking arthropods and direct contact, with similar results (Diezma-Díaz et al., 2019). Accordingly, host (age and inoculation route)- and parasite-dependent factors, such as parasite dose, are not key variables when inducing macroscopic clinical signs of chronic besnoitiosis after cattle inoculation with the tachyzoite stage.

Thus, the objective of this study was to investigate the outcome of the infection in cattle inoculated with a different invasive stage (bradyzoite stage) to that used in previous occasions (tachyzoite stage). For this purpose, different routes of administration (intravenous, subcutaneous and intradermal routes) were assayed.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All experimental procedures were approved by the Animal Welfare Committee of the Community of Madrid (Spain) following proceedings described in Spanish and EU legislation (PROEX 92/14, Law 32/2007, R. D. 53/2013) and in Council Directive 2010/63/EU.

Animals were housed in clinical facilities belonging to the Faculty of Veterinary Sciences of the Complutense University of Madrid (Registration number: ES280790000101).

2.2 | Animals, experimental design, clinical monitoring and sampling

The twelve three-month-old calves used in this study came from a Holstein Friesian dairy herd located in Madrid Province; they were free from bovine besnoitiosis and bovine viral diarrhoea (BVD). Calves were selected after assessing the absence of specific antibodies against *B. besnoiti* and other closely related coccidian parasites (*Neospora caninum* and *Toxoplasma gondii*) (Diezma-Díaz et al., 2018). In addition, prior to inoculation, a vaccination protocol against the causative agents of bovine respiratory disease (bovine parainfluenza, bovine viral diarrhoea, infectious bovine rhinotracheitis and bovine respiratory syncytial virus) and enterotoxaemia (*Clostridium perfringens* types A, B, C and D, C. *chauvoei*, C. *novyi*, C. *septicum*, C. *tetani*, C. *sordellii* and C. *haemolyticum*) was performed to guarantee the health status of the animals. Upon arrival, animals were randomly allocated into four different groups (G1, G2, G3 and G4) composed of three calves each (1, 2 and 3), and a quarantine-adaptation period began (Diezma-Díaz et al., 2018).

The inoculum consisted of 10^6 bradyzoites (see section 2.3) diluted in 2 ml PBS administered through three different inoculation routes: intravenous inoculation by single jugular venipuncture (G1), subcutaneous inoculation in the left prescapular area (G2) and intradermal inoculation in the thigh area (G3), with the dosage subdivided into ten inoculation points, five of them on each side of the animal and administered with an intradermal Dermojet HR syringe (Akra DermoJet®, Pau, France). The sites of inoculation were the same ones employed in a previous work where tachyzoites were

inoculated (Diezma-Díaz et al., 2019). Moreover, a non-infected control group was inoculated with PBS intravenously (G4).

The experimental design, clinical monitoring, samplings (Figure 1a) and clinical score for the acute (from 1 to 42 days post-infection) and chronic phase (from 43 to 90 days post-infection) (Table 1) were established in the previous trials (Diezma-Díaz et al., 2019, 2018). Fever, lymphadenopathy, cough, nasal and ocular secretion, congestive ocular conjunctiva and general oedema were monitored during the acute phase, along with ocular conjunctival cysts, orchitis and skin lesions during the chronic phase. The number of tissue cysts in the ocular conjunctiva was estimated and assigned to the following categories: 1:1–10 cysts; 2:11–20 cysts; 3:21 to >30 cysts in both eyes (Gollnick, Scharr, Schares, & Langenmayer, ; 2015). Blood samples to evaluate immune responses 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 the assay. Five millilitres of peripheral blood in Vacutainer tubes (Becton Dickinson and Company, Plymouth, UK), with and without anticoagulant, was obtained by jugular venipuncture. Vacutainers without anticoagulant were centrifuged (1,200 × *g* for 1 min) to obtain serum samples, and vacutainers with ethylenediaminetetraacetic acid (EDTA) and with lithium heparin were processed to obtain peripheral blood mononuclear cells (PBMCS) and to proceed with peripheral blood stimulation, respectively. Once a week, skin biopsies from each animal were collected from the inner part of the thigh using 6-mm-diameter dermal biopsy punch (Spengler, Issoudun, France) to perform PCR. A criterion for the humanitarian slaughtering of the animals consisting of one day of total fasting and prostration was established

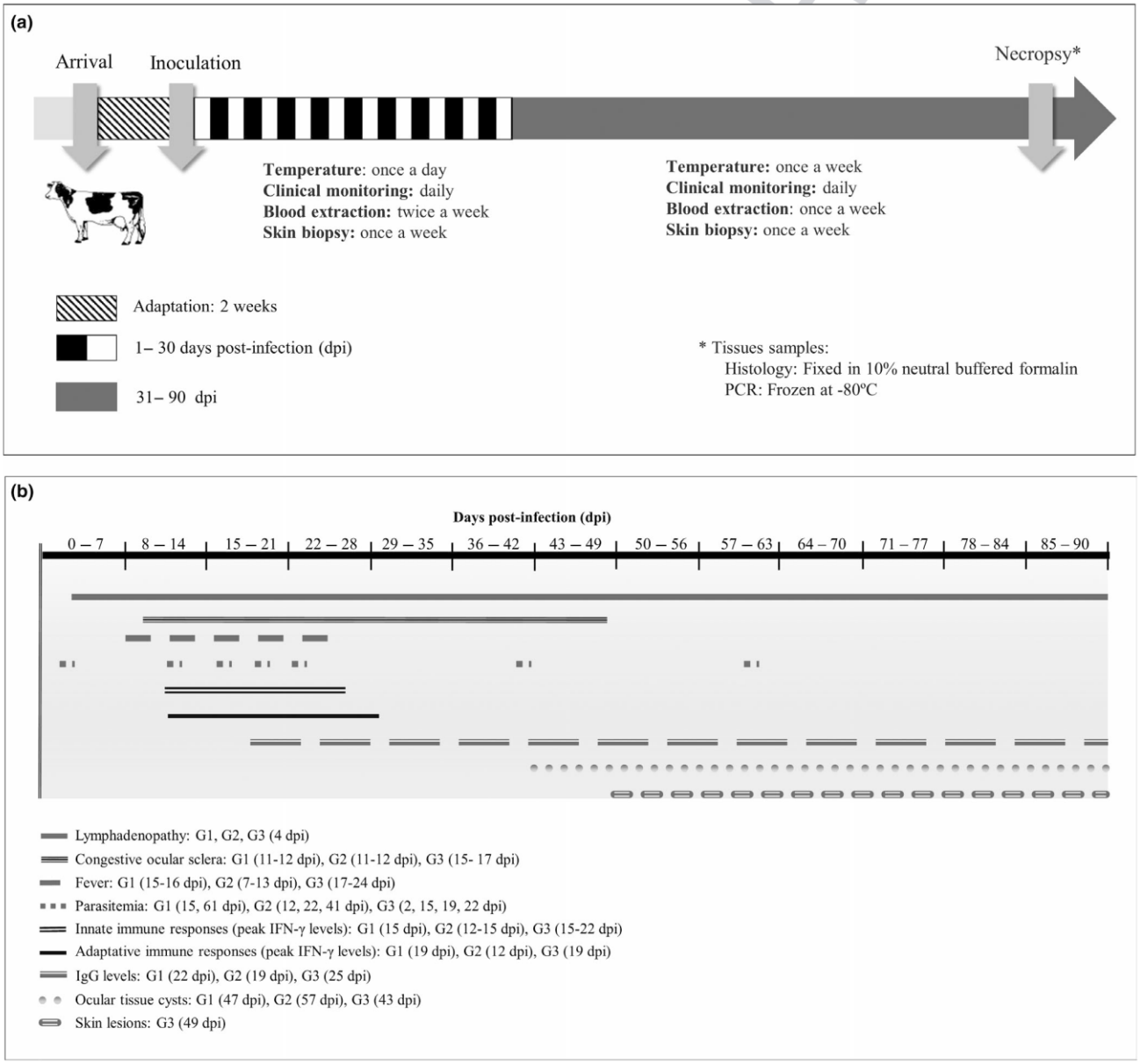

FIGURE 1 (a) Timeline, clinical monitoring and sample collection in experimentally infected calves. (b) Sequence of post-inoculation events. Clinical and laboratory parameter results

TABLE 1 Clinical score; A: Established for the acute stage of the infection (1 to 42 days pi); B: Established for the chronic stage of the infection (43 to 90 days pi)

	Days post-infection											
A	1–4	5–7	8–11	12–14	15–18	19–21	22–25	26–28	29–32	33–35	36–39	40–42
G1.1	0	1	1	2	2	2	2	2	2	2	2	2
G1.2	0	1	1	1	2	1	1	2	1	1	1	1
G1.3	0	1	1	1	2	2	2	2	1	2	2	2
G2.1	1	2	2	2	1	1	2	2	2	1	1	1
G2.2	1	2	2	2	2	2	2	2	1	1	1	2
G2.3	1	2	2	2	2	2	1	1	1	1	1	2
G3.1	0	1	1	1	1	2	2	2	1	1	1	2
G3.2	1	2	2	2	2	2	2	2	2	2	2	1
G3.3	1	1	1	1	2	2	2	2	2	2	2	2
G4.1	0	0	0	0	0	0	0	0	0	0	0	0
G4.2	0	0	0	0	0	0	0	0	0	0	0	0
G4.3	0	0	0	0	0	0	0	0	0	0	0	0
	Days post-infection											
B	43–46	47–49	50–53	54–56	57–60	61–63	64–67	68–70	71–73	74–76	77–80	81–83	84–90
G1.1	1	1	1	1	2	2	2	2	2	3	2	2	2
G1.2	1	1	1	1	1	1	1	1	1	1	1	1	1
G1.3	1	1	1	1	1	1	1	1	1	2	2	1	1
G2.1	1	1	1	1	1	2	2	1	1	1	1	2	2
G2.2	1	1	1	1	1	2	2	2	2	2	2	1	1
G2.3	1	1	1	1	1	1	1	1	1	1	1	1	1
G3.1	1	1	1	1	1	3	3	3	3	3	3	3	3
G3.2	1	1	1	2	2	2	2	2	2	2	2	2	2
G3.3	1	1	2	2	2	3	3	3	3	3	3	3	3
G4.1	0	0	0	0	0	0	0	0	0	0	0	0	0
G4.2	0	0	0	0	0	0	0	0	0	0	0	0	0
G4.3	0	0	0	0	0	0	0	0	0	0	0	0	0

Note: G: Group.
Clinical score: (Diezma-Díaz et al., 2018); *Acute stage*: 0, None (absence of infection); 1, Mild (local lymphadenopathy); 2, Moderate (fever, systemic lymphadenopathy, cough/nasal secretion and/or congestive conjunctiva); *Chronic stage*: 0, None; 1; Mild (systemic lymphadenopathy and/or congestive conjunctiva and/or conjunctival cysts of category 1 and/or ocular secretion); 2, Moderate (systemic lymphadenopathy and conjunctival cysts of category 2 and/or ocular secretion); 3, Severe (systemic lymphadenopathy and conjunctival cysts of category 3 and/or ocular secretion and/or skin lesión).

(Diezma-Díaz et al., 2018). At 90 days pi, calves were sedated with xylazine hydrochloride (Xilagesic 2%®, Calier, Barcelona, Spain) and immediately euthanized by an intravenously overdose of embutramide and mebezonium iodide (T61®, Intervet, Salamanca, Spain). Post-mortem examination of the calves was carried out immediately after euthanasia. Tissue samples for histological and molecular studies were collected (see Table 2).

2.3 | Inoculum preparation

Bradyzoites were obtained from a 4-year-old breeding bull from central Spain (Madrid). The Charolais bull showed clinical signs compatible with chronic besnoitiosis, including hyperkeratosis in the neck and scrotum, alopecia in the carpal and tarsal areas, testicular atrophy and the pathognomonic tissue cysts in the ocular conjunctiva. The animal was *B. besnoiti*-seropositive (García-Lunar, Ortega-Mora, Schares, Diezma-Díaz, & Álvarez-García, 2017) and was seronegative for *N. caninum* and *T. gondii* by Western blot, as well as BVD (BVDV Ag/Serum Plus Test, IDEXX, Maine, USA) and infectious bovine rhinotracheitis (IBR) (IBR gB X3 Ab, IDEXX, Maine, USA). The bull was killed in the slaughter house, and tissue cysts were also found in the trachea. Then, tracheal rings were collected and maintained in phosphate-buffered solution (PBS) with 2% penicillin-streptomycin-amphotericin B (Lonza, Walkersville, MD, USA).

TABLE 2 Parasite intra-organic distribution by ITS1-PCR and histopathology (HP)

	Group					
	1		2		3	
Tissue[A]	PCR[c]	HP[c]	PCR[c]	HP[c]	PCR[c]	HP[c]
Reproductive system						
[L]Testicle	–	–	–	–	2/3	–
[R]Testicle	–	–	–	–	2/3	1/3
[L]Head Epididymis	1/3	–	–	–	2/3	–
[R]Head Epididymis	1/3	–	–	–	1/3	1/3
[L]Body Epididymis	–	–	–	–	1/3	–
[R]Body Epididymis	–	–	–	–	1/3	–
[L]Tail Epididymis	1/3	–	–	–	2/3	–
[R]Tail Epididymis	2/3	–	–	–	1/3	1/3
[L]Prox vas def.[a]	–	–	–	–	2/3	3/3
[R]Prox vas def.[a]	–	–	–	–	1/3	1/3
[L]Dist vas def + Pp[b]	–	1/3	–	–	1/3	–
[R]Dist vas def + Pp[b]	1/3	1/3	–	–	–	–
Bulbourethral gland	–	–	–	–	1/3	–
Seminal vesicles	–	–	–	–	1/3	–
Penis	–	–	–	–	1/3	–
Respiratory system						
[L]Nostril	–	–	1/3	–	2/3	1/3
[R]Nostril	–	–	1/3	–	1/3	2/3
Tongue	1/3	–	1/3	–	–	–
Nasal turbinates	2/3	–	3/3	–	–	2/3
Epiglottis	2/3	–	1/3	–	2/3	1/3
Pharynx	–	–	1/3	–	1/3	–
Larynx	1/3	2/3	–	–	3/3	3/3
Trachea	–	–	–	–	1/3	2/3
[L]Lung	–	–	–	–	1/3	–
[R]Lung	–	–	–	–	1/3	–
Lymphatic system						
Submandibular (LN)	–	–	–	–	1/3	–
[L]Cervical (LN)	–	–	–	–	1/3	1/3
[L]Prescapular (LN)	–	–	–	–	1/3	–
Skin						
Facial	–	–	–	–	1/3	1/3
[L]Neck	–	–	–	–	1/3	2/3
[L]Upper-eyelid	–	–	–	–	1/3	3/3
[L]Ear pinna	–	–	–	–	1/3	–
[L]Carpal	–	1/3	1/3	–	1/3	2/3
[L]Tarsal	–	–	–	–	1/3	3/3
[L]Elbow	–	–	1/3	1/3	1/3	2/3
[L]Thigh	–	–	–	–	1/3	2/3
[L]Perineal	-	–	–	–	1/3	–
Scrotum	–	1/3	–	–	1/3	2/3

(Continues)

TABLE 2 (Continued)

	Group					
	1		2		3	
Tissue[A]	PCR[c]	HP[c]	PCR[c]	HP[c]	PCR[c]	HP[c]
Other organs						
[L]Ocular Conjunctiva	–	–	–	–	1/3	–
[L]Ocular sclera	–	–	–	–	1/3	1/3
Brain	1/3	–	–	–	3/3	–
Cerebellum	–	–	–	–	1/3	–
[R]Tendon + fascia	–	1/3	2/3	–	2/3	2/3
[R]Hoof Corium	–	–	–	–	1/3	2/3

Abberiviation: LN, Lymph node.
[L] Left/[R] Right.
(A) Parasite was not detected either by HP or PCR in bronchus, thymus, spleen, [R] tonsils, bronchial (LN), mediastinum (LN), [L] ventriculum, [L] atrium from infected animals and in all tissues from control group.
[a]Proximal vas deferens
[b]Distal vas deferens + pampiniform plexus
[c]Number (n°) of positive samples/total n° of samples

Afterwards, tracheal mucosa was separated, bradyzoites were released by teasing several tissue cysts with a scalpel in a Petri dish and grounded in a Potter-Elvehjem tissue grinder. Bradyzoites were separated from tissue debris by passage through a 40-µm sieve and then collected by centrifugation at 1,350 × *g* for 15 min at 4°C. Released bradyzoites were resuspended in PBS with antibiotics (2%) and maintained at 4°C until inoculation (Fernández-García et al., 2009). The absence of bacteria and *Mycoplasma* spp. in the inoculum was checked by culturing an aliquot in blood agar and by using a specific PCR technique, respectively (Venor™ GeM Mycoplasma Detection Kit; Minerva Biolabs, Berlin, Germany). Parasite viability was determined as described by Diezma-Díaz et al. (2018), and the bradyzoites were inoculated in cell culture to obtain a new *B. besnoiti* isolate named as Bb-Spain 4. Finally, the absence of BVDV and *Mycoplasma* spp. infections were monitored by qPCR once the new isolate was obtained (Diezma-Díaz et al., 2017). Both Bb-Spain 4 bradyzoites and tachyzoites were genotyped using microsatellite markers (see section 2.9).

2.4 | *Besnoitia besnoiti* cell culture and antigen purification

Tachyzoites of *B. besnoiti* (Bb-Spain 1 isolate) (Fernández-García et al., 2009) were grown in a MARC-145 cell monolayer and maintained with Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Madrid, Spain) supplemented with 5% foetal calf serum (Gibco, Paisley, UK) and 1% penicillin-streptomycin-amphotericin B (Lonza, Walkersville, MD, USA). After several passages, the cells were scraped and the tachyzoites were purified in cold sterile PBS at a pH 7.2 using disposable PD-10 desalting columns (Sephadex G-25; GE Healthcare, Illinois, USA). Pellets were obtained by centrifugation at 1,350 × *g* for 10 min at 4°C and stored in vials at −80°C until use (Fernández-García et al., 2009).

2.5 | Innate IFN-γ responses

IFN-γ levels were measured in serum samples from 5 days prior to infection (−5 days pi) until the end of the collection period, with a Bovine IFN-γ ELISA development kit (Mabtech AB, Sweden) according to the manufacturer´s instructions (Arranz-Solís et al., 2016).

2.6 | *Besnoitia besnoiti*-specific (adaptive) IFN-γ responses

Protein soluble extracts of *B. besnoiti* were obtained as follows for PBMC stimulation: Bb-Spain 1 tachyzoites were maintained in vitro as previously described in section 2.4. Purified tachyzoites (2×10^9) were suspended in 1 ml 10 mM Tris–HCl containing 2 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, Madrid, Spain), disrupted by ultrasound treatment (Branson Sonifier 450, Branson Ultrasonic Co., Danbury, USA) in an ice bath and then centrifuged at 10,000 × *g* for 20 min at 4°C. The protein content of the supernatant was determined using Quick Start Bradford Protein Assay (Bio-Rad ®, California, USA) and then aliquoted and cryopreserved at −80°C until use as soluble extracts for PBMC stimulation.

The PBMCs stimulation protocol was described by Sánchez-Sánchez et al. (2018). Samples were obtained twice a week during the first two weeks of the experiment and once a week thereafter. IFN-γ levels were measured in supernatants by the Bovine IFN-γ ELISA development kit (Mabtech AB, Nacka Strand, Sweden), as previously described (section 2.5.).

2.7 | Serological analysis: IgG responses

Besnoitia besnoiti-specific IgGs were determined by ELISA using lyophilized tachyzoites of *B. besnoiti* as antigen (García-Lunar et al., 2017). A RIPC value ≥ 17.34 indicates a positive result. IgG1/IgG2

ELISA were essentially carried out as described by Diezma-Díaz et al. (2018).

2.8 | Histopathology

Tissue samples and biopsies were fixed in 10% neutral buffered formalin. After seven days of fixation, the tissue samples were dehydrated using a graded series of alcohol and were embedded in paraffin used an automatic tissue processor (TP1020, Leica Microsystems, Wetzlar, Germany). Tissue sections of 4–7 μm were cut from each sample with a motorized rotary microtome (RM2255, Leica Microsystems, Wetzlar, Germany) and stained with haematoxylin and eosin (H/E) and a linear staining system (4040, Leica Microsystems, Wetzlar, Germany) for histopathological evaluation. Samples were observed with an optical microscope (BX50, Olympus, Tokyo, Japan). Photographs were taken with a digital camera (DP27, Olympus, Tokyo, Japan) and were analysed with CellsSens Entry 1.15 imaging software (Olympus, Tokyo, Japan).

2.9 | DNA extraction/ PCR determinations and microsatellite (MS) genotyping

Genomic DNA was extracted from the PBMCs, skin biopsies, tissue samples collected during necropsy and samples used for genotyping following a previously described procedure (Diezma-Díaz et al., 2017). ITS1-PCR was performed according to the method of Cortes et al. (2007) and positive samples were analysed by quantitative PCR (qPCR)that was carried out as described by Schares et al. (2011), except that the SYBR Green system was used (Frey et al., 2013).

Microsatellite (MS) genotyping was performed using the six MS loci with primers previously described (Madubata et al., 2012). Amplification of MS and sequencing to confirm the number of repeat motifs for each sample was carried out as described by Gutiérrez-Expósito et al. (2016). The six MS markers evaluated (Bt-5, Bt-6, Bt-9 and Bt-7, Bt-20, and Bt-21) were used to genotype bradyzoites and tachyzoites from Bb-Spain 4. Moreover, tachyzoites and bradyzoites from Bb-Spain 3, the isolate inoculated in the previous experimental infections in calves were also genotyped (Diezma-Díaz et al., 2017; Gutiérrez-Expósito et al., 2016). The Bb-Spain 1 isolate was employed as the control.

2.10 | Statistical analysis

Rectal temperatures, numbers of tissue cysts in the scleral conjunctiva and variations in IFN-γ levels and IgG/IgG1-IgG2 were analysed by repeated measures two-way ANOVA tests and Bonferroni post hoc tests. Differences among clinical scores, average diameters and parasite burdens from samples belonging to the same system (reproductive, respiratory, lymphatic, skin and other organs) between infected groups were assessed by non-parametric Kruskal–Wallis tests followed by Dunn's multiple range tests for all pairwise comparisons. Statistical significance for all analysis was established with $p < .05$. Finally, a chi-square test was employed to address possible differences among the positive samples by histology and PCR between groups and systems. All statistical analyses were carried out using GraphPad Prism 6.01 software (San Diego, CA, USA).

3 | RESULTS

3.1 | Microsatellite analysis

Genotyping results are shown in Table S1. MS analysis of the tachyzoite and bradyzoite stages from the Bb-Spain 3 and Bb-Spain 4 isolates and tachyzoites from Bb-Spain1 showed identical genotypes.

3.2 | Clinical signs and clinical score

All animals from G1, G2 and G3 developed lymphadenopathy approximately four days pi; this occurrence was detected in the prescapular and the precrural lymph nodes. The size decreased after one month pi. However, lymphadenopathy (precrural and/or prescapular lymph nodes) was maintained until the end of the whole experiment in all infected animals (Figure 1b/Table S2A).

A significant increase was observed in the mean temperatures from the different inoculated groups (see Figure 2a) (Table S2B) (two-way ANOVA test, $p < .001$). The onset of fever (rectal temperature ≥39.5°C) was as follows: a) the intravenously inoculated calves (G1) developed a sporadic febrile response (one day) at approximately the second week pi; b) the subcutaneously inoculated calves (G2) developed fever from the first week pi until the end of second week pi (seven days), significant differences were observed between eight to 11 days pi compared with the G1 and G3 ($p < .001$, Bonferroni post-test); and c) the intradermally infected group (G3) showed fever from 17 days pi, and it remained high until 24 days pi (eight days) ($p < .001$, Bonferroni post-test). The highest temperature values were observed in one animal from G2 (G2.3) at 11 and 13 days pi (41°C and 40.6°C, respectively). Apart from this animal, only calves from the G3 developed temperatures above 40°C. Fever was not detected in the control group (G4) throughout the experiment.

Congestive ocular scleras were observed in all infected animals (Figures 1b and 2b) from 11 to 12 days pi in G1 and G2 and later in G3 at 15–17 days pi until seven weeks pi in all infected calves.

Clinical scores recorded for the acute stage of the disease are shown in Table 1A. Group 1 showed significantly higher scores from 15 to 18 days pi and from 26 to 28 days pi compared with the uninfected group (G4) ($p < .05$; Kruskal–Wallis test, Dunn's post-test). Clinical scores in calves from G2 and G3 showed significant differences when compared with G4 at five days pi onwards until 14 days pi and from 19 to 28 days pi, respectively ($p < .05$). Lymphadenopathy was less pronounced in G1, but statistically significant differences between infected groups were not observed.

We considered that the chronic stage of the disease started on the first day that pathognomonic tissue cysts (Figure 2c) were observed in the scleral conjunctivae (Bigalke, 1968) at 43 days pi in one animal from G3 (G3.3) (Figure 1b). Four days later, visible conjunctival cysts were also observed in G3.1 and G1.1. At 54 days pi,

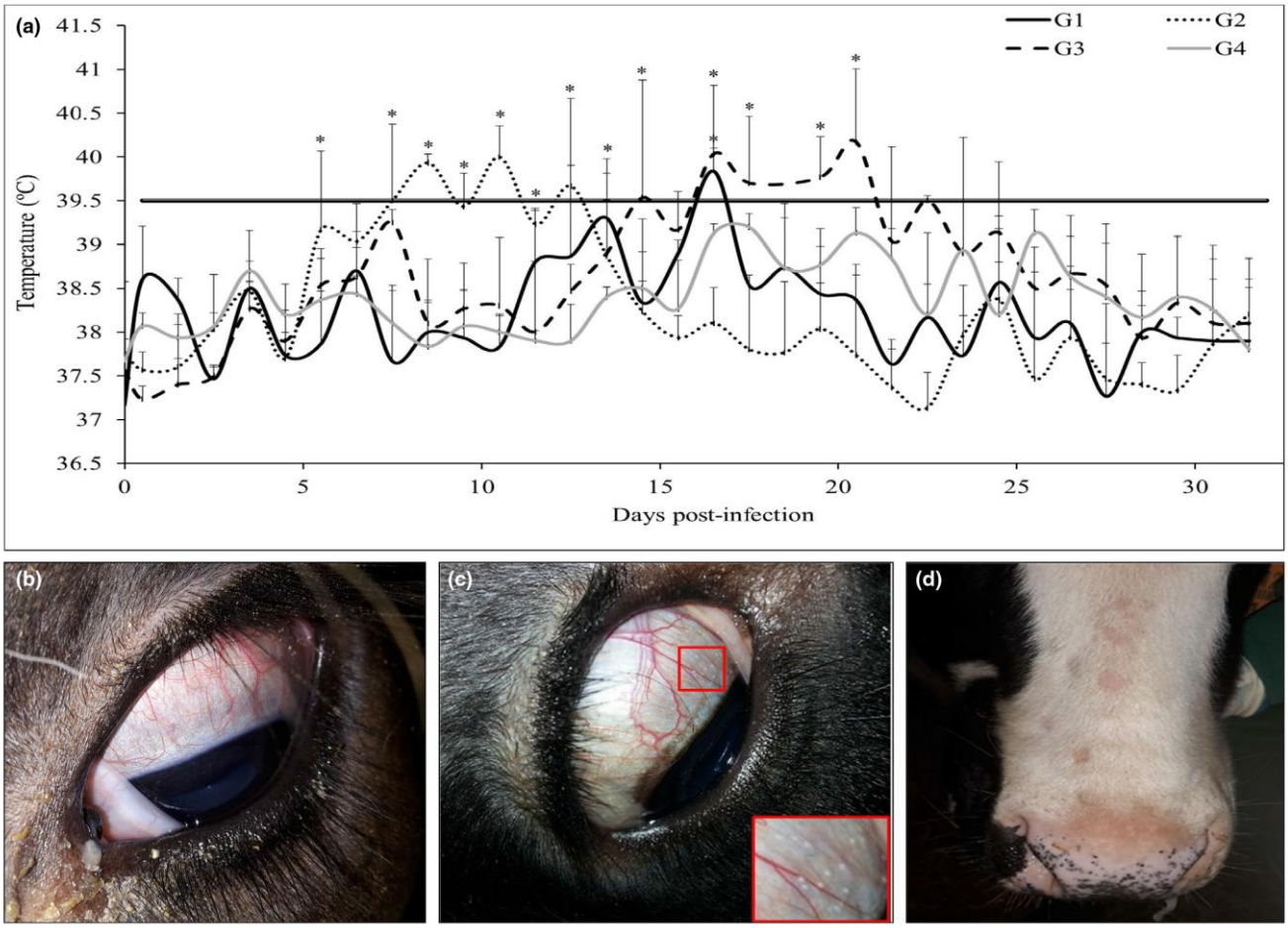

FIGURE 2 Clinical signs developed during acute (a, b) and chronic besnoitiosis (c, d). (a) Mean rectal temperatures (+*SD*) recorded during the first month post-infection. Fever was established with values ≥39.5°C. (b) Congestive ocular sclera and ocular discharge. (c) *B. besnoiti* tissue cysts developed in conjunctiva (G3.3). (d) Alopecia in the muzzle (G3.3)

two calves from G1 (2 and3) and G3.2 also showed ocular tissue cysts, and three days later, all calves from G2 had also developed scleral cysts. G3.3 and G3.1 showed the highest number of tissue cysts (category 3), followed by G1.1 and G3.2 (category 2), whereas the remaining two calves from G1 developed three ocular tissue cysts in both eyes (category 1). Regarding G2, between one and three conjunctival cysts were detected (category 1). Generally, all three infected groups showed a significant increase in the number of tissue cysts approximately 60 days pi (Table S2A). The number of tissue cysts remained constant in G1 and G2 until the end of the experiment. However, G3 displayed a second significant increase from 81 days until 90 days pi (two-way ANOVA test, $p < .001$). G3 showed significant differences compared with G1 (from 81 days pi onwards) and G2 (from 74 days pi onwards) ($p < .01$, Bonferroni post-test).

Other clinical signs characteristic of the chronic stage, such as skin lesions, were not observed apart from a few areas of alopecia developed by G3.3 in the hind legs and the muzzle (Figure 2d). These lesions were detected from six and seven weeks pi, respectively, until the end of the experiment.

Clinical scores recorded for the chronic stage of the disease are shown in Table 2B. All infected groups showed significant differences with respect to the uninfected group (G4) ($p < .01$) and showed similar clinical scores until 57–60 days pi. Afterwards, the scores increased and reached the highest category in G3 ($p < .05$), mainly influenced by the increase of the number of scleral tissue cysts.

3.3 | IFN-γ responses

3.3.1 | Innate immune responses

In all infected groups, the IFN-γ levels were significantly higher than in the uninfected group from 12 days pi until 15 days in G2; 20 days in G1 and 22 days pi in G3 ($p < .05$; two-way ANOVA). IFN-γ levels peaked twice in G1, first at two days pi (statistically significant compared with the control group; $p < .05$) and again at 15 days pi ($p < .01$). Group 2 presented the highest levels of IFN-γ from 12 ($p < .001$) to 15 days pi ($p < .05$; two-way ANOVA). Finally, G3 showed higher IFN-γ levels from 15 to 22 days pi, with a maximum at 19 days pi ($p < .001$). These levels decreased to basal values

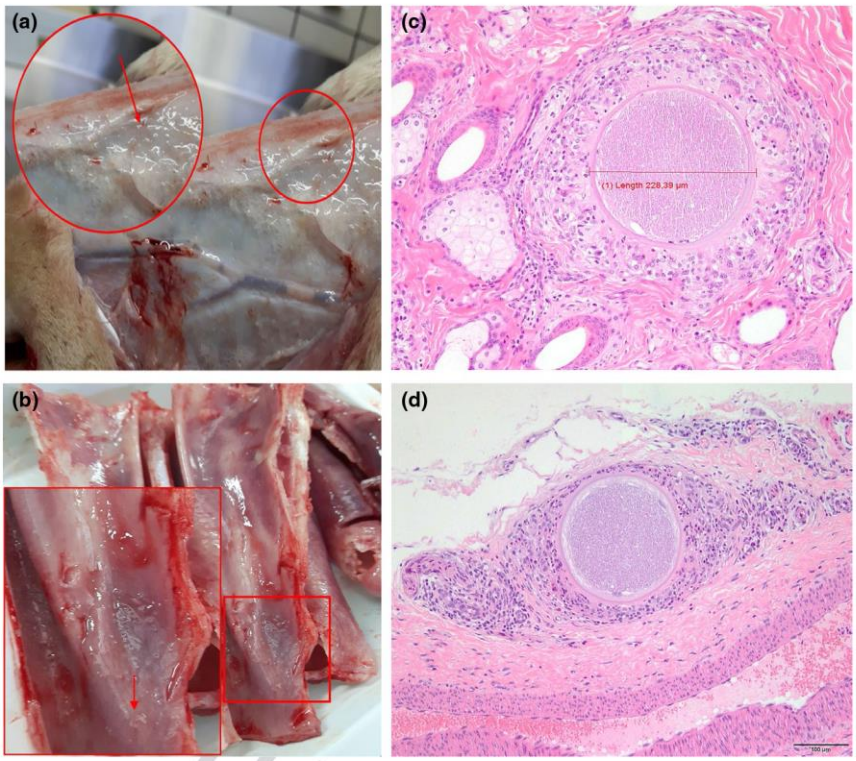

FIGURE 3 Macroscopic lesions detected at necropsy (G3.3): Tissue cysts in subcutaneous tissue (a) and nasal turbinate (b). *B. besnoiti* tissue cysts by histology (haematoxylin and eosin). Viable tissue cyst surrounded by pericystic inflammatory infiltrate from skin sample (c) and from vas deferens (d) (G3.3) (magnification, 10×)

in all three infected groups from day 25 pi and remained low thereafter (Figure 4a).

3.3.2 | Adaptive immune responses

When the PBMCs were stimulated with a *B. besnoiti*-soluble extract, a significant increase of IFN-γ levels in the culture's supernatants was observed in G1 and G3 at 19 days pi and in G2 at 12 days pi ($p < .001$; two-way ANOVA test). The kinetics of IFN-γ levels within each group also showed that maximum IFN-γ levels were reached at 19 days pi for all infected groups. However, the levels reached by G3 were significantly lower compared with G1 and G2 ($p < .01$; Bonferroni post-test). In addition, average levels of IFN-γ were significantly higher in G1 and G2 compared with G3 at 26 days pi, and in G1 compared with G3 at 33 days pi ($p < .01$; Bonferroni post-test). At 33 days pi, the levels started to decrease and remained higher in G1 and G2 compared with G3 (Figure 4c). IFN-γ levels recorded prior to inoculation did not vary throughout the experimental study in G4.

3.4 | Humoral immune responses (IgG levels)

Besnoitia besnoiti-specific antibody responses throughout the experimental study are shown in Figure 3c. The first group that seroconverted was G2 at 19 days pi, followed by G1 at 22 days pi and G3 at 25 days pi. Differences among all infected groups and the uninfected group appeared from 19 days pi onwards ($p < .01$). Antibody levels significantly increased, reaching a maximum at 61 to 68 days pi ($p < .001$; two-way ANOVA test).

Non-significant differences were observed between infected groups. However, G3 showed an increase in IgG response at the end of the experiment (87 days pi). In contrast, antibody levels decreased in G1 and G2 ($p < .05$, Bonferroni post-test).

Higher IgG1 levels were observed in the infected groups compared with G4 from 19 days pi for G2/G3 ($p < .05$; two-way ANOVA) and from 22 days pi for G1 ($p < .01$) (Figure 4d). At 33 days pi, significant lower IgG1 levels were found in G2 with respect to G1 and G3, whereas G3 showed higher IgG1 levels than G1 and G2 ($p < .05$). Non-significant differences were found among infected groups until 82–90 days pi, when IgG1 levels were significantly higher in G1 than in G2 and G3 ($p < .05$). IgG2 antibody levels were higher in all infected groups than in non-infected animals from 33 days pi onwards (G1: $p < .01$; G2-G3: $p < .05$; two-way ANOVA) (Figure 4e). No significant differences were observed in the IgG2 levels between infected groups, except for 90 days pi when the IgG2 levels were significantly higher in G1 than in G2 ($p < .05$).

3.5 | Macroscopic lesions

At the time of necropsy, ascites was present in G1.1, G3.1 and G3.3. Macroscopic cysts in ocular conjunctiva were visible in all infected animals and were more numerous in calves from G3 (Table S2A). One animal from G3 (G3.3) also showed skin lesions in facial and carpal areas, multifocal cysts in subcutaneous tissue and respiratory mucous membranes, mainly, in the nasal turbinates and tracheal mucosa (Figure 3a and b).

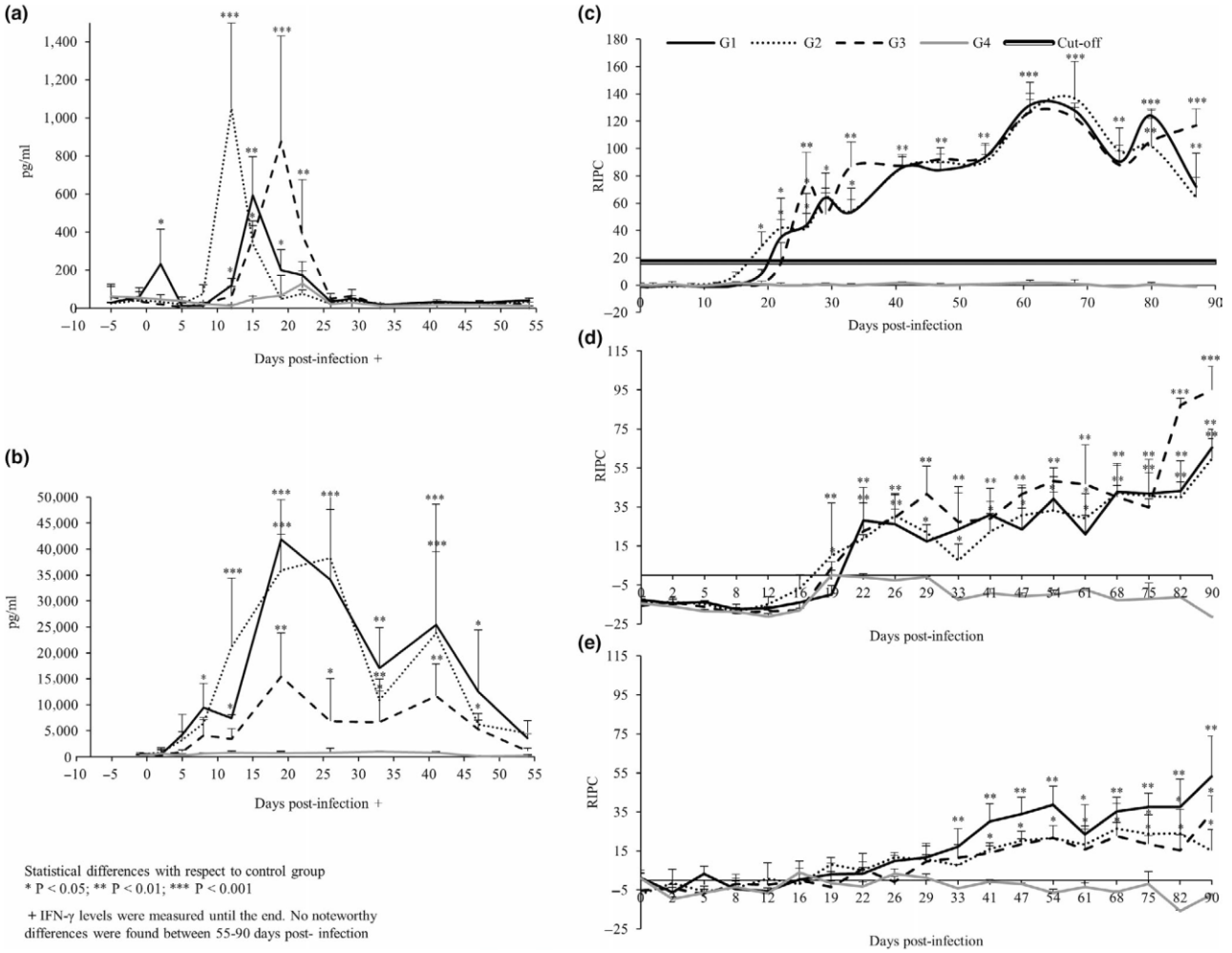

FIGURE 4 Cellular (a, b, e) and humoral (c, d) immune responses developed by inoculated calves. (a) innate IFN-γ responses measured in serum samples; (b) *B. besnoiti* –specific IFN-γ response measured after stimulation of PBMCs; (c) *B. besnoiti* –specific IgG responses; (d) Mean Relative Index (+*SD*) IgG1 antibody levels; (e) Mean Relative Index (+*SD*) IgG2 antibody levels

3.6 | Detection of tissue cysts in samples collected

As shown in Table 2, *Besnoitia* spp. tissue cysts were detected by histology in 49 samples. Most positive samples belonged to animals from G3 (*n* = 41), followed by G1 (*n* = 7) and finally by G2 (*n* = 1). The number of positive skin samples (40.8% of 49 positive samples) was statistically higher than for respiratory (26.5%) and reproductive (18.36%) tract samples, as well as for other organs and lymphatic system (14.3%) ($p < .01$, chi-square test). Regarding the tissue cyst size, the average diameter was 181.2 µm (±60.4 µm) (Figure 3d), and differences between tissue samples and infected groups were not significant.

3.7 | Detection of *Besnoitia besnoiti* DNA in blood and tissues by ITS1-PCR/qPCR

Parasite DNA was not detected in the skin biopsies. Parasitaemia was detected sporadically in calves of G1 at 15 and 61 days pi (*n* = 1); G2 at 12, 22 and 41 days (*n* = 1); and G3 at 2 (*n* = 1), 15 (*n* = 1), 19 (*n* = 3), and 22 (*n* = 2) days pi.

A total of 624 tissues samples collected at necropsy were analysed by ITS1-PCR. Four hundred sixty-eight samples belonged to infected animals and 156 to control animals, which were PCR negative. As shown in Table 2, *Besnoitia* DNA was detected in 82 samples (17.5% of 468). Statistically, the calves from G3 showed the highest number of PCR-positive tissues (*n* = 57; 69.51%) compared with G1 (*n* = 13; 15.8%) and G2 (*n* = 12; 14.6%) ($p < .01$). In G3, 40 positive samples out of 57 positives (70.2%) came from G3.3, which had also developed skin lesions (Table 2, Table S3). Parasite DNA was mostly detected in the respiratory (35.4%) and reproductive (30.5%) tracts, followed by other organs (15.8%), mainly the ocular sclera, tendon and hoof corium as well as skin (14.6%) and lymphatic system (3.7%). Moreover, parasite DNA was detected in samples where no tissue cysts were found by histological examination, for instance, in brain samples (Table 2).

The amount of parasite/ng bovine DNA in the ITS1-PCR-positive samples are shown in Table S3. Significant differences were observed

among the infected groups. The parasite load in G3 was higher than in calves inoculated by intravenous (G1) or subcutaneous (G2) routes ($p < .0001$; Kruskal–Wallis, Test Dunn). Moreover, samples from the upper respiratory tract and skin from G3 showed the highest parasite load with respect to the other systems ($p < .001$).

4 | DISCUSSION

We have successfully developed a model for chronic bovine besnoitiosis in calves, in which all infected animals developed macroscopic tissue cysts. This is the first time that bradyzoites have been inoculated by three different routes (intravenous, subcutaneous and intradermal routes) in cattle under the same experimental conditions. Remarkably, more severe clinical signs were detected in calves inoculated by the intradermal route.

Two recent experimental infections carried out in calves formed the basis for the present work, as identical experimental designs were followed. When tachyzoites were inoculated by intravenous (10^6, 10^7 and 10^8 tachyzoites) (Diezma-Díaz et al., 2018), subcutaneous and intradermal routes (10^6 tachyzoites) (Diezma-Díaz et al., 2019), mild to moderate acute besnoitiosis was evident, followed by the absence of the clinical signs and macroscopic lesions characteristic of the chronic phase and scarce intra-organic distribution of the parasite. The absence of the clinical signs that are characteristic of chronic besnoitiosis hampers the employment of these experimental models based on the inoculation of the tachyzoite stage, regardless of the inoculation route in future drug and vaccines efficacy trials. Accordingly, the other parasite stage described in the intermediate host, the bradyzoite, was inoculated in the present work. Prior to the experimental inoculations, good health status of the calves and appropriate quality controls of the inoculum were monitored, in contrast to the only two previous experimental infections carried out in the past with this parasite stage in immunologically depressed animals (Bigalke, 1968; Diesing et al., 1988). Moreover, experimental designs followed in the past differed notably in the number of animals, inoculation routes and immunosuppression therapies employed. We again studied three possible routes of inoculation (intravenous, subcutaneous and intradermal route), which could happen in nature since parasite can be transmitted by bloodsucking arthropods or through lacerations or wounds during direct contact between infected and non-infected cattle (Álvarez-García et al., 2013). The dose of 10^6 bradyzoites was selected according to previous studies done with tachyzoites to compare results obtained with the same parasite dose (Diezma-Díaz et al., 2019; Diezma-Díaz et al., 2018).

Herein, the acute phase of the infection was classified as mild to moderate, as no severe clinical signs such as oedema or orchitis were detected. The outcome of the acute infection was similar in all infected groups. Lymphadenopathy preceded fever (Figure 1b), began at approximately four days pi and was maintained until the end of the trial. However, lymphadenopathy was only evident until the first month pi in tachyzoite-inoculated calves, which was probably associated with a lower stimulation of the immune system (Diezma-Díaz et al., 2019; Diezma-Díaz et al., 2018). Lymphadenopathy was followed by fever, which is a clinical sign associated with *Sarcocystidae* parasite infections and first replication cycles of the parasite (Benavides et al., 2014). Remarkably, all infected animals showed a febrile response after a prolonged incubation period compared with previous works (Diezma-Díaz et al., 2019, 2018). Fever appeared in the subcutaneous group at 7 days pi, in the intravenous group at 15 days and finally, in the intradermal group at 17 days pi. In contrast, calves inoculated with tachyzoites by the intravenous route showed a 12-hr incubation period (Diezma-Díaz et al., 2018). Indeed, parasitaemia was more frequently detected during the febrile stage (Bigalke, 1968; Diezma-Díaz et al., 2018) (Figure 1b). In the present study, parasitaemia preceded fever and was more frequently detected in G2 and G3, which could explain why the fever lasted for one week in G2 and G3 in contrast to calves inoculated with tachyzoites by the same inoculation routes, where fever was scarcely detected (Diezma-Díaz et al., 2019; Diezma-Díaz et al., 2018). Based on the present findings and previous assays, the acute phase of bovine besnoitiosis in experimental infections seems to be easily induced regardless of the variables studied (Basson et al., 1970; Bigalke, 1968; Diesing et al., 1988; Janitschke, De Vos, & Bigalke, 1984; Shkap, 1986). However, the parasite stage, route of inoculation and immunosuppressive treatments seem to influence on the severity and duration of the clinical signs (Diesing et al., 1988; Diezma-Díaz et al., 2019; Diezma-Díaz et al., 2018).

The chronic phase of the disease was successfully reproduced and varied from mild to moderate clinical scores in the intravenously and subcutaneously inoculated groups to a moderate to severe score in the intradermally inoculated group. Thus, the most successful host- and parasite-dependent variable combination in inducing the chronic stage of the disease was the bradyzoite stage inoculated by the intradermal route as with the acute stage of the disease. Previous attempts with inoculated tachyzoites failed to induce macroscopic lesions and microscopic tissue cysts were only detected in intravenously inoculated calves (Diezma-Díaz et al., 2019; Diezma-Díaz et al., 2018). Herein, the first macroscopic conjunctival tissue cysts were observed at 43 days pi (18 days post-seroconversion) in intradermal inoculated animals and, subsequently, at 49, 54 and 57 days pi in the remaining infected groups (Figure 1b). Moreover, only skin lesions were observed in one intradermally inoculated calf at approximately 49 days pi (24 days post-seroconversion). The chronobiology of the chronic phase agrees with the observations reported in natural infections during a cohabitation trial in adult cattle (Gollnick et al., 2015), where the first macroscopic parasitic cysts were detected in the sclera of infected animals between 19 and 23 days post-seroconversion. Next, skin lesions were found in the proximal part of the hind legs at approximately 25 days post-seroconversion in one cow, which showed clinical signs characteristic of the acute besnoitiosis. These skin alterations were clearly visible within 166 days after seroconversion (Gollnick et al., 2015).

Herein, parasite detection was correlated with the severity of the clinical signs observed. Accordingly, the highest number of samples with tissue cysts and the highest parasite burden corresponded

to animals from G3 inoculated by the intradermal route, in particular, the calf with skin lesions developed the highest number of scleral tissue cysts. Our findings agree with the observations made in natural infections, where the highest number of microscopic tissue cysts has been largely reported in chronically infected animals which showed severe clinical signs and lesions characteristic of chronic besnoitiosis (McCully, Basson, Van Niekerk, & Bigalkie, 1966; Pols, 1960). As expected, the parasite showed tropism for skin, eyes and mucous membranes of the upper respiratory and genital tracks, in agreement with previous reports in naturally (Diezma-Díaz et al., 2017; Frey et al., 2013; Gentile et al., 2012; Manuali et al., 2011; Nobel, Neumann, Klopper, & Perl, 1981) and experimentally infected cattle (Diezma-Díaz et al., 2019; Diezma-Díaz et al., 2018). The cyst morphology and size were normal and compatible with that described for mature viable cysts (Frey et al., 2013; Langenmayer et al., 2015). The tissue cyst rupture may be observed from 30 days pi onwards (Basson et al., 1970) and could explain the sporadic parasitaemia observed at later stages of the disease (41 and 61 days pi) (Figure 1). Whether the presence of *Besnoitia* in the testes, which was detected in both tissue cysts and parasite DNA, could affect the reproductive function, as stated for naturally infected bulls, remains to be elucidated (Kumi-Diaka, Wilson, Sanusi, Njoku, & Osori, 1981; Nieto-Rodríguez et al., 2016; Pols, 1960). However, the low parasite load detected is unlikely to cause sterility, as naturally infected breeding bulls with impaired reproductive function normally present a high number of mature tissue cysts (Esteban-Gil et al., 2016; Kumi-Diaka et al., 1981).

The immune response kinetics were similar in all infected groups and comparable to previous experimental infections in calves (Diezma-Díaz et al., 2019; Diezma-Díaz et al., 2018). After the calves were infected, an innate IFN-γ response was developed, followed by an adaptive IFN-γ response and seroconversion. The most evident differences were observed when we employed tachyzoites or bradyzoites as inoculum. A delay in the innate IFN-γ response was observed in bradyzoites with respect to tachyzoite inoculation (12–20 vs. 4–7 days pi), which could also be associated with a delay in the detection of specific antibody (17 vs. 19–25 days pi). Again, the antibody titres were similar between infected groups, except for calves inoculated by intradermal route, with an increase of antibody levels observed at the end of the trial, possibly due to tissue cysts rupture that may have favoured the re-exposure of parasite antigens to the immune system (Frey et al., 2013). Differences between inoculation routes were confirmed again (Diezma-Díaz et al., 2019). The parasite exposure to the immune system by subcutaneous or intravenous routes led to an earlier innate IFN-γ response and higher adaptive IFN-γ response, which might have contributed to a better control of the infection compared with the intradermal inoculation. Cellular immune responses are thought to play a key role in the control of the infection regardless of the parasite stage inoculated (Álvarez-García, García-Lunar, Gutiérrez-Expósito, Shkap, & Ortega-Mora, 2014; Diezma-Díaz et al., 2018). Indeed, in previous experimental infections accomplished with bradyzoites, the best results were obtained under immunosuppressive therapies. Bigalke (1968) inoculated bradyzoites in two immunocompetent bulls by the intranasal route and reproduced the acute and chronic phases of the disease with scant detection of cysts. Later, Diesing et al. (1988) inoculated bradyzoites in seven animals by different routes. Five splenectomised animals either died or were euthanized. Two calves immunosuppressed by cortisone administration and inoculation by intraperitoneal route and by subcutaneous and intravenous route showed severe acute and chronic clinical signs. However, the immunosuppression therapies are not ideal for use in developing a reproducible experimental model of infection, since animals are then more vulnerable to other transmissible diseases.

Based on our results, the bradyzoite stage appears to be a principal key variable in determining the outcome of the chronic infection since all inoculated animals developed tissue cysts regardless of the inoculation route. In *Sarcocystidae* parasites, the bradyzoite differentiation is an immune evasion mechanism that allows the parasite to persist in the host for a long life (Hemphill & Gottstein, 2006; Jeffers, Tampaki, Kim, & Sullivan, 2018). Indeed, *B. besnoiti* tachyzoites display different antigenic patterns and protein compositions compared with bradyzoites (Fernández-García et al., 2013; García-Lunar, Regidor-Cerrillo, Gutiérrez-Expósito, Ortega-Mora, & Álvarez-García, 2013). Moreover, in the closely related *N. caninum*, bradyzoite antigens are less immunogenic compared with tachyzoite antigens (Jiménez-Ruíz et al., 2013). Taking into account the chronobiology of acute besnoitiosis and the finding of proliferative zoites during the acute infection, the rapidly dividing tachyzoite state is assumed to be responsible for the acute phase (Álvarez-García et al., 2013). Consequently, in the present experimental model, bradyzoites should initially switch into tachyzoites that could explain the prolonged incubation period and the delay of the onset of fever compared to these aspects in previous experimental trials (Diezma-Díaz et al., 2019; Diezma-Díaz et al., 2013). Reactivation of chronic besnoitiosis has been experimentally demonstrated in immunosuppressed hamsters infected with *Besnoitia jellisoni* (Frenkel & Wilson, 1972). Moreover, in *T. gondii*, an immunosuppressive environment (e.g. lack of IFN-γ) eases the bradyzoite to tachyzoite differentiation (Lyons, McLeod, & Roberts, 2002), which agrees with the lowest IFN-γ response and the most frequent parasitaemia and severe clinical signs observed in this study.

The inoculation route could also be important to facilitate the establishment of a chronic infection since a higher intra-organic dissemination of the parasite was detected in calves inoculated by the intradermal route as also happens in others bovine pathogens such as bluetongue virus (Umeshappa et al., 2011). Whether different lymphatic drainage could have favoured these results remains unclear. Lymph nodes in the posterior thigh are expected to have a relevant role since horizontal transmission is thought to frequently occur after mating. However, the main differences observed among the different inoculation routes could be related to the different effector mechanisms of the host early innate immune response that the bradyzoites would initially face and to the vascularization that facilitates that parasite dissemination and induces an early immune response. Thus, a more rapid and efficient immune response is

expected to occur against bradyzoites inoculated by the intravenous route, followed by the subcutaneous route and intradermal route in agreement with our results. Extracellular parasites in blood face a full-blown systemic response. As reported by Rojo-Montejo et al. (2012) in *N. caninum*, while many parasites remain extracellular, these may elicit an enhanced humoral immune response, unlike that associated with a higher cellular internalization of the parasite. Although parasites in subcutaneous tissue are rapidly absorbed through blood vessels, they might be driven by macrophages to draining lymph nodes to be processed by T-cells (Hunsaker & Perino, 2001). Finally, bradyzoites in the dermis are first exposed to a complex immune cell population in the skin, to smaller calibre blood vessels that may make parasite dissemination difficult, with the parasites finally being processed in the draining lymph node facilitated by the enrichment of dendritic cells (Kabashima, Honda, Ginhoux, & Egawa, 2018; Romani, Thurnher, Idoyaga, Steinman, & Flacher, 2010). In addition, dendritic cells are major effector innate cells in the dermis that could display a 'Trojan horse' mechanism to facilitate parasite dissemination since is a conserved strategy among Toxoplasmatinae parasites (Collantes-Fernández et al., 2012; Lambert, Hitziger, Dellacasa, Svensson, & Barragan, 2006).

Two critical issues arise concerning the employment of bradyzoites as innocula that may limit the reproducibility of this experimental model: a) health status of the donor animals and b) isolate variability since *N. caninum* (Regidor-Cerrillo et al., 2013, 2010) and *T. gondii* (Verma, Ajzenberg, Rivera-sanchez, Su, & Dubey, 2015) isolates display clear differences in virulence. Since bradyzoites cannot be maintained in vitro, they must be isolated from infected animals under sterile conditions and immediately inoculated. Thus, the health status of the donor animals is crucial since common pathogens such as BVD is widespread in cattle and could contaminate the inocula. While the health status of the donor is crucial, isolate variability appears to be of minor importance based on the low parasite intraspecific variability reported in *B. besnoiti*. Microsatellite genotyping performed in this work (Table S1) is in agreement with previous studies (Diezma-Díaz et al., 2017; Diezma-Díaz et al., 2018; Gutiérrez-Expósito et al., 2016), thereby proving a genetic homogeneity of all *B. besnoiti* isolates.

In conclusion, we have developed a model for bovine besnoitiosis where the chronic stage of the disease was successfully reproduced. The parasite stage (bradyzoite) and the inoculation route (intradermal) were crucial parasite- and host-dependent variables, respectively, that influenced the outcome of the infection. In future research, tachyzoite-bradyzoite switching in in vitro models should be investigated to obtain a parasite inoculum that could facilitate a reproducible experimental model.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

ORCID

Gema Álvarez-García https://orcid.org/0000-0002-6627-7577

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SUPPORTING INFORMATION

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