Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado Integrado em Medicina Veterinária

Dissertação

The first competitive ELISA for a sensitive and specific diagnosis of besnoitiosis using a monoclonal antibody against Besnoitia besnoiti

Daniela Alcântara de Melo Nascimento

Orientador(es) | Gereon Rudolf Matthias Schares

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A dissertação foi objeto de apreciação e discussão pública pelo seguinte júri nomeado pelo Diretor da Escola de Ciências e Tecnologia:

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Évora 2019

Para ti, da tua Casquinha e agora também colega.

“Success comes from curiosity, concentration, perseverance and self-criticism” Albert Einstein

Acknowledgements

I would like to express my appreciation and gratitude and thank those significant people who have been part of my journey and had an invaluable contribution to achieve my goal, without them this thesis would not have been possible:

To my supervisor, Dr. Gereon Schares, for the daily ongoing guidance and dedication despite the busy schedule, prompt availability to help me with my doubts and for the useful critiques during my internship at Friedrich-Loeffler-Institut (FLI) and after. Ich kann Ihnen nicht genug danken.

To my supervisor, Dr. Helder Cortes, for being a tremendous mentor to me, for the crucial support, friendship, encouragement, guaranteed kind words and for believing in me since the beginning.

To the lab assistants of Institute of Epidemiology at FLI, specially, to Andrea Bärwald and Alrik Kunisch, the great technical assistance and patience with me during six months at FLI are greatly appreciated. I am extremely grateful. Dankeschön.

To the Vice President of FLI and Head of Department of Epidemiology at FLI, Prof. Dr. Franz J. Conraths, and to my University teachers, specially to, Dra. Joana Reis and Dra. Ludovina Neto, I am deeply thankful for the encouragement and kindness.

To the Erasmus + Programme, I am thankful for the grant that partially supported my stay during my internship at FLI.

To the wonderful and irreplaceable friends that I met in the University of Evora, specially, to Pimby, Inês Maria, Frazão, Valiente, Carol, Conguita, Piu, Carina and Boto, I am so thankful for the friendship, happy memories (that are so many), for the amount of laughter and even for the painful study sessions and tragic mornings.

To my best friend, Trutarini, for being my everyday sun light and my favourite human.

To my boyfriend, Tiago, for the never-ending support, I'm deeply grateful for all the love, care and friendship.

Finally but most importantly, I must express my very profound gratitude to my Family for making sure I was healthy and happy every day since ever, especially to Mãe, Pai, Eva, Yara, Vanina, Tai and Samuel, thank you for your constant support, unconditional love and for giving me the conviction that I can achieve anything. Words cannot express how grateful I am.

Abstract

Besnoitia (*B.) besnoiti* is a cyst-forming apicomplexan parasite responsible for bovine besnoitiosis, a re-emerging disease in Europe with both cutaneous and systemic manifestations. The significant morbidity, lack of treatment and production losses associated make specific, sensitive, rapid and economic diagnosis imperative.

Thirteen monoclonal antibodies (mAbs) were previously produced against a membrane-enriched extracts of *B. besnoiti* tachyzoites (Apure-BbELISA Ag) and now characterized by Immunoblot assay and Indirect enzyme linked immunosorbent assay (iELISA). Hence, in order to meet the needs for a diagnostic method that would be accurate and easy to apply in the area of animal health, a study was undertaken to develop the first mAb-based competitive ELISA (Bb-cELISA1) at Friedrich-Loeffler-Institut (FLI) in Germany.

A total of 948 cattle sera were used to evaluate the Bb-cELISA1. The results were compared with previous iELISA results and revealed 99 per cent sensibility and 99.7 per cent specificity.

Keywords: Monoclonal antibody, competitive ELISA, bovine besnoitiosis, *Besnoitia besnoiti,* mAb-cELISA1

Resumo

O primeiro ELISA de competição para um diagnóstico sensível e específico de besnoitiose utilizando um anticorpo monoclonal contra *Besnoitia besnoiti*

Besnoitia (B.) besnoiti é um parasita apicomplexa formador de quistos, responsável pela besnoitiose bovina, uma doença re-emergente na Europa com manifestações cutâneas e sistémicas. A morbilidade, inexistência de tratamento e perdas produtivas associadas, tornam imperativo um diagnóstico específico, sensível, rápido e económico.

Treze anticorpos monoclonais (mAbs) anteriormente produzidos face a um extrato de membrana enriquecida de taquizoítos de *B. besnoiti* (Apure-BbELISA Ag) foram agora caracterizados através de Immunoblot e Indirect enzyme linked immunosorbent assay (iELISA). Consequentemente, com o objetivo de encontrar um método diagnóstico que seja exato e fácil de aplicar na área da saúde animal, o trabalho foi realizado para desenvolver o primeiro mAb-based ELISA competitivo (Bb-cELISA1) no Friedrich-Loeffler-Institut (FLI) na Alemanha.

Foram utilizados 948 soros de bovino para avaliar o Bb-cELISA1. Os resultados foram comparados com resultados anteriores de iELISA e reveladas sensibilidade de 99,1 por cento e especificidade de 99,7 por cento.

Palavras-chave: Anticorpos monoclonais, ELISA de competição, Besnoitiose bovina, *Besnoitia besnoiti*, mAb-cELISA1

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List of abbreviations, siglas and symbols

βME	Beta-mercaptoethanol
Ab	Antibody
Ag	Antigene
APS	Ammonium Persulfate
Bb-cELISA1	First mAb-based competitive enzyme-linked immunosorbent assay
B. besnoiti	*Besnoitia besnoiti*
cELISA	Competitive enzyme-linked immunosorbent assay
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal bovine serum
FLI	Friedrich-Loeffler-Institut
HCl	Hydrochloride
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
H_2SO_4	Sulfuric acid
IBR	Infectious bovine rhinotracheitis
iELISA	Indirect enzyme-linked immunosorbent assay

IFAT	Immunofluorescence assay
Ig	Imunoglobulin
ITS-1	Internal transcribed spacer region 1
Iv	Index value
LMW	Low Molecular Weight
mAb	Monoclonal antibody
MAT	Microagglutination test
Min	Minutes
NC	Negative control
N. caninum	*Neospora caninum*
OD	Optical density
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween20 0.05%
PBS-TG	Phosphate buffered saline with Tween20 0.05% and fish gelatin liquid
PC	Positive control
PCR	Polymerase Chain Reaction
PI	Percentage inhibition
PVDF	Polyvinylidene difluoride
RNA	RiboNucleic Acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBS	Tris-buffered saline
TBS-T (0.05%)	Tris-buffered saline with Tween20 0.05%
TEMED	Tetramethylethylenediamine
T. gondii	*Toxoplasma gondii*
TMB	Tetramethyl benzidine
<	Less than
>	Greater than

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Preface

The present dissertation was written following a six-month internship, from November 2018 to April 2019, at Institute of Epidemiology, Friedrich-Loeffler-Institut (FLI) Greifswald-Insel Riems, Germany.

The work of the FLI focusses on farm animal health and welfare and on the protection of humans from zoonosis. The FLI does applied research in different scientific fields.

Under the supervision and guidance of Doctor Gereon Schares, a recognized specialist in parasitology, the author was able to learn and practice laboratory techniques such as cell culture maintenance, enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Immunoblot assay, Immunofluorescence assay (IFAT) and meanwhile develop a study of mAbs characterization in order to develop the first mAb based competitive ELISA (Bb-cELISA1) with highly sensitivity and specificity.

1. Literature Review

1.1. *Besnoitia besnoiti*

Besnoitia besnoiti is a cyst-forming obligate intracellular apicomplexan protozoan parasite. *B. besnoiti* has been placed in the Sarcocystidae family and in the Toxoplasmatinae subfamily along with other parasite species of medical and veterinary relevance, such as *Toxoplasma gondii* and *Neospora caninum*, the causative agents of toxoplasmosis and neosporosis, respectively (Marotel, 1912).

B. besnoiti is the aetiological agent of bovine besnoitiosis, a chronic and debilitating disease of cattle. This disease causes morbidity and severe economic losses, mostly by infertility in bulls, but also by a decline in milk production and occasionally secondary abortions in cows and skin lesions (Pols, 1960; Bigalke, 1968).

The complete life cycle of *B. besnoiti* remains unkown; cattle and antelopes are known as natural intermediate hosts of *B. besnoiti* but the definitive host of this parasite has not been identified, yet. Two different intermediate host stages are known: tachyzoites and bradyzoites. Most likely, this parasite needs in addition to intermediate hosts also definitive hosts to fulfil its probably heteroxenous life cycle with a predator as definitive host and a prey species as intermediate host in which cysts are formed. It has been suggested, that wild ruminants and rodents can possibly be considered as reservoirs of the parasite (European Food Safety Authority, 2010).

It has been shown that *Stomoxys calcitrans* and Tabanidae species can act as mechanical vectors to transmit infection from chronically or asymptomatic infected cattle to other animals (Bigalke, 1968). Although low antibody levels during winter when there is no biting fly activity suggests that a transcutaneous infection via insects is essential, still more investigations are required to confirm the role of biting flies in the transmission of bovine besnoitiosis (Liénard et al., 2011; Sharif et al., 2019). A very present and concerning issue is that the global warming can promote the development of biting flies and cause extension of their activity period and thus may promote the transmission of *B.*

besnoiti. A further possibility of mechanical transmission is iatrogenically, during prophylactic or treatment procedures (Pols, 1960; Bigalke, 1968; Kutz et al., 2009).

There is epidemiological evidence of direct transmission between animals, possibly via mucosal contact by licking (Cortes et al., 2014). The presence of cysts in the genital mucosae suggests that transmission during natural mating is also a possibility (Cortes et al., 2014; Hornok et al., 2014). In addition, the recovered and subclinically infected animals are considered an important source of infection since they remain lifelong carriers (Álvarez-García et al., 2013). There are no evidences of vertical transmission, however the negative impact on milk production often affects the development of the calf (Cortes et al., 2006).

B. besnoiti infection may pass through an (1) acute stage (11–24 days post infection) a (2) subacute stage (between the end of acute disease and the macroscopic observation of the first tissue cysts) and (3) the chronic stage (>34 days post infection) (Cortes et al., 2014). The severity of the disease can vary between mild and severe with a mortality rate lower than 10 % (European Food Safety Authority, 2010).

B. besnoiti tachyzoites rapidly proliferate in macrophages, fibroblasts and endothelial cells in blood vessels and are responsible for the acute stage of the disease (Schulz, 1960; Cortes et al., 2005). Chronic besnoitiosis refers to the stage in which *B. besnoiti* tachyzoites switch into slowly dividing bradyzoites (cystozoites), which are contained in thick walled tissue cysts mainly found in the cutis, subcutis, fascia, conjunctiva, and the genital and respiratory mucosa (Bigalke, 1968).

1.2. Introduction of Bovine besnoitiosis

Bovine besnoitiosis has been described in the sub-Sahara Africa (Bigalke & Prozesky, 2004) and Asia (Olias et al., 2011). In the past decades, several outbreaks in Portugal (Cortes et al., 2004; 2005; 2006), France (Alzieu et al., 2007; Jacquiet et al., 2010; Liénard et al., 2011), Germany (Schares et al., 2009; Rostaher et al., 2010)), Spain (Juste et al., 1990; Fernández-García et al., 2009; 2010; Gutiérrez-Expósito et al., 2013), Croatia

(Beck et al., 2013), Hungary (Hornok et al., 2014), Italy (Gazzonis et al., 2007; 2014; Gentile et al., 2012), Switzerland (Basso et al., 2013) and Belgium (Vanhoudt et al., 2015) have been reported. Since 2010 bovine besnoitiosis is considered a re-emerging disease and endemic in Europe according to the European Food Safety Authority (EFSA) and there is evidence of an increased number of cases and geographic expansion of the *B. besnoiti* infection and the associated disease. More recently in 2016, besnoitiosis was diagnosed in an Irish dairy herd, it was the first outbreak in Ireland or the United Kingdom and the most northern European outbreak described, yet (Ryan et al., 2016).

Bovine besnoitiosis can affect all cattle breeds, animals of all ages and both sexes, however the clinical disease is quite unlikely to affect calves of less than six months old (Bigalke, 1968).

At the acute phase is characterized by nonspecific signs that make clinical diagnoses difficult: hyperthermia (above 40°C), tachycardia, tachypnea, serous nasal and ocular discharges, anorexia, weight loss, generalized weakness, superficial adenomegaly, anasarca of a variable degree with joints edema that may cause pain during movement and lead to lameness, loss of milk production, acute orchitis and painful testes (Schulz, 1960). Differential diagnoses include infectious bovine rhinotracheitis (IBR), bluetongue virus, malignant catarrhal fever and *Mycoplasma bovis* infection (Ryan et al., 2016).

During chronic disease the dermal lesions are always present making a diagnosis based on clinical signs easier when compared with the acute phase. Dramatic thickening, hardening and folding or wrinkling of the skin, always accompanied by hyperkeratosis, hyperpigmentation and alopecia are visible (Figure 1); bulls can develop necrotizing orchitis, which may result in permanent infertility (Pols,1960). Differential diagnosis include photosensitization, ringworm, scabies, trace element deficiencies and cutaneous lymphoma (Cortes et al., 2014).

We can define three clinical classes after infection by *B. besnoiti*: (1) a small proportion of animals developing typical clinical signs, (2) a group of seropositive animals with

sclero-conjunctival cysts and (3) the largest group, i.e. a group of seropositive but asymptomatic animals (Pols, 1960).

Figure 1 - A case of besnotiosis in a cow presenting severe besnoitiosis skin lesions (Adapted from Cortes et al., 2006)

1.2.1. Diagnostic methods

Several techniques have been reported for the diagnosis of bovine infection with *B. besnoiti*, these include direct detection of the parasite (the presence of cysts on the conjunctiva and vulva) and/or its deoxyribonucleic acid (DNA) in tissue samples (histopathology and polymerase chain reaction (PCR)) and indirect detection based on serology (iELISA, IFAT and Immunoblot assay) (Cortes et al., 2014).

Nowadays, in animals suffering from chronic disease the diagnosis of bovine besnoitiosis can be achieved by the direct detection of the parasite and/or its DNA. In this phase specific antibodies can be detected but not in all cases (Hemphill & Leitão, 2014). For the diagnosis of clinical and subclinical disease, the detection of antibodies against *B. besnoiti* in sera seems to be the most appropriate approach.

After the development of tissue cysts has started, a histopathologic analysis may represent a good method for diagnosis, due to the very high number of cyst in the skin of sick

animals. However, in cases where the cysts are absent on a histological slide of the skin, the overall cattle may harbour still tissue cysts in a high number (Cortes et al., 2014). It was recommended to examine a skin punch biopsy (8 mm diameter) from the 'Rump' region since this skin region seems to represent the optimal site for taking skin biopsies for detection or isolation of *B. besnoiti* (Schares et al., 2016).

A conventional and real-time fluorescent polymerase chain reaction ("ITS1 rDNA PCR") for detection of *B. besnoiti* infections in bovine skin biopsies have been developed by Cortes et al. (2007a). It was based on the sequence of the internal transcribed spacer region 1 (ITS-1) of the ribosomal RiboNucleic Acid (RNA) gene of *B. besnoiti* and have been used to detect the parasite in skin biopsies through the amplification of parasite specific DNA sequences. A real time PCR assay was also established for the quantitative detection of *B. besnoiti* in chronically infected cattle by Schares et al. (2011). This technique is highly sensitive and specific but, unfortunately, PCR assays are not routinely used in many diagnostic laboratories.

Indirect ELISAs (iELISAs) available as commercialized or in-house tests can be recommended as fast and inexpensive mass screening test for seroepidemiological examinations of bovine besnoitiosis. Schares et al. (2013) have developed a method to detect *B. besnoiti* antibodies by iELISA, using a so-called "APure BbELISA antigen", produced by sequential immunoaffinity chromatography, which had a diagnostic sensitivity of 100 % and a diagnostic specificity of 99.8 %, relative to reference tests. This APure BbELISA antigen can be employed with diagnostic purposes without the need of Immunoblot assay to confirm and have been found appropriate diagnostic techniques in the acute stage of the disease (García-Lunar, 2016).

Immunoblot assays are frequently used as confirmatory assays to validate findings by other serological techniques for the diagnoses of bovine besnoitiosis. Techniques based in tachyzoite and bradyzoite extracts separated in SDS-PAGE under non-reduced and reduced conditions have shown diagnostic specificity and sensitivity values close to 100 % (Fernández-García et al., 2009; Schares et al., 2010). However, it is an expensive and time-consuming tool and it is only available in some specialized laboratories.

A microagglutination test (MAT) developed by Waap et al. (2011, 2014) have shown to have a large-scale and low cost applicability. The capability for detection of antibodies, without the need of specific secondary antibodies, makes this technique attractive for wildlife studies. However, cross-reactions with similar parasites can still be seen and is recommended a confirmatory test.

Diagnostic problems are the lack of sensitivity in the detection of antibodies mainly during the acute and sometimes the chronic stage, as well as a lack of serological specificity. There are commercial tests for which high proportions of false-positive reactions are reported, probably because as a consequence of the cross-reactions with similar parasites, e.g *N. caninum*, *T. gondii* or *Sarcocystis* spp (Shkap et al., 2002; Cortes et al., 2006; Schares et al., 2010). This expresses problems in the differentiation of the diseases serologically, which is necessary in countries where more than one infection coexist.

1.2.2. Control measures

More than one hundred years after the first reference of the disease (Cadéac, 1884), and the first reference to its parasitic etiology (Besnoit and Robin, 1912) (both cited by Pols, 1960), this parasitic disease has been persisting mysteriously. There are no efficient pharmaceutical compound available for the treatment of besnoitiosis in cattle and no exact recommendation to control it. Arylimidamides have shown some promise, sulfamides and tetracyclines are commonly used to diminish the severity of clinical signs, nevertheless, this therapeutic is not effective *in vivo* and relapses can happen (Cortes et al., 2007b; 2011; Jacquiet et al., 2010). The use of attenuated live vaccines are not authorized in most of European countries because of the lack of scientific data that guarantee efficacy and safety. There is a risk of introducing the parasite into uninfected herds and of inducing carriers among the vaccinated animals. In South Africa, the vaccine is based on tachyzoites of an isolate from blue wildebeest grown in cell cultures (Bigalke et al., 1974). In Israel, the vaccine in use contains live attenuated parasites derived from cell cultures (Pipano, 1997).

Control measures should be performed in three steps: (a) a serological analysis before introducing an animal into a besnoitiosis-free cattle herd, (b) a systematic evaluation of infection status of individual cattle in case besnoitiosis is emerging in a previously free herd with the aim to separate or to cull all seropositive animals, and (c) the protection of animals against ectoparasites (Jacquiet et al., 2010). To increase the awareness, it is worth promoting all the information about the disease not only to veterinarians but also to cattle breeders and farmers (Jacquiet et al., 2010).

1.3. Monoclonal antibodies background

Kohler and Milstein (1975) have revolutionized the regulation of immune responses with their vision for antibodies as tools for research on vaccine production and prevention, diagnostic and treatment of diseases. Because of the hybridoma technology, monoclonal antibodies homogeneous and highly specific are being developed against single epitopes and can be produced in unlimited quantities (Kohler & Milstein, 1975; 1976).

Thirteen mAbs were previously produced in the Institute of Epidemiology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany, against an enriched membrane extract of *B. besnoiti* (APure BbELISA) that were prepared following previous procedures (Schares et al., 1998; Schares et al., 2013; Wouda et al., 1998).

The enriched membrane extract had been labelled by surface biotinylation and analysis by a subsequent immunoprecipitation showed that at least some of the extracted antigens are located on the tachyzoite surface (Schares et al., 2013).

To produce mAbs against APure BbELISA antigen two BALB/c mice were immunized with three subcutaneous injections of 50 µg of the ELISA antigen (enriched membrane extract) using GERBU MM as an adjuvant (GERBU Biotehnik GmbH, Heidelberg Germany). The second and third injections were made four and eight weeks after the initial immunization with the same amount of the antigen preparation, for both mice. Three days after the last immunization, spleen cells (lymphoblasts) of Mouse 1 were fused with Sp2/0 myeloma cells and hybridoma cells grown as described (Schares et al., 1999). Eleven

months after the initial immunization, Mouse 2 was injected again with 50 µg of the antigen preparation, finally spleen cells (lymphoblasts) of the animal were fused with Sp2/0 myeloma cells. The euthanasia and fusion of the mouse spleen cells with SP2/0 myeloma cells happened three days after the last immunization. Then the hybridomas supernatants were screened for antibodies by immunoblot and the positive hybridomas were cloned and re-cloned if possible twice in order to insure their clonality (Figure. 2).

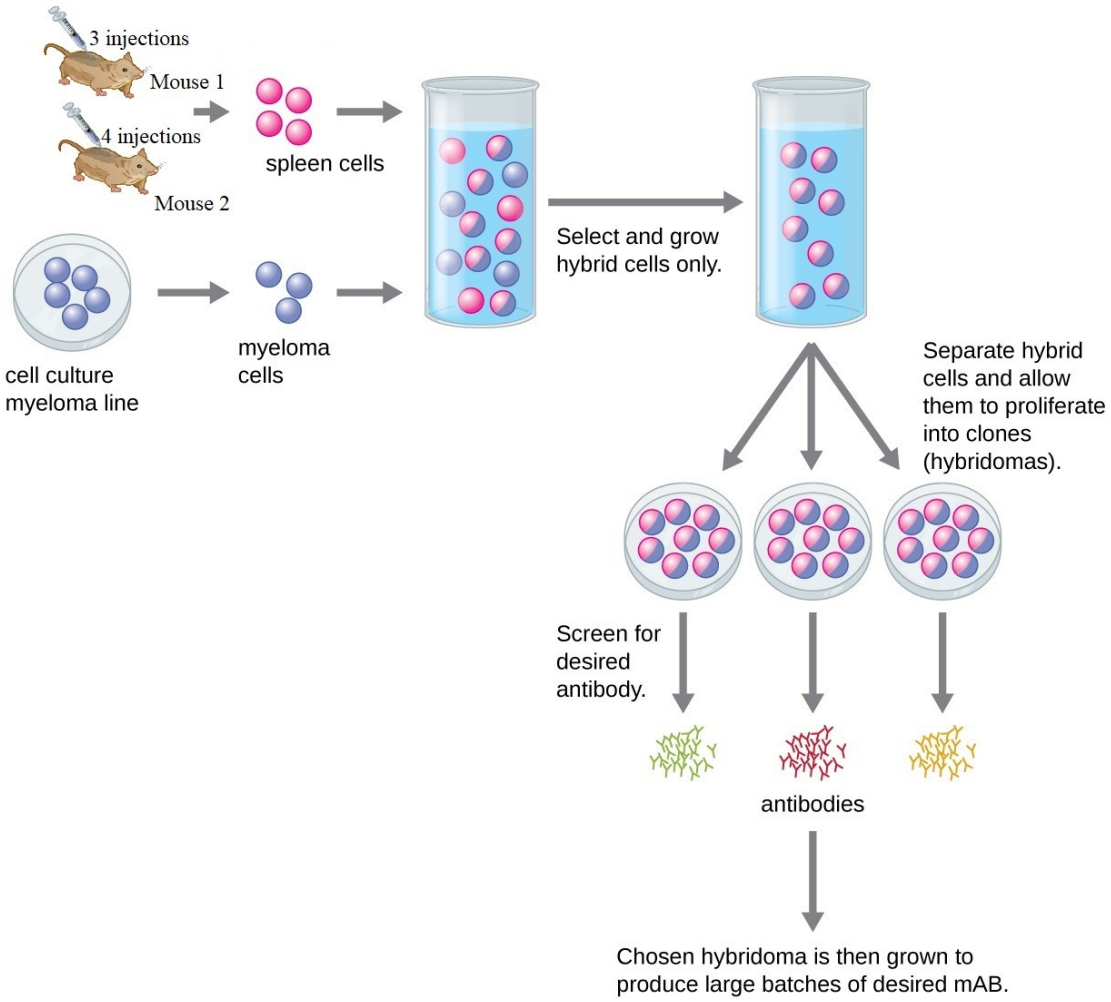

Figure 2 - A summary of the monoclonal antibody production. (Adapted from https://bio.libretexts.org/Bookshelves/Microbiology/Book%3A_Microbiology_(OpenStax)/20%3A_Laboratory_Analysis_of_the_Immune_Response/20.1%3A_Practical_Applications_of_Monoclonal_and_Polyclonal_Antibodies?fbclid=IwAR3M-0348DHY792lZ_gT3Be7YCFwrZwWNpbvRdPVaIrPNNohHw0pBedeOM, consulted at September 2019)

The mouse experiments reported in this publication were approved by the "Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei" of the German Federal State of Mecklenburg-Vorpommern (Permit 7221.3-2.5-004/10).

Isotyping was performed with a commercially available kit called "IsoQuick™ Strips and Kits for Mouse Monoclonal Isotyping" (Sigma-Aldrich, Deisenhofen, Germany), in order to characterize the mAbs by their immunoglobulin class and subclass (heavy chain) and light chains. The results are expressed in Table 1.

Table 1 - Isotyping results of mAbs

mAb designation	Hybridoma supernatant designation	Heavy Chain	Light Chain
1	1/18-8/2-8	IgG1/IgG2a	Kappa
2	1.17.8	IgG2a	Kappa
3	1/24-9/9-4	IgG1	Kappa
4	1/24-9/2-10 (B11)	IgG1	Kappa
5	1/17-7/15-3/1-3/3-13 (24-9)	IgG1	Kappa
6	B 9.2.5	IgG2a	Kappa
7	1/2-6/1-9	IgG1	Kappa
8	7.5.5 III	IgG1	Kappa
9	9.23.11 I	IgG1	Kappa
10	B 2.5.7	IgG1	Kappa
11	3.10.8 III	IgG1	Kappa
12	B 6.21.5	IgG2a	Kappa
13	B 5.5.11	IgG1	Kappa

Of the thirteen hybridomas, nine produced Imunoglobulin (Ig) G1 antibody (Ab) and three produced IgG2 Ab. MAb 1 produced both IgG1 and IgG2 which suggests that hybridoma cells were not clonal, yet.

Regarding the light chain isotype, all thirteen hybridomas produced kappa Ab.
Most of the monoclonal antibodies were class IgG and subclass 1. That fact can be explained by the humoral immune response that in response to protein antigens

predominantly produce IgG1 subclass, whereas polysaccharide antigens predominantly give rise to IgG2 antibodies; the adjuvant used can also effect the prevalence of IgG1; and the used myeloma parental line was maybe producing IgG1 (Köhler & Milstein, 1975; Stevens et al., 1983; Siber et al., 1980). After isotyping, all hybridoma supernatants were stored at 4°C until used.
For simplicity reasons, in this study the mAbs will be referred by the mAb designation and not by the hybridoma designation (Table 1).

2. Statements and definition of tasks

A number of serological methods have been developed to diagnose bovine besnoitiosis. Despite the valuable efforts made in order to find a gold standard diagnostic method, a specific, sensitive, rapid and economic technique has not been established yet.

The accurate impact of this re-emerging disease has not been determined so far because of the lack of prevalence and incidence studies, therefore an efficient diagnostic method would contribute as well to the implementation of control strategies in the already affected and also on risk areas.

The identification of subclinical forms are more challenging when compared with the identification of clinical cases, thus serology is considered an appropriate diagnostic tool. The identification of serologically positive animals is of major relevance to elaborate proper control measures as mentioned before.

Clinical cases represent the iceberg's tip of besnoitiosis and are relatively sporadic. Most affected cattle are asymptomatic and subsequently constitute a reservoir that must be identified as early as possible (Bigalke, 1968; Jacquiet et al., 2010).

The following sections focus on several essential techniques to achieve a characterization of the mAbs with the target of a mAb based cELISA (Bb-cELISA1). For this purpose, thirteen mAbs were characterized by iELISA and Immunoblot assay and, finally in order

to evaluate the sensitivity and specificity of the Bb-cELISA1, positive and negative reference cattle sera were analysed.

This type of cELISA, (also known as inhibition ELISA) offers distinct advantages that overcome the limitations of diagnosis: the antibodies used are homogeneous, with specificity for a single epitope or a small protein region. They are also less likely to interact with antibodies to closely related proteins and cross-reactivity of secondary antibodies is eliminated.

3. Materials and Methods

3.1. Optimization of the Indirect ELISA

Enzyme immunoassays allow the measurement of both Ab and antigen (Ag), the latter may comprise of an unlimited range of molecules (Kurstak, 1986).

Indirect ELISA can detect specific antibodies and allows the quantification of mAbs in a microtiter plate coated with a specific Ag. In this study it was important to know if the hybridomas produced mAbs reactive with *B. besnoiti* tachyzoite antigen.

In this assay the samples containing the antibody, i.e. hybridoma supernatants were added to the antigen coated plates and the binding antibodies were visualized later-on using a Horseradish peroxidase (HRP) conjugated secondary Ab that hydrolyzed the substrate (Figure 3). The amount of antigen-antibody complexes is assessed by photometric measurement at 450 nm, after stopping the color reaction using sulfuric acid. The measured amount of color is proportional to the amount of specific antibody in the hybridoma supernatant, but only in the linear range of the assay (Coligan, 1996).

In this study the specificity and concentration of the HRP conjugated secondary Ab were optimized, with the final aim to evaluate the reactivity of the thirteen mAbs in the supernatants from the hybridoma to *B. besnoiti* tachyzoite antigen.

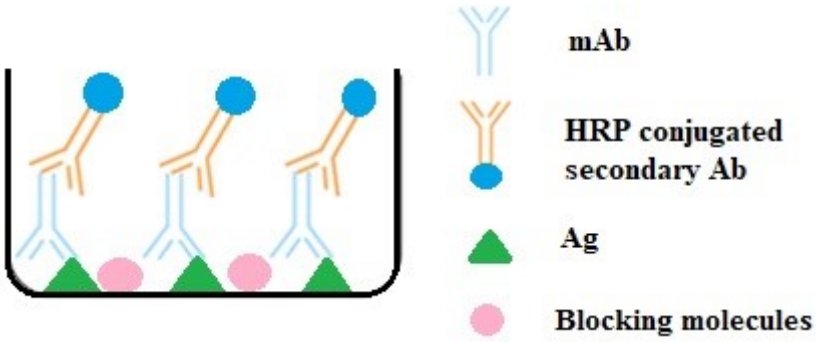

Figure 3 - Indirect ELISA diagram

3.1.1. Reagents, solutions and materials

All solutions were prepared fresh except the substrate buffer.

- Phosphate buffered saline with Tween20 0.05% (v/v) (PBS–T)
- PBS–T with 20% horse serum: pH 7.2. Was used as sample dilution buffer and blocking solution.
- PBS–T with 1% horse serum.
- Bicarbonate buffer: weekly new 0.1 M; pH 8.3. Was used as coating buffer.
- Sodium hydrogen carbonate. Was used as substrate buffer.
- Hydrogen peroxide (H_2O_2) 30%
- Tetramethyl benzidine (TMB) (Sigma-Aldrich, Taufkirchen, Germany). Was used as chromogenic substrate
- 2M Sulfuric acid (H_2SO_4)
- MAbs (undiluted): 1; 3; 4; 5; 7; 8; 9 and 11 on the iELISA optimization and from 1 to 13 on the final iELISA
- APure-BbELISA Ag (final concentration 200 ng/ml, 150 µl)
- Peroxidase-Conjugated AffiniPure Sheep Anti-mouse IgG Fcγ Fragment Specific (Jackson ImmunoResearch Laboratories, West Grove, USA)
- Peroxidase-Conjugated AffiniPure Rabbit Anti-mouse IgG + IgM (H+L) (Jackson ImmunoResearch Laboratories, West Grove, USA)

- As negative control (NC) was used Dulbecco's Modified Eagle's Medium (DMEM) and as positive control (PC) was used the fusion supernatant that was collected from the fused cells one to three days after fusion, both undiluted.
- Nunc-Immuno™ MicroWell™ 96 well solid plates (Nunc-Immuno (Polisorb), Thermo scientific, Langenselbold, Germany)
- ELISA reader (Tecan Sunrise, Tecan, Crailsheim, Germany)

3.1.2. Execution

To establish the optimal dilution of the HRP conjugated secondary Ab, the APure-BbELISA Ag stock was briefly centrifuged and diluted in the coating buffer at a dilution of 1:600 and used to sensitize a 96-well microtiter plate at 37°C for one hour. The wells were washed three times with PBS–T and the unbound active sites were blocked with PBS–T, 20% horse serum, followed incubation at 37°C. After 30 minutes (min), some mAbs, PC and NC sera were added and incubated again for 30 min. The unbound antibody was washed away three times with PBS-T and the wells were then incubated for 30 min with Peroxidase-Conjugated AffiniPure Rabbit Anti-mouse IgG (H+L) comparing two different dilutions (1:500 and 1:5000) in PBS–T, 1% horse serum. Unbound HRP conjugated Ab was washed away three times and the substrate solution was added (100 µl TMB and 1.2µl H_2O_2 30% in 10 ml of substrate buffer). Color development was stopped, after 15 min, by the addition of sulfuric acid 2M 50µl /well. Optical density (OD) was measured at 450 nm by the ELISA reader.

The test results were normalized and, for each well the index value (Iv) was calculated (Iv = (OD-NC) / (PC-NC)), where OD is the optical density obtained in each well, PC is the optical density of the positive control and NC the optical density of the negative control). The results are in Table 5 (in the "Results" chapter of this study).

With the establishment of the optimal dilution of the HRP conjugated Ab, the selection of the optimal HRP conjugated Ab regarding the specificity was followed. The protocol was similar with the exception that, it was performed comparing two different HRP conjugated Ab: Peroxidase-Conjugated AffiniPure Rabbit Anti-mouse IgG (H+L) and Peroxidase-Conjugated AffiniPure Sheep Anti-mouse IgG Fcγ Fragment Specific, both at

the dilution of 1:500 in PBS–T with 1% horse serum. The test results were normalized and interpreted as mentioned above and are expressed in Table 6 (in the "Results" chapter of this study).

Finally, an iELISA was performed with all the supernatants of the hybridoma in optimal conditions. The protocol was performed as mentioned above with the optimal conditions of the HRP conjugated Ab: Peroxidase-Conjugated AffiniPure Sheep Anti-mouse IgG Fcγ Fragment Specific at the dilution of 1:500 in PBS–T, 1% horse serum.

3.2. SDS-PAGE and Western blotting

The aim of the present study was the definition of the specificity and reactivity of each mAb. For that purpose we performed SDS-PAGE and Western blot analyses of several *B. besnoiti*, *T. gondii* and *N. caninum* antigens, to analyze in Immunoblot the reaction of the mAbs.

We used Polyvinylidene difluoride (PVDF) membranes with blotted antigens of *B. besnoiti* tachyzoites, *B. besnoiti* bradyzoites and APure-BbELISA Ag. Because of the possible cross-reactions of *Besnoitia* antisera with *N. caninum* and *T. gondii* antigens we analyzed also the reaction of the mAbs to the membranes of *N. caninum* tachyzoites and *T. gondii* tachyzoites.

The SDS-PAGE (Lämmli, 1970) combines the high-resolution power of the electrophoretic mobility of the respective proteins in a polyacrylamide gel with the capability of sodium dodecyl sulfate (SDS), an anionic detergent, to break down protein aggregates into their individual polypeptide chains and charges them negatively. SDS-PAGE and the subsequent Western blotting has become a standard tool in every laboratory in which proteins are analyzed and purified (Towbin et al., 1979).

The first step is the preparation of the gels, running the gel electrophoresis which was followed by the protein electrophoretic transfer from the SDS-PAGE to PVDF membranes using a semidry Western blot transfer system. The method used in the laboratory based on a protocol developed by Towbin et al. (1979).

SDS-PAGE was carried out using 12.5 % polyacrylamide gels. All Ag based on entire zoites had either been obtained by cell cultivation or by collecting from heavily infected skin (in the case of *B. besnoiti* bradyzoites). Tachyzoites pellets were conserved at -80°C and bradyzoites (tissues) were conserved at -20 ºC; they were diluted in both reducing and non-reducing sample buffer. For reducing conditions beta-mercaptoethanol (βME) was added to the sample buffer to reduce the disulphide bridges of proteins, so they can be better separated by size in SDS-PAGE gels. However, certain antibodies cannot detect proteins in their reduced forms and in that case we also needed to perform SDS-PAGE under non-reducing conditions, which means not using βME in the sample buffer. For both reducing and non-reducing conditions, samples of 100 µl containing 4 x 10^8 *B. besnoiti* tachyzoites, 4 x 10^7 *T. gondii* tachyzoites, 4 x 10^7 *B. besnoiti* bradyzoites, 8 x 10^7 *N. caninum* tachyzoites, and 85 µg of APure-BbELISA Ag were used for the production of each gel.

3.2.1. Reagents, solutions and materials

- Solution A (collection buffer): 6.06 g Tris-Hydrochloride (HCl), 4 ml 10% SDS, fill up 100 ml bidistilled water; pH 6.8
- Solution B (running buffer): 18.17 g Tris-HCl, 4 ml of 10% SDS, fill up 100 ml bidistilled water; pH 8.8
- Solution C (acrylamid): 30 ml Acrylamid, 0.80 g N,N`-Methylen-bis-acrylamid. Fill up 100 ml bidistilled water
- Tetramethylethylenediamine (TEMED)
- Ammonium Persulfate (APS) 40% (w/v).
- Antigen: All tachyzoites were grown in Marc-145 cell (rhesus monkey (*Macaca mulatta*), foetal kidney cells, permanent) monolayers with DMEM supplemented with 5% fetal calf serum (FCS) and 1% Penicilin/Streptomycin (Biochrom, Berlin, Germany) maintained at 37 °C/5% carbon dioxide (CO_2). To use the tachyzoites as Ag in serological tests, at least one day prior to harvesting, a change for DMEM without FCS was made to avoid that *N. caninum* specific antibodies frequently present in FCS may cause false positive reactions (Dubey et al., 2017).

Tachyzoites were separated from host cells by vigorous pipetting with cold phosphate buffered saline (PBS) washes, filtered with 5 µm hydrophilic syringe filters (Sartorius Lab Instruments, Göttingen, Germany) and centrifuged for 10 min at 974 X g at 4°C. Pellets were resuspended in PBS and the procedure was repeated five times. Finally, tachyzoites were counted using a Neubauer improved cell counting chamber (NanoEnTek Inc., Seoul, Korea) and the pellet stored at -80°C until used.

The bradyzoites were mechanically released from the skin by mortar and pestle of an infected skin sample, washed with cold PBS five times, counted using a Neubauer chamber and at the end a pellet was stored at -80°C until used.

The APure-BbELISA was produced previously by sequential immunoaffinity chromatography using polyclonal rabbit antisera raised against *N. caninum* or *B. besnoiti* by experimental infection of rabbits, coupled to rProtein A Sepharose 4B (Schares et al., 2013).

- LMW (Low Molecular Weight) marker, (SERVA Eletrophoresis GmbH, Heidelberg, Germany):
    - Phosphorylase B 97.4 kD
    - Albumin bovine (BSA) 67.0 kD
    - Albumin egg 45.0 kD
    - Carbonic anhydrase 29.0 kD
    - Trypsin inhibitor (soybean) 21.0 kD
    - Cytochrome C 12.5 kD
    - Trypsin inhibitor (bovine lung) 6.5 kD
- Non-reduced Sample buffer 5x: 3.75 g Tris, 10 g SDS, 35 ml Glycine, 25 mg Bromophenol Blue; 100 ml water; pH 6.8 adjusted with HCl
- Reduced Sample buffer 5x: 3.75 g Tris, 10 g SDS, 25 ml βME, 35 ml Glycine, 25 mg Bromophenol Blue, 100 ml water; pH 6.8 adjusted with HCl
- Running buffer 5x: Tris 15 g, Glycine 72 g, SDS 5 g
- PVDF membranes (Immobilon-P, Millipore, Darmstadt, Germany) - 1x / gel; 7.2x 8.5cm
- Methanol

- 1x Towbin buffer: 25 mM Tris, 192 mM Glycin, 10% Methanol, 0,1% SDS; pH 8,3
- PBS–TG (blocking solution): PBS 0.05% (v/v), Tween20 2% (v/v), fish gelatine liquid (Serva, Heidelberg, Germany)
- India Ink staining solution for PVDF membranes: 5 ml concentrated acetic acid, 0.5 ml fountain pens (Pelikan); 500 ml PBS-T
- Filter paper (Hahnemühle FineArt, Dassel, Germany) - 7.5 x 8.5cm
- "Trans-Blot Turbo" equipment (Bio-Rad laboratories, California, EUA)
- ThermoMixer (Eppendorf, Hamburg, Germany)

3.2.2. Execution

The running gel is prepared for two gels at a time because of the fast polymerization (description in Table 2) and added approximately five ml between the glasses, above is covered with bidistilled water and the polymerization lasts one hour. After the collection gel is prepared (description in Table 2) and added above, the comb is carefully inserted.

Table 2 - Running gel and collection gel – Volume of solutions for the preparation of SDS-PAGE gels

Running gel – volume for 2 gels		**Collection gel – volume for 4 gels**	
Solution B (ml)	2.5	Solution A (ml)	1.5
Solution C (ml)	4.2	Solution C (ml)	0.7
Bidest water (ml)	3.3	Bidest water (ml)	3.6
Total (ml)	10	Total (ml)	5.8
10.0 µl of TEMED and 25.0 µl of APS 40% are added		16.0µl of TEMED and 8.0 µl of APS 40% are added	

Thereafter, the Ag is diluted in 1x sample buffer (reduced and non-reduced) and incubated for 10 min at 95°C in the ThermoMixer. Then the BIO-RAD chamber is filled in with 1x

running buffer and the Ag is added to the preparative slot (usually 100 µl) as well as the marker (5 µl) which is added into a separate small slot. The electrophoresis is performed at 120 V, 500 mA, 150 W for approximately one hour and 20 min.

The PVDF membrane are activated in methanol and then placed in 1x Towbin buffer and shaken. The filter and the gel are placed in the 1x Towbin buffer. Methanol is known to improve the adsorption of protein to the membrane support, prevent gel swelling, and protein distortion during electroblotting (Towbin & Gordon, 1984).
The blotting sandwich is composed by three filters, one PVDF membrane, one gel and above more three filters, care was taken to remove all air bubbles. The blot sandwich is placed in "Trans-Blot Turbo" equipment and the semi-dry electro blotting is performed at 25 volts/gel for 30 min.

After the transfer two strips of the membrane, coated with the marker proteins and the antigen, were cut off and the transferred proteins visualized using an India ink stain to check the effectiveness and homogeneity of the transfer across the membrane, as previously reported (Hancock & Tsang, 1983).

Finally, it is important to block the remaining binding sites by immersing the membrane in a blocking solution. Blocking a blot serves two important purposes: help mask any potential nonspecific binding sites on the membrane itself and promote renaturation of antigenic sites (Towbin & Gordon, 1984).
When dry, the membrane and respective inked strips can be properly stored at -20°C.

3.3. Immunoblot assay

We analyzed the reaction of the mAbs to PVDF membranes containing total lysates of *B. besnoiti* tachyzoites and bradyzoites, as well as APure-BbELISA Ag. To detect cross-reactions we analyze the reaction of the mAbs to membranes with total lysate antigens from *N. caninum* tachyzoites and *T. gondii* tachyzoites. Those procedures were carried out with the Immunoblot assay under reducing and non-reducing conditions.

After SDS-PAGE the transferred proteins were bound to the surface of the membrane, providing access to immunodetection by specific mAbs. Any remaining binding sites were blocked by immersing the membrane in a blocking solution. After probing with the mAbs (primary Ab) the antibody-antigen complexes were detected using the HRP conjugated (secondary ab) and 4-chloro-1-naphthol as a substrate.

In this assay the mAbs were applied undiluted. The used control sera were diluted at 1:100 in PBS–TG and are described in table 3.

3.3.1. Reagent, solutions and materials

- PBS–TG: PBS 0.05% (v/v), Tween20 2% (v/v), fish gelatine liquid. Used as blocking solution and buffer.
- Tris-buffered saline (TBS); pH 7.4. Used for washing and buffer.
- TBS-T (0.05%): TBS, Tween 20 0.05%; pH 7.4. This buffer was used for washing.
- HRP conjugated secondary Ab: Peroxidase-conjugated AffiniPure sheep Anti-mouse IgG Fcγ Fragment Specific 1:500 diluted in PBS–TG.
- mAbs.
- Substrate solution: 40 ml TBS, 40 µl H_2O_2 30% (v/v), 30 mg 4-chloro-1-naphthol [Sigma] in 10 ml methanol.
- PVDF membranes containing: *B. besnoiti* tachyzoites, *B. besnoiti* bradyzoites, *N. caninum* tachyzoites, *T. gondii* tachyzoites and APure-BbELISA Ag.
- Positive control and negative control (Table 3)

Table 3 - Controls used for each PVDF membrane in Immunoblot assay

Membrane	Positive control	Negative control
B. besnoiti tachyzoites	*B. besnoiti* positive bovine serum.	*N. caninum* positive bovine serum
B. besnoiti bradyzoites		
APure-BbELISA Ag		
N. caninum tachyzoites	*N. caninum* positive mouse serum	DMEM
T. gondii tachyzoites	Pool from six mice *T. gondii* positive serum	DMEM

3.3.2. Execution

The strips were firstly wetted in PBS–TG and then the mAbs, the Positive control and the Negative control were added. After the strips were shacked for one hour and the solution removed, they were washed five times with TBS-Tween (0.05%). The HRP conjugated secondary Ab was added to incubate, then the solution was removed and the strips washed three times with TBS-Tween (0.05%) and two times with TBS.

Finally, the substrate solution was added to the strips. The reaction was stopped after aspirating the substrate solution and after a final wash with double-distilled water was made.

3.4. Meta-periodate treatment

MAbs were developed against the enriched membrane extract of *B. besnoiti* tachyzoites. For the characterization of mAbs we tried to determine if they are recognizing carbohydrate or protein epitopes.

To determine the possible involvement of carbohydrates in the formation of epitopes that bind to the generated mAbs, a meta-periodate treatment was performed according to

Woodward et al. (1985). The meta-periodate treatment consists in a mild periodate oxidation at acid pH that enable the cleavage of carbohydrate vicinal hydroxyl groups preventing the modification of polypeptide chain structures (Woodward et al., 1985).
This method is particularly useful in the Immunoblot assay because the modification of epitopes take place after electrophoretic separation of proteins eliminating the problems associated with changes in electrophoretic mobility which result from enzymatic and chemical deglycosylation procedures which are applied prior to the SDS-PAGE (Woodward et al., 1985).

3.4.1. Reagents, solutions and materials

- Natrium-Acetat-buffer: 1.361 g Na-Acetat in 100 ml water; pH 5.5 adjusted with acetic acid
- Natrium-meta-Periodate-buffer: 10 mM Natrium-periodate in Natrium-acetat-buffer; pH 5.5 adjusted with acetic acid
- Stopping solution: 50 mM Natrium-Borohydrid in PBS
- Nitrocellulose membrane (Optitran, Schleicher & Schuell, Dassel, Germany): 7.2x 8.5cm
- As Positive Control was used a *B. besnoiti* positive bovine serum and as Negative Control was used a *N. caninum* positive bovine serum

3.4.2. Execution

The procedure started with a preparation of a SDS-PAGE gel containing APure-BbELISA Ag with a subsequent Western blotting. The protocol was the same mentioned previously in this study, except with one modification: it was important to activate the nitrocellulose membrane with bidistilled water instead of methanol. After the Western blotting, the nitrocellulose membrane was cut into 26 strips. Subsequently, thirteen strips were incubated in Natrium-Acetat-buffer and another thirteen strips stayed in Natrium-meta-periodate-buffer for one hour in the dark, at room temperature. After one hour, the stopping solution was added and the strips were incubated for 30 min in the dark. Finally, the Immunoblot assay to test the mAbs was performed with the protocol previously

described in this study. As PC was used a *B. besnoiti* positive bovine serum and as NC was used a *N. caninum* positive bovine serum.

3.5. Optimization of the Competitive ELISA

The final aim of this study is the development of a mAb-based competitive ELISA. For this purpose the selected mAbs to optimization of the assay were mAb 3 and mAb 11. These were the selected mAbs because they expressed high index values in iELISA and most importantly they revealed no cross-reactions in immunoblot assay with *N. caninum* and *T. gondii* PVDF membranes under reducing and non-reducing conditions.

The results of the assay depends on the blocking of mAb binding to the coated Ag in the presence of positive sera, therefore the mAbs are evaluated for its ability to compete with *B. besnoiti* positive sera for binding to the Ag.

The mAbs dilution was optimized at two-fold serial dilutions at 1:10; 1:20; 1:30 and 1:40, therefore four different plates were performed with both mAbs in each half of the plate. The PC concentration used in the four plates was optimized at two-fold serial dilutions from 1:2 up to 1:32,768 for each mAb. Regarding the NC, the dilution was already optimal and performed at two-fold always at 1:2 for each mAb. All dilutions were made with PBS–T, 20% horse serum. As NC a *N. caninum* positive bovine serum was used and as PC a *B. besnoiti* positive bovine serum was applied.

3.5.1. Reagents, solutions and materials

All solutions were prepared fresh except the substrate buffer.

- PBS–T: PBS, 0.05% (v/v) Tween 20
- PBS–T, 20% (v/v) horse serum: pH 7.2. Was used as sample buffer and blocking solution.
- PBS–T, 1% (v/v) horse serum.
- Binding buffer: weekly a new 0.1 M Bicarbonate; pH 8.3 was used as coating buffer

- Sodium hydrogen carbonate. Was used for substrate buffer.
- H_2O_2, 30% (v/v)
- TMB: Used as chromogenic substrate
- Sulfuric acid, 2M H_2SO_4
- MAbs (3 and 11)
- APure-BbELISA Ag: Ag was diluted in bicarbonate buffer (1:600)
- Peroxidase-Conjugated AffiniPure Sheep Anti-mouse IgG Fcγ Fragment Specific diluted 1:500 in PBS–T, 1% horse serum.

3.5.2. Execution

The protocol used was similar to the iELISA protocol but still with a few changes (Figure 4).

The APure-BbELISA Ag was briefly centrifuged, diluted in the coating buffer and used to sensitize a 96-well microtiter plate at 37°C for one hour. The wells were washed three times with PBS–T and the unbound active sites were blocked with PBS–T, 20% horse serum, followed incubation at 37°C. After 30 min the PC and NC sera, diluted in PBS–T, 20% horse serum, were added always in two-fold. After 30 min of incubation, the unbound antibody was washed away three times with PBS-T and the wells were then incubated for 30 min with mAbs 3 and 11 at the correspondent dilution depending on the plate. Then the wells were washed three times with PBS-T and the wells were then incubated for 30 min with HRP conjugated secondary Ab diluted at 1:500 in PBS–T, 1% horse serum. Unbound HRP conjugated secondary Ab was washed away three times and the substrate solution was added (100 µl TMB and 1.2µl H2O2 30% (v/v) in 10 ml of substrate buffer). Color development was stopped, after 15 min, by the addition of sulfuric acid. Optical density (OD) was measured at 450 nm by the ELISA reader and analyzed with software for an enzyme immunoassay (Tecan Group Ltd.). We calculated the inhibition relative to the negative and positive controls, therefore the ODs were converted to percentage inhibition (PI) values by the following formula:

$$\mathrm{PI}(\%) = \frac{(\mathrm{OD\ in\ PC\ test\ well\ [1{:}2\ to\ 1{:}32{,}768])} - (\mathrm{OD\ in\ PC\ [1{:}2]})}{(\mathrm{OD\ in\ NC}) - (\mathrm{OD\ in\ PC\ test\ well\ [1{:}2\ to\ 1{:}32{,}768])}}\ x\ 100$$

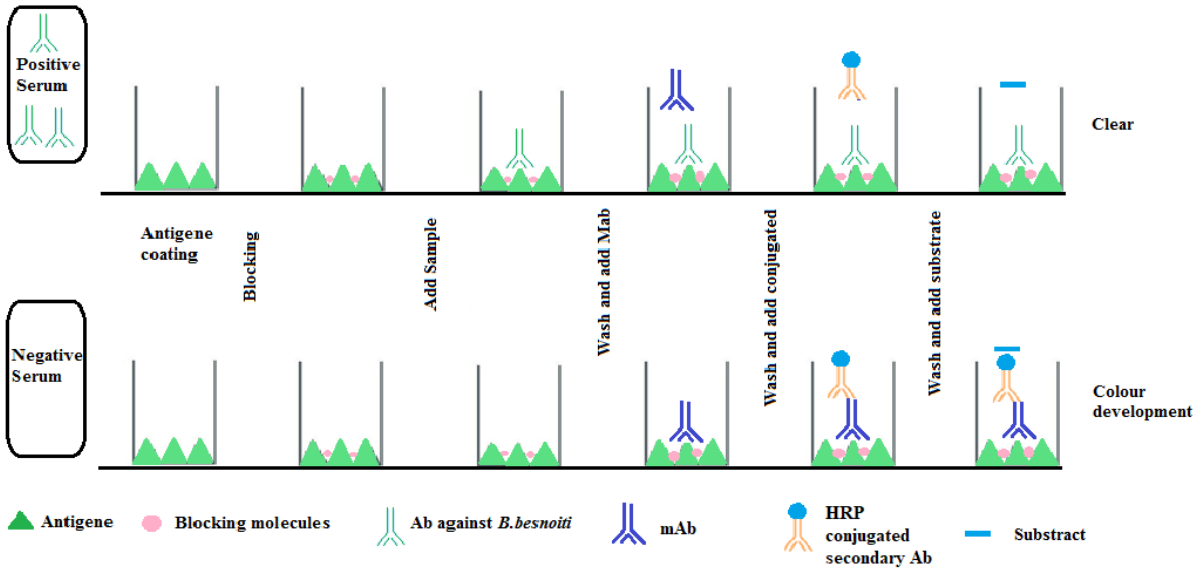

Figure 4 - Competitive ELISA diagram

3.6. Analysis of samples by Competitive ELISA

In cattle serum, Ab against *B. besnoiti* can be detected by virtue of their ability to block the binding of a mAb to the target epitope in a mAb-based competitive ELISA assay. A total of cattle sera (n= 948) were analyzed by Bb-cELISA1 with optimal conditions with the aim of evaluate the sensitivity and specificity of this test.

In this analysis, reference positive and reference negative cattle sera were included. These sera came from four different farms and were all tested for besnoitiosis with an indirect ELISA ("reference test"), for some cases an additional analysis by IFAT was performed. One farm had clinical cases of acute and chronic neosporosis, another farm had cases of sarcocystiosis and two others had clinical cases of chronic besnoitiosis.
After tested for besnoitiosis, 723 samples were selected as a reference negative population and 225 samples as a reference positive population.

The assays were carried out with mAb 3, this mAb proved to be optimal earlier when diluted at 1:40.

As NC were used a *N. caninum* positive bovine serum and blocking solution. As PC was used a *B. besnoiti* positive bovine serum. The PC optimal dilution was established earlier to be 1:100 and 1:1000. All cattle sera analyzed were diluted 1:2 as well as the NC in PBS–T, 20% horse serum. All controls were done in fourfold.
The materials and the protocol used in this analysis were exactly as described previously for Competitive ELISA (Figure 4).

After the assay, to distinguish a positive serum of a negative serum, was done a visual evaluation of the plate where a besnoitiosis positive serum expressed a clear well and a besnoitiosis negative serum expressed a well with colour development, in case of uncertainty the test was repeated.

A comparative analysis of the "reference test" and the Bb-cELISA1 was carried out with the aim to evaluate the performance of Bb-cELISA1. An statistical package was used to find out the optimal cutpoints using Youden's index that is equal to sensitivity plus specificity minus one.

4. Results

4.1.Immunoblot assay.

After SDS-PAGE and Western blotting to PVDF it was possible to visualize in Immunoblot the protein bands recognized by each mAb. After the proteins had been transferred to a nitrocellulose membrane, the involvement of carbohydrates in epitope formation could be assessed with a meta-periodate treatment.

Usually, the Immunoblot assay was implemented with PVDF membranes after SDS-PAGE had been performed under reduced and non-reduced conditions, in order to evaluate the reactivity and specificity of the mAbs for different parasitic stages (*B. besnoiti* tachyzoites and *B. besnoiti* bradyzoites), antigen preparations (APure BbELSA Ag) or other species (*N. caninum* tachyzoites and *T. gondii* tachyzoites).

After immunoblotting the relative molecular masses of the reactive bands were determined by comparison with a LMW-SDS Marker standard. The reactivity and the relative

molecular masses are expressed in the Table 4 and, the reactive bands are displayed in Figures 5, 6, 7, 8 and 9.

To assess the involvement of carbohydrates, APure-BbELISA Ag was separated under non-reduced conditions in SDS-PAGE and Western blotted to a nitrocellulose membrane. The membrane was cut in 26 strips and thirteen control strips were incubated only with Natrium-Acetat-buffer (first strip of the pair) and other thirteen strips were mildly oxidated with Natrium-meta-periodate-buffer (second strip of the pair). An immunoblot was performed and the mAbs were incubated with each pair of trips. The results are displayed in Figure 10. The effect of the meta-periodate treatment on the strips is presented in Table 5.

Table 4 - Serological characterization of the mAbs: protein bands detected by Immunoblot assay after SDS-PAGE under reducing and non-reducing conditions and transfer to PVDF membranes

Species	*B. besnoiti*						***T. gondii***		***N. caninum***	
Stage (prep-aration)	T		B		T (APure-BbELISA Ag)		T		T	
mAb	r[b]	nr[b]	R	nr[b]	r[b]	nr[b]	r[b]	nr[b]	r	nr[b]
1	88.7	-	-	-	-	87.6; 42.3	82.2	-	-	-
2	-	> 97.4	-	-	-	> 97.4	-	-	-	-
3	42.5	> 97.4 (22.5; 21.0; 15.9)	> 97.4	> 97.4	42.9 (42.1)	> 97.4; 21.6; 16.8 (22.8; 22.1)	-	-	-	-
4	42.1	> 97.4	> 97.4	> 97.4	42.9 (42.1)	> 97.4	-	-	-	-
5	-	81.7	-	-	-	85.5; 80.6; 70.3 (60.4; 36.1)	-	-	-	-
6	75.7 (45.0)	80.6	-	-	77.1; 34.6; 30.5; (56.9; 20.4; 38.0)	84.4; 80.0; 38.8; 19.6 (74.6; 63.7; 25.9)	43.5	-	-	-
7	-	81.7	-	-	-	85.5; 81.1; 70.3 (60.4; 36.1)	-	-	-	-
8	40.1 (37.2; 30.6; 26.8; 26.6)	41.3 (39.5; 37.7; 26.8)	41.3	-	40.9 (36.0)	> 97.4; 43.2; 40.1 (86.5; 82.2; 36.1; 34.8)	-	-	-	-
9	40.1 (37.2)	41.3 (39.5; 37.7)	41.7	-	40.9	> 97.4; 43.2; 40.1 (86.5; 82.2; 37.0; 36.1; 34.8)	-	-	-	-
10	40.1 (37.2; 30.6; 30.2; 26.8; 27.2)	41.3 (39.5; 37.7; 29.0; 27.1)	41.3	-	40.9 (37.2; 36.0)	> 97.4; 43.2; 40.1 (86.5; 82.2; 36.1; 34.8)	36.4 (36.1; 25.6)	46.2; 44.4; (42.5; 40.1)	39.8	40.2; 38.6 (33.8)
11	-	34.9 (39.5; 37.7)	-	-	-	39.7; 37.9; 36.1; 33.9	-	-	-	-
12	33.1 (24.5)	32,7 (29.9)	33.9; 31.9	28.5 (32.0)	33.1; 30.6	33.4; 30.8 (27.6; 26.2; 22.8)	23.4	25.9	-	24.5
13	-	22.5; 21.0; 15.9	-	-	-	21.2; 16.2 (22.5; 21.8)	-	-	-	-

T, tachyzoite; B, bradyzoite; r, reducing conditions; nr, non-reducing conditions
[b] Values in parenthesis indicate the molecular size of faint reactivity
- No reactivity observed

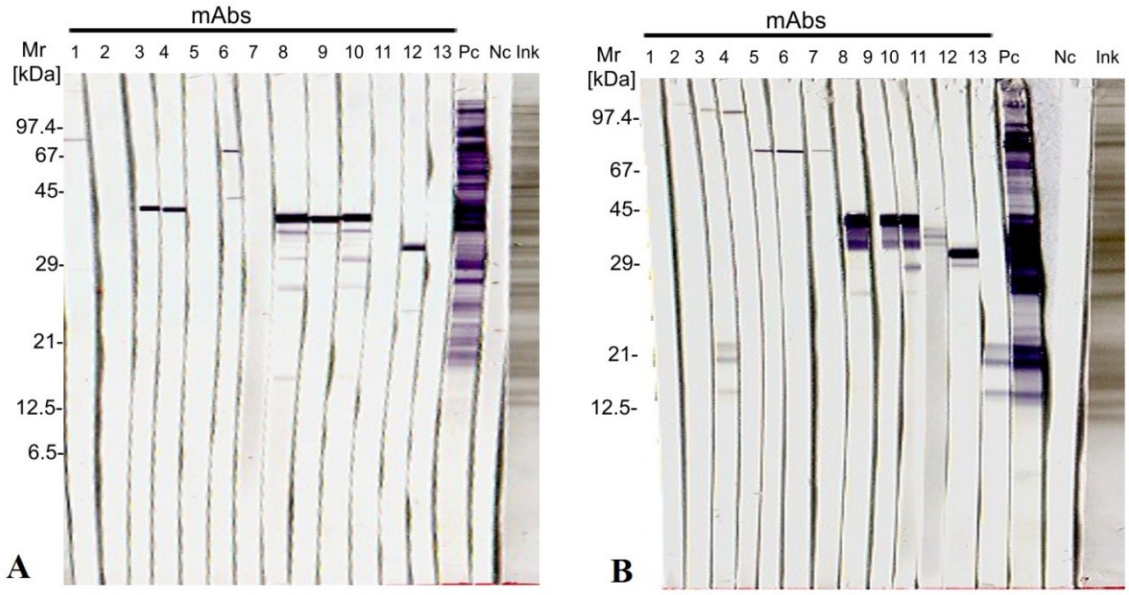

Figure 5 - Immunoblot reactions of the mAbs against *Besnoitia besnoiti* tachyzoites after SDS-PAGE under (A) reducing and (B) non-reducing conditions and Western blotting to PVDF membranes

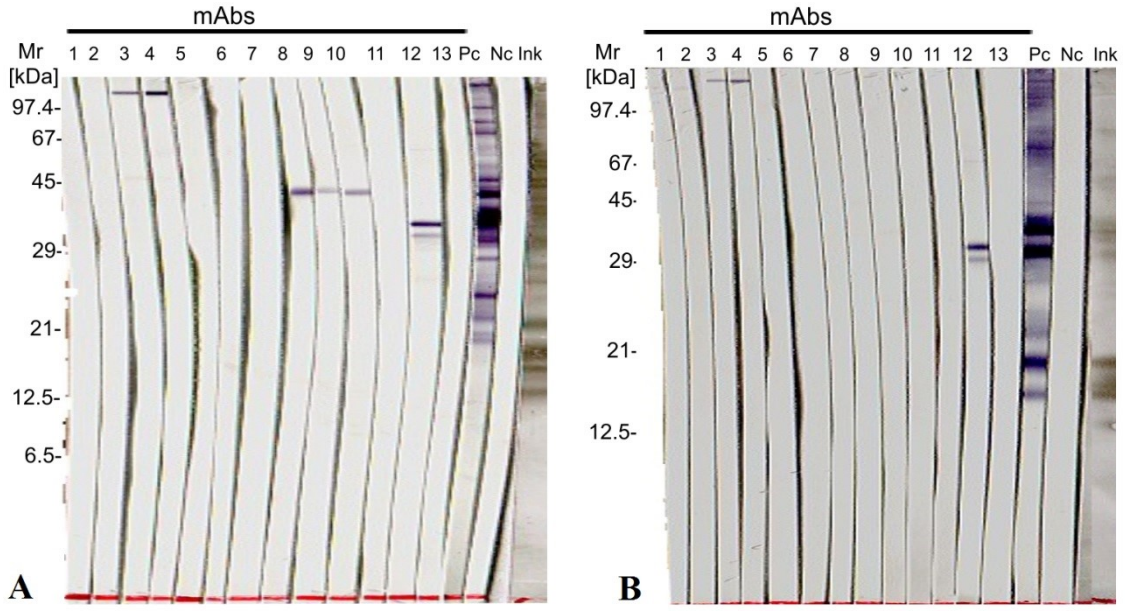

Figure 6 - Immunoblot reactions of the mAbs against *Besnoitia besnoiti* bradyzoites after SDS-PAGE under (A) reducing and (B) non-reducing conditions and Western blotting to PVDF membranes

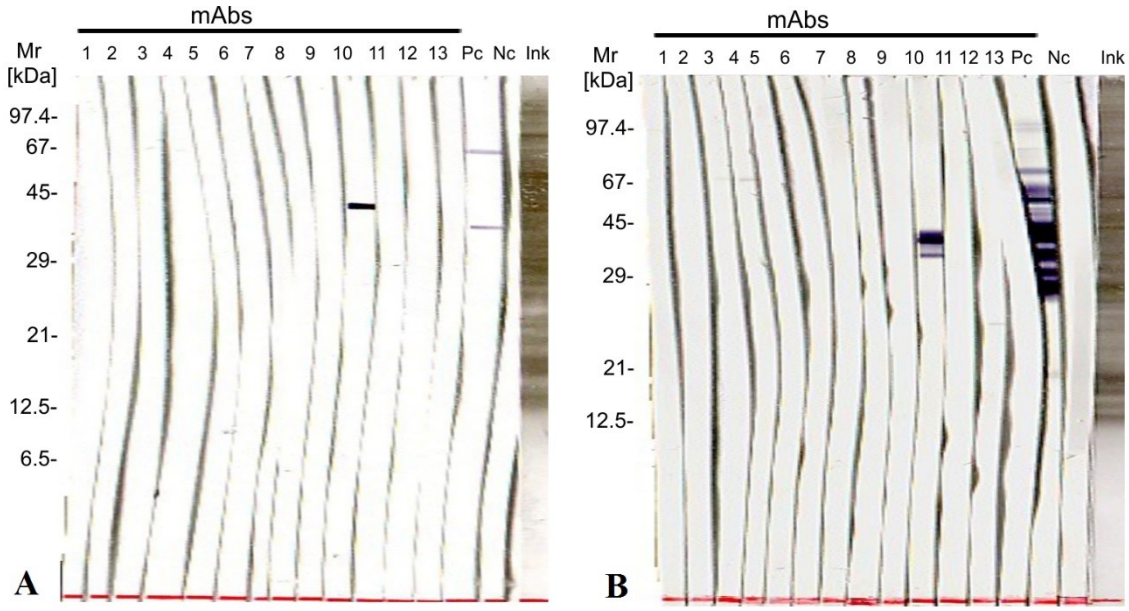

Figure 7 - Immunoblot reactions of the mAbs against *Neospora caninum* tachyzoites after SDS-PAGE under (A) reducing and (B) non-reducing conditions and Western blotting to PVDF membranes

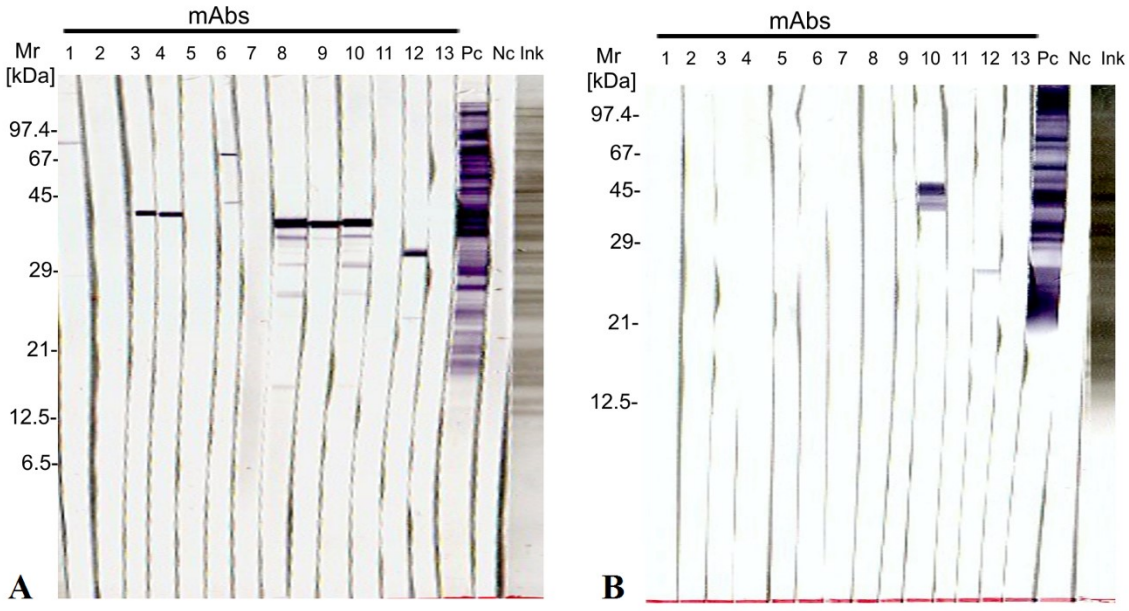

Figure 8 - Immunoblot reactions of the mAbs against *Toxoplasma gondii* tachyzoites after SDS-PAGE under (A) reducing and (B) non-reducing conditions and Western blotting to PVDF membranes

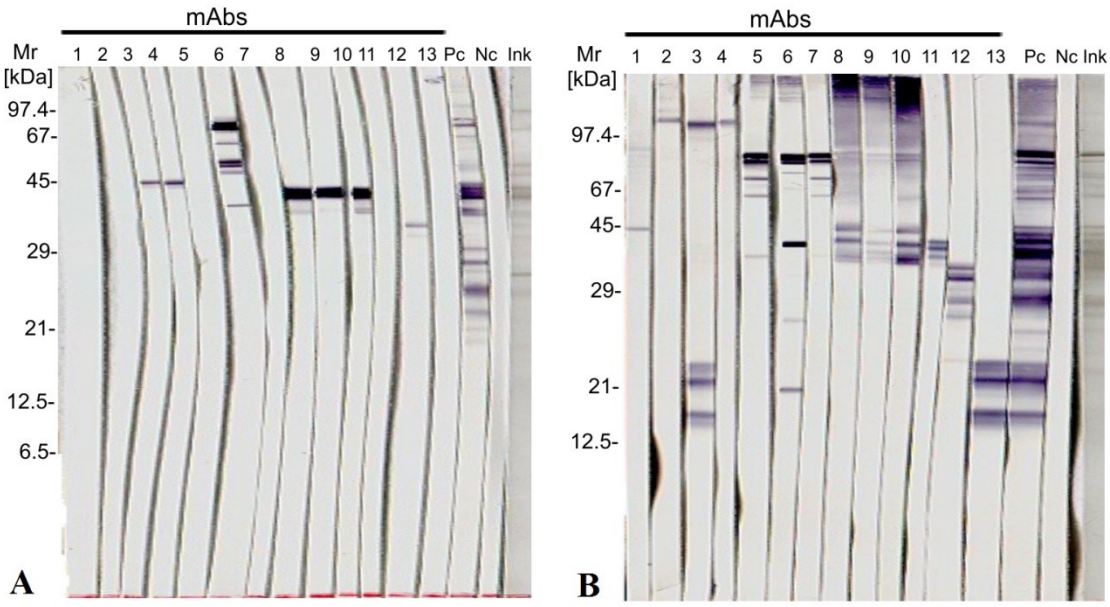

Figure 9 - Immunoblot reactions of the mAbs against APure-BbELISA Ag after SDS-PAGE under (A) reducing and (B) non-reducing conditions and Western blotting to PVDF membranes

4.2. Meta-periodate treatment

After the preparation of the SDS-PAGE gel containing APure-BbELISA Ag, subsequent Western blotting under non-reducing conditions and a transfer to nitrocellulose membranes were performed with a mild periodate oxidation explained earlier. The results are provided in Figure 10.

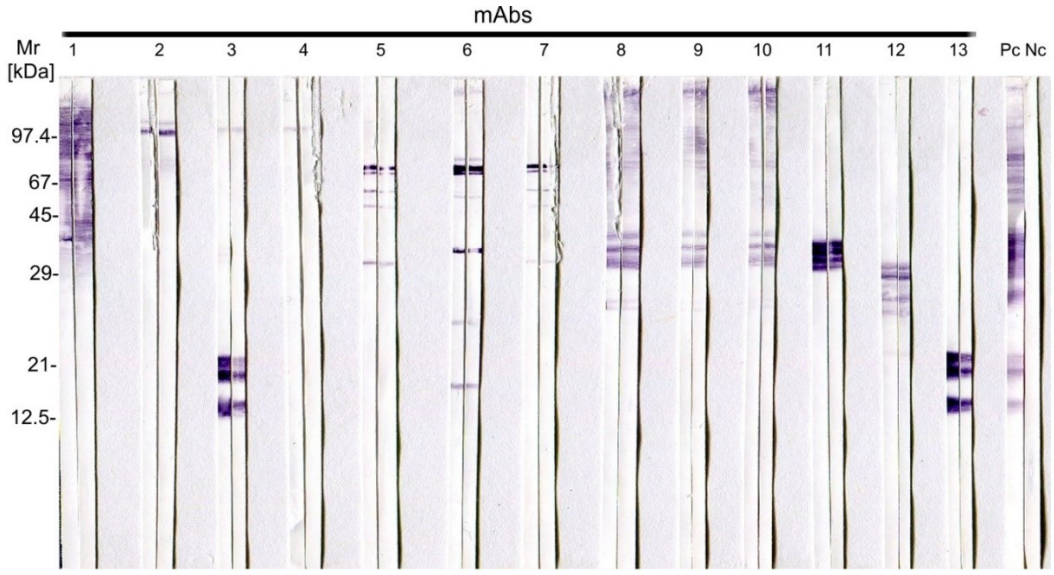

Figure 10 - Immunoblot reactions of the mAbs against APure-BbELISA Ag after SDS-PAGE under non-reducing conditions, transfer to nitrocellulose membranes and meta-periodate treatment

Table 5 - Serological characterization of the mAbs: effects detected by Immunoblot in nitrocellulose membranes after SDS-PAGE under non-reducing conditions, Western blotting and meta-periodate treatment

mAb designation	Effect of meta-periodate treatment on APure-BbELISA Ag [b]
1	-
2	-
3	21.6; 16.8 (22.8; 22.1)
4	-
5	-
6	-
7	-
8	-
9	-
10	-
11	-
12	-
13	21.6; 16.8 (22.8; 22.1)

[b] Values in parenthesis indicate the molecular size of bands in which a reduction in reactivity after meta-periodate treatment was observed;

- No effect observed

4.3 Optimization of the Indirect ELISA

Firsts iELISAs were performed with a few mAbs to confirm antibody dilution and evaluate the optimal dilution of HRP conjugated secondary Ab. One HRP conjugated secondary Ab tested was Peroxidase-Conjugated AffiniPure Rabbit Anti-mouse IgG (H+L) which was applied in two different dilutions: 1:500 and 1:5000. The index values calculated using values obtained for positive and negative control are shown in Table 6.

Table 6 - Indirect ELISA index values revealing the reactivity of some mAbs to the Apure-BbELISA Ag as determined by with Peroxidase-conjugated AffiniPure Rabbit Anti-mouse IgG (H+L) at two different dilutions (1:500 and 1:5000)

	Dilution	
mAb designation	**1:500**	**1:5000**
1	0.113	-0.109
3	1.153	0.686
4	0.250	-0.041
5	0.084	-0.055
7	0.097	-0.114
8	0.580	0.002
9	0.190	-0.084
11	0.601	0.262

After identifying the optimal HRP conjugated secondary antibody dilution it was essential to establish the optimal HRP conjugated secondary Ab regarding the specificity for mouse immunoglobulins. Prior lab experience indicated that HRP conjugated anti-mouse IgG (H+L) also reacted with bovine IgG which would interfere with bovine IgG in competitive ELISA. Therefore we tested some mAbs in the iELISA with the non-specific HRP conjugated secondary Ab (Peroxidase-Conjugated AffiniPure Rabbit Anti-mouse IgG (H+L)) and with a specific HRP conjugated Ab (Peroxidase-Conjugated AffiniPure Sheep Anti-mouse IgG Fcγ Fragment Specific). The index values were calculated and are presented in Table 7.

Table 7 - Indirect ELISA index values for some mAbs representing their reactivity with Apure-BbELISA antigen as determined using two different peroxidase-conjugated secondary antibodies, diluted 1:500

	HRP conjugated secondary Ab	
mAb designation	**Peroxidase-Conjugated AffiniPure Rabbit Anti-mouse IgG (H+L)**	**Peroxidase-Conjugated AffiniPure Sheep Anti-mouse IgG Fcγ Fragment Specific**
1	0.113	0.503
3	1.153	1.016
4	0.250	0.122
5	0.084	0.053
7	0.097	0.035
8	0.580	0.410
9	0.190	0.154
11	0.601	0.845

The optimal assay conditions established resulted in increased test sensitivity for the purpose of the study so all thirteen mAbs were finally analyzed through iELISA and Index Values were calculated and shown in Table 8.

Table 8 - Indirect ELISA index values for individual mAbs tested with the Peroxidase- Conjugated AffiniPure Sheep Anti-mouse IgG Fcγ Fragment Specific at 1:500 dilution

mAb designation	iELISA Index Values
1	0.503
2	0.118
3	1.016
4	0.122
5	0.053
6	0.012
7	0.035
8	0.410
9	0.154
10	0.479
11	0.845
12	0.019
13	0.670

4.4. Optimization of the Competitive ELISA

After analyzing the mAbs in the indirect ELISA we were able to conclude that the mAbs 3 and 11 were the ones that showed the strongest reactivity with the APure-BbELISA Ag. Both mAbs were used to optimize the conditions of the cELISA.

To determine the optimal mAb and its concentration required for an optimal cELISA, four different dilutions were tested: 1:10; 1:20; 1:30; 1:40 in four different plates. In addition, the performance of competitive ELISAs and optimal dilutions of the Positive Control were assessed by a serial titration from 1:2 up to 1:32,768.

For each PC titer, the percentage of inhibition was calculated and normalized by OD values of negative and positive controls, the inhibition curves were determined for the four different mAb dilutions (1:10; 1:20; 1:30; 1:40) for mAb 3 (Figure 11) and for mAb 11 (Figure 12).

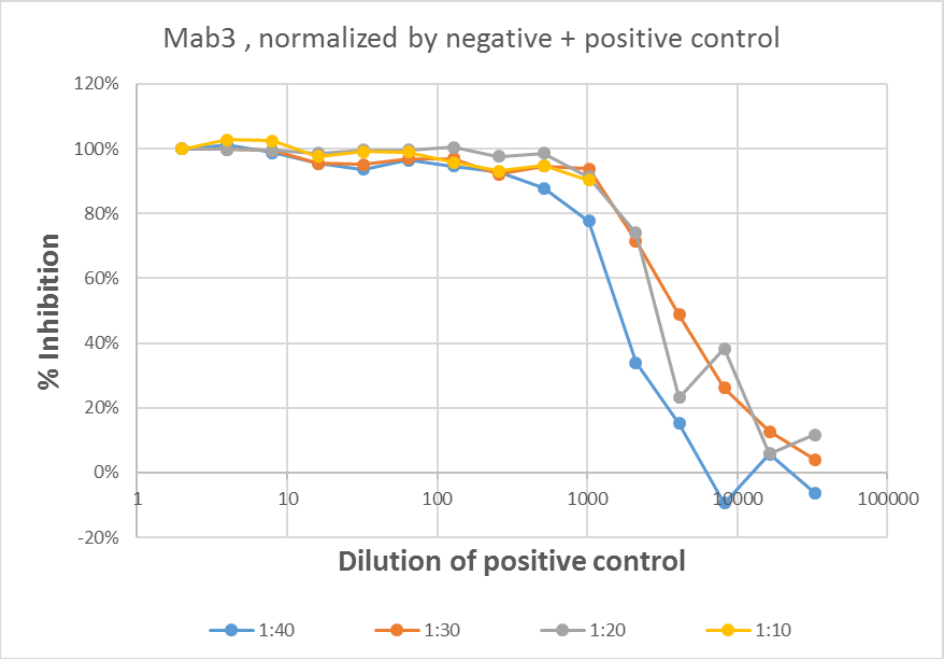

Figure 11 - Inhibitive curves as determined by a titration of the positive control serum and after using four different dilutions of the mAb 3 in the competitive ELISA using Apure-BbELISA antigen to sensitize plates.

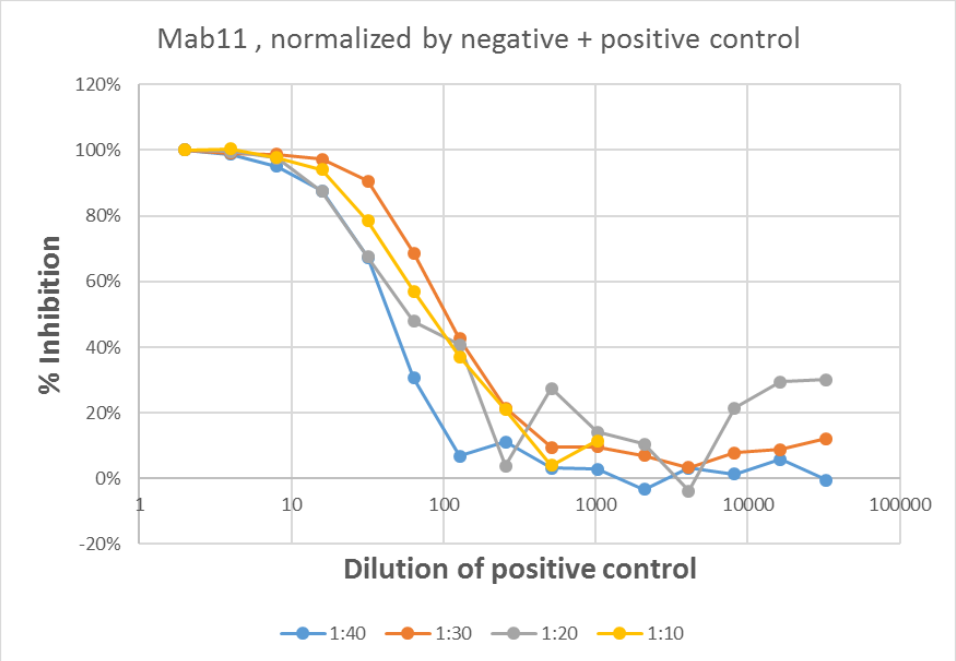

Figure 12 - Inhibitive curves as determined by a titration of the positive control serum and after using four different dilutions of the mAb 11 in the competitive ELISA using Apure-BbELISA antigen to sensitize plates

The dilution of the mAb preparation (hybridoma supernatant) had only a limited effect on the outcome of the competitive ELISAs. To save mAb preparation, it was decided to use a 1:40 dilution as optimal for the establishment of the competitive ELISA. In case of mAb 3 the positive serum could be diluted much higher than in case mAb 11 used in competitive ELISA. Based at the outcome of the titration experiments the Positive Control serum was used at 1:100 and used for the normalization of results. An additional 1:1000 diluted control was applied in each assay as a weak positive control. These determinations were essential to proceed to the next step of analysis of cattle sera.

4.5. Analysis of samples by Competitive ELISA

A total of 948 cattle sera confirmed to be *B. besnoiti* positive or negative by iELISA were used. These sera had been collected from four different farms (i.e. a farm with acute chronic cases of *N. caninum* infections, a farm with a sarcocystiosis problem and two farms with cases of chronic besnoitiosis). All sera had been tested by iELISA, previously. Sera from herds with neosporosis and sarcocystosis as well as *B. besnoiti* negative sera from herds with bovine besnoitiosis were regarded as "reference negative". The *B. besnoiti* positive sera were selected from animals present in a herd with bovine besnoitiosis and regarded as "reference positive".

A comparative analysis of the results from the "reference test" and Bb-cELISA1 was carried out to achieve an accurate assess to the sensitivity and specificity of the diagnostic test (Table 9).

Table 9 - Comparison of results obtained between 948 reference negative and positive cattle sera

	Reference negative sera	**Reference positive sera**	**Total**
Negative	722	2	724
Positive	1	223	224
Total	723	225	948

To summarize the performance of the Bb-cELISA1, R, version 3.5.3 (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org) was used to define an optimal cut-off and to determine diagnostic sensitivity, specificity, and positive and negative predictive values (package “optimal.cutpoints”).

Youden's index was used to estimate with a confidence interval of 95%: the inhibition cut-off was 49%, resulting in sensitivity of 99.1% and specificity of 99.9%; the positive predictive value was 99.6 % and the negative predictive value was 99.7%; there was only one false positive and two false negative results (table 10).

In Figure 13 are expressed the inhibition percentages of the negative cattle sera (n= 224) and the inhibition percentages of the positive cattle sera (n=724) in the Bb-cELISA1.

Table 10 - Optimal cutpoins with an inhibition cut-off of 49%

Inhibition cut-off	**49%**
Sensitivity	99.1%
Specificity	99.9%
Negative Predictive Value	99.7%
Positive Predictive Value	99.6%
False Positive	1
False Negative	2

The competitive ELISA Bb-cELISA1 almost perfectly separates negative and positive reference sera when 49% was used as the cut-off. In Figure 13 we can see that most of the positive reference sera showed a percentage of inhibition close to 100%. In contrast, most of the negative reference sera showed percentage of inhibition values around 15%.

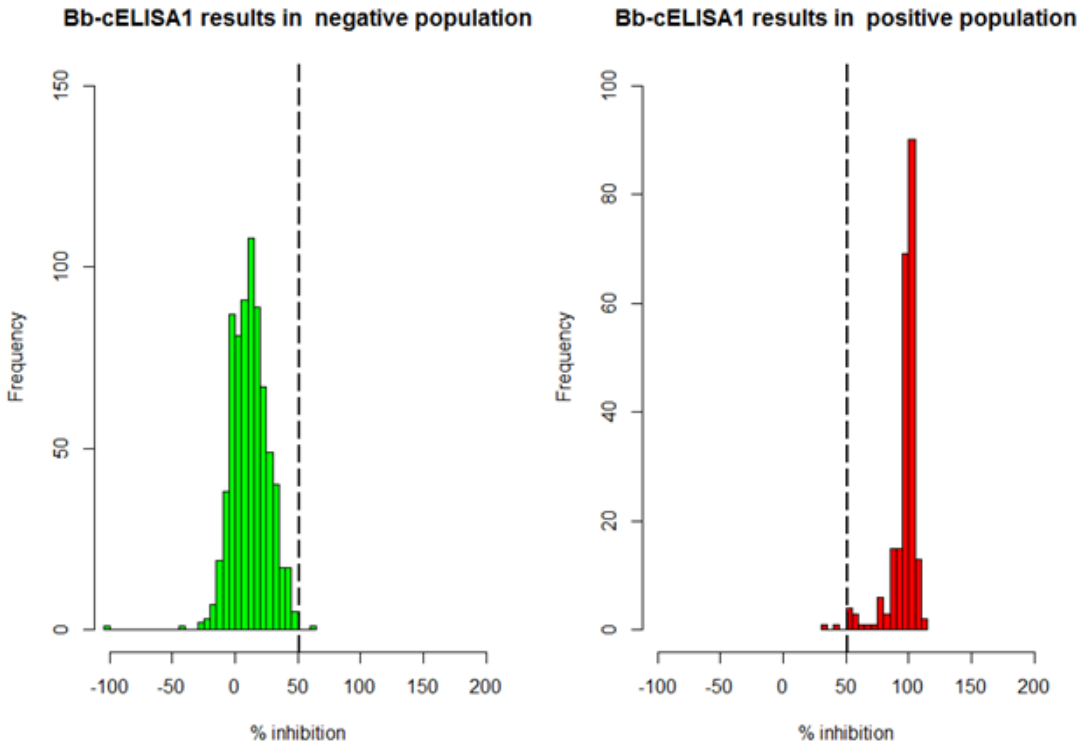

Figure 13 - Distribution of inhibition percentages in Bb-cELISA1 of 224 *B. besnoiti* negative sera (on the left) and 724 *B. besnoiti* positive sera (on the right) against mAb 3

Discussion

Although the mortality rate due to infections with *B. besnoiti* is low, the morbidity reaches 10% of the herd in the first three years and, diseased males are unfertile, thus having a relevant economic importance and, remaining a lack of knowledge on it epidemiology. Thus, there is a major necessity to develop specific research programs in epidemiology, diagnosis and control as well. Bovine besnoitiosis can have massive effects both during the acute and chronic phases of infection, that can compromise the welfare of the infected animal.

Essentially, the economic losses occur because the hides of chronically infected animals may become valueless for tanning, the carcasses may need to be condemned in the slaughterhouse, diseased bulls become sterile, milk production is insufficient, and cows, when pregnant, abort during the acute infection.

Despite more than 100 years from the recognition of bovine besnoitiosis and research, some aspects related to prevalence and incidence of infection in endemic areas, routes of transmission and risk factors associated with the infection, remain uncertain. Serological tests are the key for the implementation and monitoring success of control programs and, the most widely used approach relies on clinical inspection and diagnostic analysis.
The mAb technology has been improving significantly the diagnosis of protozoal and parasitic diseases since the limitations of polyclonal antibodies are overcome (Siddiqui, 2010). Because of the exquisite specificity of monoclonal antibodies, highly specific diagnostic methods may be developed.

This study was initiated in order to meet the needs of a specific and sensitive serological diagnostic method and the lack of epidemiologic studies that would be essential to control this re-emerging disease and prevent the spread of bovine besnoitiosis, nationally and internationally.

In the present thesis, we characterized thirteen mAbs that were previously generated against an enriched membrane extract of *B. besnoiti* tachyzoites (the so-called APure-

BbELISA Ag) in order to develop the first mAb-based competitive ELISA (Bb-cELISA1). This aim was successfully accomplished.

In order to establish an indirect ELISA to assess the reactivity of mAbs against APure-BbELISA Ag, some mAbs were analyzed with different HRP conjugated secondary Abs at different dilutions. The results showed that mAbs are more reactive with APure-BbELISA Ag, when the indirect ELISA was performed with a conjugate dilution of 1:500 (Table 7) but regarding the type or specificity of the HRP conjugated secondary Ab (i.e. either a H+L or a Fcγ specificity) no marked differences were noted on the results with the tested mAbs (Table 8). As an optimal HRP conjugated secondary Ab, we decided to select the Peroxidase-conjugated AffiniPure Sheep Anti-mouse IgG Fcγ fragment specific secondary antibody because its specificity for the mouse IgG Fcγ fragment ensured minimal cross reactivity, with bovine and horse serum proteins either, used as analyte (bovine serum) or used as blocking reagent (horse serum) in the assay. Therefore, the ability of mAbs to bind the APure-BbELISA Ag was evaluated by the optimal Indirect ELISA established and, mAbs 3 and 11 showed relatively high Index Values of 1.016 and 0.845, respectively. We concluded that these mAbs were the most reactive and for the purpose of this study they were selected to be appropriate ones to optimize the conditions for a competitive ELISA.

To study the mAb reactivity and specificity of these and the remaining mAbs in more detail, several immunoblot assays were performed. Some Ags were transferred to PVDF membranes in both reducing and non-reducing conditions in order to evaluate specificity of the mAbs regarding the stage (*B. besnoiti* tachyzoites and *B. besnoiti* bradyzoites) and regarding the genus (*T. gondii* tachyzoites and *N. caninum* tachyzoites). It was decided to use also non-reduced *B. besnoiti* antigens because a reducing buffer (containing β-mercaptoethanol) in SDS-PAGE destroys disulphide bonds and potentially relevant epitopes in both *B. besnoiti* tachyzoites and bradyzoites (Schares et al., 2010). Our results indicate that this decision was important, because, in general, relevant differences on the reactivity and localization of protein bands were observed between reduced and non-reduced conditions.

In order to evaluate the reactivity of the mAbs, an Immunoblot assay was performed in both, reducing and non-reducing conditions. The PVDF membrane containing the APure-BbELISA Ag that was used to immunize the mouse prior to mAbs production. According to the protein bands visualized, results showed that all thirteen mAbs reacted positively with APure-BbELISA Ag. However, marked differences were visualized when the Ag was reduced by βME (i.e. denatured and disulfide bonds were broken). Interestingly, overall the mAbs bound more frequently when disulfide bonds are intact (i.e., when the APure-BbELISA ag was used under non-reduced conditions).

Although the mouse used for mAb production had been immunized against an enriched membrane extract of tachyzoites (APure-BbELISA Ag), a few mAbs reacted also with *B. besnoiti* bradyzoite antigen (mAbs 3, 4, 8, 9, 10 and 12): mAbs 3, 4 and 12 showed strong reactions with *B. besnoiti* bradyzoite antigens under both conditions, reduced and non-reduced. A possible explanation is that some tachyzoite antigens or their epitopes are shared with those of the bradyzoite stage of *B. besnoiti*. This hypothesis was already mentioned by Schares et al (2013) and such cross-reacting epitopes have been previously reported for *T. gondii* (Darcy et al., 1990).

In both reducing and non-reducing conditions, our immunoblot results showed that mAbs 1, 2, 5, 6, 7, 11 and 13 reacted with *B. besnoiti* tachyzoites extracts and not with *B. besnoiti* bradyzoite antigens, which suggested that these mAbs are putative markers for monitoring a tachyzoite-to-bradyzoite stage conversion.

MAbs 3 and 4 recognized a band with a molecular mass of approximately 42 kDa under reduced conditions in both the total *B. besnoiti* tachyzoites and the APure-BbELISA Ag extract. One of the proteins with a corresponding relative molecular weight had previously been identified as a surface protein by surface biotinylation of tachyzoites (Schares et al., 2013). Whether the proteins identified here by mAbs 3 and 4 are indeed located at the surface needs further clarification. Because this protein band was not visualized under non-reduced conditions the result may suggest that this epitope is not available to bind to these mAbs when the disulphide bonds are intact (non-reduced conditions).

The mAbs 8, 9 and 10 reacted with a band with a molecular mass of approximately 41.3 kDa under non-reduced conditions in *B. besnoiti* tachyzoites and with a protein of approximately 40.1 kDa under reduced conditions in *B. besnoiti* tachyzoites and under

non-reduced conditions in the membrane extract (i.e. the APure-BbELISA antigen) which may indicate that this protein band also corresponds to a surface protein previously described (Schares et al., 2013). In this band, epitope binding seems to be not related to the presence of disulfide bonds in the protein band as reaction was present in both, reducing and non-reducing conditions.

In some of the bands recognized by mAbs in *B. besnoiti* bradyzoites, a high relative molecular mass (> 97.4 kDa) was observed, in both reduced and non-reducing conditions (mAbs 3 and 4). In contrast, in *B. besnoiti* tachyzoites antigen such bands (>97.4 kDa) were visible only in non-reduced conditions (mAbs 2, 3 and 4). The same was true if the APure-BbELISA Ag was examined, as only under non-reduced conditions bands > 97.4 could be visualized (mAbs 2, 3, 4, 8, 9 and 10). This could suggest that disulfide bonds are involved either in epitope building or in a multimerization of proteins.

In general, no major differences were observed after the meta-periodate treatment suggesting that the majority of epitopes recognized by the mAbs were not related to a glycoprotein. However, the bands that showed significant differences after the meta-periodate treatment were those recognized by mAbs 1, 3 and 13. These results suggest, that there is an involvement of carbohydrates in the formation of epitopes that bind to these mAbs. Regarding mAbs 3 and 13, both seemed to react with the same protein bands in the nitrocellulose membrane (bands of 21.6, 16.8, 22.8, and 22.1 kDa) and, in both the reactivity was similar after the meta-periodate treatment, showing a faint reaction regarding proteins with a molecular mass of 22.8 kDa and 22.1 kDa but persisting strong reactions with 16.8 kDa and 21.6 kDa.

Surprisingly, mAb 1 reacted much stronger with APure-BbELISA Ag on the nitrocelullose membrane as compared to the reaction on PVDF membrane resulting in a diffuse banding pattern in case of nitrocellulose. MAbs 5 and 6 reacted differently in the nitrocelullose membrane lacking one protein band of high molecular weight (>94.4 kDa). Possibly, these differences can be explained by differences in the binding characteristics between nitrocellulose and PVDF membranes.

As the aim was the development of a specific test, the study of cross-reactions with other taxonomically related coccidian parasites was crucial, therefore PVDF membranes of *T. gondii* tachyzoites and *N. caninum* tachyzoites were analyzed and the results showed that

mAbs 1, 6 and 12 reacted with *T. gondii* tachyzoites and mAb 10 reacted strongly with both *T. gondii* tachyzoites and *N. caninum* tachyzoites. The observed reactions on the PVDF membranes by mAb 6 and 12 were very faint and, not considered significant. After these results we can conclude that none of the mAbs 1, 6 or 12 would be specific to detect Abs against *B. besnoiti* in animal serum. As mAbs 3 and 11 were the most reactive to APure-BbELISA Ag, they were selected to be ideal for the optimization of a competitive ELISA. This decision was further confirmed taking into consideration the absence of cross-reactions with *T. gondii* tachyzoites and *N. caninum* tachyzoites membranes in immunoblot.

The competitive ELISA to detect antibody reactions by using a specific monoclonal antibody as competitor largely relies on its affinity for the epitope (i.e. the antigenic determinant) and its specificity. The optimization of the conditions under which the mAbs were used in cELISA was essential in this study. In fact, the ability of mAbs to compete with bovine serum antibodies depends not only on the specificity and the avidity of their binding to the antigen but also on the relative amounts of the competing antibodies (i.e. mouse vs. bovine antibody concentration). Thus, mAbs 3 and 11 were tested separately at different dilutions with sequentially dilutions of the bovine positive control serum (1:2 up to 1:32,768). As expected, the results showed that as much as we diluted the positive control, i.e. a lower concentration of epitope specific bovine serum antibodies caused a lower inhibition. In the case of mAb 3 a dilution of 1:1000 of the bovine positive control was still able to efficiently inhibit the mAb (with a percentage of about 80%). In contrast, in the case of the mAb 11 a dilution of 1:1000 of the bovine positive control was inhibiting the mAb reaction for less than 10 %. It can be concluded that the binding of mAb 11 to its epitope is stronger than of mAb 3. In order to optimize the test in terms of sensitivity, we selected mAb 3 to establish the Bb-cELISA1. In this test, the positive control serum was used at dilutions of 1:100 on each plate to normalize the results. In addition, the positive control was also used at a dilution of 1:1000, to confirm the analytic sensitivity of the test on each ELISA plate. Surprisingly, it was noticed that different mAbs dilutions did not show a significant variation of inhibition curves (Fig. 11 and 12). Therefore, it was decided to use mAb 3 at the dilution of 1:40, to save as much of the hybridoma supernatant.

In order to ascertain that the Bb-cELISA1 can effectively detect *B. besnoiti* specific antibodies, in infected cattle sera, 948 reference samples were analyzed with the optimal conditions (mAb 3 at a dilution of 1:40 and, the positive control serum dilutions of 1:100 and 1:1000). In addition, on each plate, a negative control serum was tested at a dilution of 1:2 (i.e. the dilution at which samples were tested, too). To assess the diagnostic sensitivity, sera from herds with bovine besnoitiosis were used. In the past it has been shown that *N. caninum* and *Sarcocystis* spp. infections may cause false positive results in *B. besnoiti* ELISAs. Thus, we used sera from herds with bovine neosporosis and herds with the presence of *Sarcocystis* infection to confirm diagnostic specificity of the Bb-cELISA1.

To validate the Bb-cELISA1, statistic parameters were determined. To summarize the performance of the Bb-cELISA1, several statistic parameters were calculated by a "Computing Optimal Cutpoints in Diagnostic Tests" managed with "R". Based on the Youden's index an optimal inhibition cut-off of 49% was determined, resulting in an estimate of the diagnostic sensitivity of 99.1% and a diagnostic specificity of 99.9%. At this cut-off the test had a positive predictive value of 99.6 % and the negative predictive value of 99.7%; there was only one false positive and two false negative results (Table 10).
In summary, the statistical analysis revealed that the Bb-cELISA1 had a high diagnostic sensitivity and a high diagnostic specificity for the detection of bovine antibodies against *B. besnoiti*. Thus, these results suggest that the Bb-cELISA1 could be used as a stand-alone test for herd monitoring programs, without the need for a confirmatory test.

Summarizing, mAb 3 is an IgG1 isotype antibody with a Kappa light chain. It was produced by the hybridoma cell line 1/24-9/9-4 and showed high reactivity against the APure-BbELISA Ag when tested in iELISA. Under non-reducing conditions the epitope characterization showed a high molecular mass protein band (> 97.4 kDa) and three further reaction bands at 22.5 kDa, 21.0 kDa and 15.9 kDa in tachyzoite antigens. Under reduced conditions a strong protein band of 42.5 kDa was noticed in the *B. besnoiti* tachyzoite antigen. Regarding the *B. besnoiti* bradyzoite antigen, a high molecular mass

protein band (> 97.4 kDa) was recognized, both, under reducing and non-reducing conditions. A diffuse band of proteins was detected in the membrane of APure-BbELISA Ag in non-reducing conditions, strong reactions were visualized with the following molecular masses > 97.4 kDa, 21.6 kDa, and 16.8 kDa and two further reactions (22.8 kDa and 22.1 kDa). No reactions were visualized in *T. gondii* tachyzoites and *N. caninum* tachyzoite antigens.

In comparison to the iELISA, the Mab-based Bb-cELISA1 has the advantage of only requiring an Anti-mouse HRP-conjugated secondary Ab, instead of the need of a variety of HRP-conjugated secondary Ab specific, each specific for the tested species. Thus, the Bb-cELISA1 assay can be applied in testing different animal species. This fact can be helpful for further research on the life cycle of *B. besnoiti*, still remaining so mysterious.

This study represents the first attempt on a Mab-based cELISA test for *B. besnoiti* infection in cattle. The aim to establish such test was successfully accomplished. However, the method still needs to be optimized. In order to save time, it would be better to reduce steps of the cELISA protocol (e.g., the possibility to block the plate with a blocking solution in which the mAb is already included, needs to be evaluated). This option eliminates the step of applying the mAb after the animal serum would save approximately 30 minutes. As an alternative, the addition of the diluted mAb directly to the diluted serum seems to be possible. This was suggested by Lunt et al. (1988). We think that it would be interesting to explore these options. Moreover, there is further the possibility that this test can be applied with the same efficiency, when the ELISA plate is coated with crude *B. besnoiti* Ag instead of the purified APure-BbELISA Ag. This modification may provide a more economic test, as time consuming and costly antigen purification can be avoided.

Mabs have several applications and thirteen mAbs were characterized in this study. The ability of mAbs to capture the antigen could also be used for antigen detection by sandwich (or capture antigen) ELISA or another serological test that might be more practical to use in the field.

Moreover, monoclonal antibodies may be helpful by giving us some information about the exact role that antibodies play in resistance to *B. besnoiti* and the mechanisms by which the parasite survives.
In further studies an IFAT could be performed since this allows the visual localization of the epitopes that each of the mAbs recognizes in and on parasitic stages, this technique was not performed in this study.

Conclusion

Since 2010, bovine besnoitiosis is considered a re-emerging disease and endemic in Europe according to EFSA and there is evidence of an increased number of cases and geographic expansion of the *B. besnoiti* infection and the associated disease. Because clinical cases represent the iceberg's tip and are relatively sporadic in endemic herds, diagnosis must identify asymptomatic cattle that subsequently constitute a reservoir of bovine besnoitiosis.

Thirteen mAbs were previously generated against an enriched membrane extract of *B. besnoiti* (APure BbELISA). This study describes several techniques essential to achieve a complete as possible characterization of the mAbs using iELISA and Immunoblot assay. The aim to establish a specific, sensitive, rapid and economic technique was successfully accomplished with a diagnostic sensitivity of 99.1% and a diagnostic specificity of 99.9%. However, the method still needs to be improved, in order to reach a more rapid and economic technique.

The accurate impact of this re-emerging disease has not been determined so far and, studies on the prevalence and incidence may be an important contribution. Our results suggests that the Bb-cELISA1 could be used as a stand-alone test for herd monitoring programs, without the need for a confirmatory test and contribute as well to the implementation of control strategies in the already affected and also at risk areas.
To conclude, the Bb-cELISA1 represents a valuable tool for the diagnosis and control of bovine besnoitiosis and for epidemiology studies of *B. besnoiti* infections in a variety of host species.

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