

UNIVERSIDADE FEDERAL DE PELOTAS
Programa de Pós-Graduação em Biotecnologia



Dissertação

Avaliação de um ELISA competitivo para detecção de anticorpos contra *Babesia bovis*

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**Avaliação de um ELISA competitivo para detecção de anticorpos
contra *Babesia bovis***

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RESUMO

GÖTZE, Marcelo Mendes. **Avaliação de um ELISA competitivo para detecção de anticorpos contra *Babesia bovis*** 2010. 53f. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A babesiose bovina, causada por *Babesia bovis* e *Babesia bigemina*, é a doença mais importante transmitida por carrapatos *Rhipicephalus (Boophilus) microplus* em áreas tropicais e subtropicais da América do Sul. O diagnóstico definitivo pode ser feito através da detecção de eritrócitos infectados em esfregaços sanguíneos, porém a parasitemia em sangue periférico é frequentemente muito baixa para que esse método seja utilizado de forma confiável para fins de diagnóstico. Por esse motivo, vários testes sorológicos, incluindo a fixação de complemento, hemaglutinação indireta e imunofluorescência indireta (IIF) têm sido usados para detectar anticorpos em bovinos infectados. Embora estes testes permitam a detecção de animais persistentemente infectados, eles têm limitações na especificidade e/ou sensibilidade. O IIF tem sido o mais sensível, mas a reatividade cruzada entre as espécies, interpretação subjetiva, e baixa produção tem limitado a sua utilidade. Os ELISA têm encontrado ampla aplicação no diagnóstico de doenças infecciosas. O formato cELISA (competitivo) pode fornecer um nível adicional de especificidade, pois o anticorpo é dirigido para um epítipo único específico para o organismo a ser detectado. Por estas razões, este estudo teve como objetivo avaliar a sensibilidade e especificidade do cELISA comparado com IIF e nested PCR (nPCR) para diagnóstico de babesiose causada por *B. bovis*. Para tanto, amostras de sangue bovino foram coletadas no Brasil e na Argentina e processadas para o diagnóstico de *B. bovis*. Utilizou-se o nPCR como teste padrão para a validação do cELISA, e a IIF como teste comparativo. O cELISA para diagnóstico de *B. bovis* apresentou-se de fácil processamento, com altos níveis sensibilidade e especificidade, além da rapidez no processamento de amostras em larga escala, sendo de grande utilidade para casos de surtos de babesiose bovina.

Palavras-chave: *Babesia bovis*, diagnóstico, ELISA competitivo, nested PCR, imunofluorescência indireta.

ABSTRACT

GÖTZE, Marcelo Mendes. **Evaluation of diagnostic tests for *Babesia bovis*** 2010. 53f. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Bovine babesiosis caused by *Babesia bovis* and *Babesia bigemina*, is the most important disease transmitted by *Rhipicephalus (Boophilus) microplus* in tropical and subtropical areas in South America. Definitive diagnosis can be made by detecting infected erythrocytes in blood smears. However, the parasitemia in peripheral blood is often too low for this method to be used for diagnostic purposes. For this reason, several serological tests, including complement fixation, indirect hemagglutination and indirect immunofluorescence (IIF) have been used to detect antibodies in infected cattle. Although these tests allow the detection of persistently infected animals, they have limitations in specificity and/or sensitivity. The IIF has been the most sensitive, but cross-reactivity between species, subjective interpretation, and low production has limited its usefulness. The enzyme linked immunosorbent assay (ELISA) have found wide application in the diagnosis of infectious diseases. The cELISA format (competitive) can provide an additional level of specificity, because the antibody is directed to a single epitope, specific for the organism to be detected. For these reasons, this study aimed to evaluate the sensitivity and specificity of cELISA compared to IIF and nested PCR (nPCR) for diagnosis babesiosis caused by *B. bovis*. Therefore, blood samples were collected from cattle in Brazil and Argentina, and processed for the diagnosis of *B. bovis*. The nPCR was used as the gold standard to validate the cELISA and the IIF as a comparative test. The cELISA for the diagnosis of *B. bovis* presented is easily processed with high levels of sensitivity and specificity. It is easily performed in a high number of samples, making it useful in cases of outbreaks of bovine babesiosis.

Key-words: *Babesia bovis*, diagnostic, competitive ELISA, *nested* PCR, indirect immunofluorescence.

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1. INTRODUÇÃO

A babesiose bovina, causada por *Babesia bovis* e *Babesia bigemina*, é a doença mais importante transmitida por carrapatos *Rhipicephalus (Boophilus) microplus* em áreas tropicais e subtropicais da América do Sul (Gluglielmone, 1995). A doença é responsável pela causa de grandes perdas econômicas na pecuária bovina. As perdas diretas estão relacionadas com a morbidade e a mortalidade de bovinos; as indiretas, com o custo do tratamento e do controle.

O diagnóstico clínico deve ser sempre confirmado pelo laboratorial específico, em virtude da diferença de sensibilidade aos medicamentos e da possibilidade de diagnóstico errôneo devido a outras doenças com sinais clínicos semelhantes. O diagnóstico definitivo da doença pode ser feito através da detecção de eritrócitos infectados em esfregaços sanguíneos, porém a parasitemia em sangue periférico é frequentemente muito baixa para que este método seja utilizado de forma confiável para fins de diagnóstico (Böse, et al., 1995; Goff, et al. 2006). Por este motivo, vários testes sorológicos, incluindo a fixação de complemento (Mahoney, 1962), hemaglutinação indireta (Goodger, 1971), e indiretos como imunofluorescência (Goff et al., 1982) têm sido usados para detectar anticorpos em bovinos infectados. Embora esses testes permitam a detecção de animais persistentemente infectados, eles têm limitações na especificidade e/ou sensibilidade.

A utilização da reação em cadeia da polimerase (PCR) para a detecção de *B. bovis* têm o potencial de proporcionar resultados qualitativos com alta sensibilidade. Entre as ferramentas moleculares, a PCR é o teste mais comumente utilizados, incluindo PCR outros instrumentos derivados, tais como o multiplex e *nested* PCR (nPCR) (Fahrimal et al., 1992; Figueroa et al., 1992, 1993). A alta sensibilidade e especificidade da técnica de nPCR torna útil para validar os resultados de outras técnicas de diagnóstico e para estudos epidemiológicos (Figueroa et al., 1993; Goff et al., 2006, 2008, Silva et al., 2009).

Os testes ELISA têm encontrado ampla aplicação no diagnóstico de doenças infecciosas. O teste pode ser automatizado para grande escala, e tem o potencial de especificidade melhorada em função dos esforços desenvolvidos na preparação e caracterização do antígeno (Goff et al., 2003). O formato cELISA (competitivo) pode fornecer um nível adicional de especificidade, já que utiliza um anticorpo dirigido

1 para um epitopo único sabidamente específico para o agente a ser detectado. Por
2 estas razões, foi produzido um cELISA (Goff et al., 2003) com base na capacidade
3 de anticorpos séricos inibirem um anticorpo monoclonal (MAb) diretamente contra
4 um epitopo localizado na RAP-1 (rhoptry-associated protein 1) de *Babesia bovis*
5 (Suarez et al., 1991, 1993, 1994; Hotzel et al., 1996, 1997; Boonchit et al., 2006).
6 Esse cELISA foi avaliado em condições laboratoriais com 135 amostras positivas e
7 141 amostras negativas. As amostras positivas eram de animais infectados
8 experimentalmente (119 amostras) ou de regiões endêmicas de Porto Rico (16
9 amostras). Amostras negativas eram de regiões livres de *R. B. microplus* (141
10 amostras). ROC análise revelou um teste com especificidade e valores preditivos
11 positivos de 100 % e sensibilidade de 91,1%. O cELISA foi distribuído em 4
12 diferentes laboratórios e processou-se 100 amostras definidas, incluindo positivas e
13 negativas. A concordância entre laboratórios variou de 94% a 88%, não
14 apresentando variação significativa entre os laboratórios. Esse experimento
15 demonstrou os atributos do cELISA para aplicações internacionais (Goff et al., 2006).

16 Devido aos prejuízos causados pela babesiose bovina no Rio Grande do Sul
17 e em países de fronteira, como Argentina e Uruguai, torna-se desejável um teste que
18 possibilite de forma rápida e segura o diagnóstico dessa enfermidade. Dessa forma,
19 este estudo teve como objetivo comparar o testes de cELISA com IIF e nPCR para
20 diagnóstico de *B. bovis*.

21

2. ARTIGOS

2.1. ARTIGO I

BOVINE BABESIOSIS: EPIDEMIOLOGICAL ASPECTS, DIAGNOSIS AND VACCINATION

Artigo científico formatado segundo as normas da revista (Pesquisa Veterinária
Brasileira)

Este artigo constitui-se de uma revisão sobre babesiose bovina, dando um enfoque para aspectos epidemiológicos, diagnóstico e vacinação. A redação da revisão teve contribuição dos pesquisadores Ignacio E. Echaide e Susana de Torioni Echaide, do Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Rafaela, Santa Fe, Argentina, pois foi elaborado durante uma visita de 2 meses àquela instituição. O pesquisador Leandro Quintana Nizoli, da Universidade Federal de Pelotas, também teve participação na redação da revisão.

***BOVINE BABESIOSIS: EPIDEMIOLOGICAL ASPECTS, DIAGNOSIS
AND VACCINATION***

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ABSTRACT

Bovine babesiosis is a tick-borne, hemoprotezoan disease responsible for substantial morbidity and mortality in cattle throughout the world. In South America, the disease is caused by *Babesia bovis* and *Babesia bigemina*. Definitive diagnosis of bovine babesiosis can be made by detecting infected erythrocytes in stained blood films. However, the parasitemia in peripheral blood is often too low to allow this method to be used reliably for diagnostic purposes. Serological testing is used as a tool to determine the babesial infection status of individuals and herds in control programs and, in the absence of treatment, as an indicator of persistently infected hosts. Live vaccines based on attenuated strains are used to control bovine babesiosis in several endemic countries, including Argentina and Brazil. Although these vaccines generally confer a long lasting protective immunity, they have a number of drawbacks that could be circumvented by a subunit vaccine. The identification and characterization of such subunit vaccine candidates is an active field of research. However, no practical candidates are yet forthcoming. The focus of this review will mainly be on the epidemiologic aspects, diagnosis, as well treatment and vaccination of bovine babesiosis.

Key words: *Babesia bovis*, *Babesia bigemina*, bovine babesiosis

1 INTRODUCTION

2
3 Bovine babesiosis is a tick-borne, hemoprotezoan disease responsible for
4 substantial morbidity and mortality in cattle throughout the world (McCOSKER, 1981).
5 Although it has been estimated that most of the world's cattle population (currently
6 1.371 billion (FAOSTAT Data, 2004) are exposed to babesiosis, this figure probably
7 do not represent a true reflection of the number of animals at risk.

8 Three-quarters of the world's cattle population are reared in regions where
9 babesiosis is enzootic and in many subtropical and tropical countries the disease
10 results in significant economic losses (UILENBERG, 1995). Costs due to babesiosis
11 are incurred not only from mortality, ill-thrift, abortions, loss of milk/meat production
12 and draft power and from control measures (such as acaricide treatments, purchase
13 of vaccines and therapeutics), but also through its impact on international cattle trade
14 (BOCK et al., 2004).

15 In South America, the two prevalent species are *Babesia bovis* and *Babesia*
16 *bigemina*. *B. bovis* is classically known as a small *Babesia*, while *B. bigemina* is
17 larger and can extend to the full diameter of an erythrocyte (POTGIETER, 1977).
18 Both species show considerable morphological variation, making it difficult to identify
19 one from the other on morphological grounds alone (CALLOW, 1984; DE VOS &
20 POTGIETER, 1994).

21 The world distribution of bovine babesiosis due to *B. bovis* and *B. bigemina* is
22 between 32°S and 40°N (McCOSKER, 1981) where it is transmitted mainly by
23 *Rhipicephalus (Boophilus) microplus* tick larvae (MAHONEY & MIRRE, 1979). This
24 tick specie is the only vector of bovine babesiosis in South America
25 (GUGLIELMONE, 1995). *Bos indicus* x *Bos taurus* cattle are generally more resistant
26 to *R. (B.) microplus* infestations than *B. taurus* breeds, (LEMOS et al., 1985;
27 SUTHERST et al., 1988; GUGLIELMONE et al., 1992). The symptoms of the acute
28 form of the disease include anaemia, fever, haemoglobinuria, ataxia, and sometimes
29 death (BOCK et al., 2004). After acute or clinical infections, recovered animals
30 frequently sustain subclinical infections which are microscopically undetectable.
31 These carrier animals, which are not clinically ill, may continue to infect the tick vector
32 (CALDER et al., 1996).

33 Definitive diagnosis can be made by detecting infected erythrocytes in stained
34 blood films, but the parasitemia in peripheral blood is often too low to use this method

reliably for diagnostic purposes. Serological testing is used as a tool to determine the babesial infection statuses of individuals and herds in control programs and, in the absence of treatment, is the best indicator of persistently infected hosts.

Live vaccines based on attenuated strains are used to control bovine babesiosis in several affected countries, including Argentina (WILKOWSKY et al., 2008). Although these vaccines generally confer a long lasting protective immunity, they have a number of drawbacks that could be circumvented by a subunit vaccine (UILENBERG, 1995; BROWN & PALMER, 1999; JENKINS, 2001). Several recombinant *B. bovis* and *B. bigemina* antigens have been reported to confer at least partial protective immunity (BROWN AND PALMER, 1999; JENKINS, 2001). The focus of this review will mainly be on the epidemiologic aspects, diagnostic, as well treatment and vaccination of bovine babesiosis.

EPIDEMIOLOGICAL ASPECTS

In the Brazilian territory, both species of *Babesia* are thought to be highly prevalent in most of this region. However, some microregions of the Northeastern and South regions are exceptions (areas South of parallel 32°S are free of *R. (B.) microplus* ticks). In Argentina, these protozoans are prevalent North of parallel 30°S, where their natural vector exists (BONO et al., 2008). The limiting factors in the distribution of tick vectors are humidity and temperature.

An important factor in disease outbreaks of babesiosis are the increasing development of acaricide resistance as well as the spread of (infected) ticks under favorable climatic conditions into areas normally free of babesiosis. Climate changes, due to global warming, may also result in the extension of the distribution areas of these ticks and thus of babesiosis (DE WAAL & COMBRIK, 2006).

An outbreak of babesiosis due to *B. bovis* was reported by SCHILD et al. (2008) in Santa Vitória do Palmar municipality, considered a *R. (B.) microplus* free area, in Southern Brazil. Animals from endemic areas were introduced to the herd and 40 out 393 animals died. It was suggested that the mean temperatures between 23.2°C and 23.6°C and the relative humidity between 75.3% and 77.5% observed in the period allowed the occurrence of one tick generation, which caused infection by *B. bovis* in cattle without immunity.

1 The Friesian breed is one of the major components of the dairy cattle
2 population in the world, and such cattle suffer heavy losses from this disease when
3 situated in babesiosis enzootic areas (GUGLIELMONE et al., 1992). Calves less than
4 2 months old born to previously unexposed cows are susceptible to infection and
5 effects of the disease while offspring of immune cows are resistant (CALLOW, 1984;
6 DE VOS et al., 1987).

7 After the age of 2 months a natural innate resistance or non-specific immunity,
8 that is not dependent on the immune status of the cow, provides additional
9 protection. This immunity generally persists for at least a further 4-6 months
10 (GUGLIELMONE et al., 1992; GOFF et al., 2001). Infection during this period induces
11 a long-lasting immunity, whereas primary infection later in life can produce severe
12 illness (MAHONEY et al., 1973, 1979). Surviving animals develop persistent
13 infections and serve as reservoirs for continued transmission.

14 Therefore, calves exposed to babesiosis during the first 6-9 months of age,
15 rarely show clinical symptoms and develop a solid long-lasting immunity
16 (DALGLIESH, 1993), leading to the development of an endemic stable disease
17 situation (CALLOW, 1977). It is also during this period that the risk of vaccine
18 reactions is very small and is therefore utilized as a window for vaccination.

19 However, when some animals fail to become infected for a considerable
20 period after birth, an endemic unstable situation arises, and they may develop
21 severe, life threatening disease if exposed later in life (DE WAAL & COMBRIK,
22 2006). According to BERENS et al. (2007) recovered bovines can remain infected for
23 years and experimental evidence showed that a single animal can be co-infected with
24 genetically and antigenically distinct strains.

25 *Bos indicus* cattle and their crosses are more resistant to the clinical effects of
26 infection with *B. bovis* and *B. bigemina* than *Bos taurus* cattle. Thus, because *B.*
27 *indicus* cattle and their crosses the inoculation rate of *Babesia spp.* is lowered
28 because fewer ticks are likely to attach per day, and because a smaller proportion of
29 ticks that feed on infected cattle will in turn be infected (GUGLIELMONE et al., 1989;
30 JONSSON et al., 2008). Therefore, it was postulated that, in some situations the
31 introduction of *B. indicus* blood would reduce the level of inoculation of *Babesia*
32 *species* to the point of converting an enzootic stability situation into instability
33 (GUGLIELMONE, 1995; UILEMBERG, 1995), increasing the risk of babesiosis
34 outbreak.

According to JONSSON (2006) purebred *B. indicus* cattle will carry 10% to 20% as many ticks as pure *B. taurus* cattle, and crossbred cattle will carry somewhere in between, more or less proportional to their *B. indicus* content, when exposed to the same environmental conditions of infestation. Although the genes involved in this expression have not been identified, it is widely known that these phenotypic characteristics are hereditary. In addition, GUGLIELMONE (1994) reported that the outbreaks in dairy cattle are associated with excessive tick control, and the use of annual pasture and rotational grazing, whereas in beef cattle herds outbreaks were associated with the use of acaricides with long residual periods.

R. (B.) microplus infection occurs in all 26 Brazilian states and its eradication is not feasible. The cattle population at risk numbers over 144 million, distributed over an area of approximately 8 million km², with a highly varied climate. This area includes several regions, each with its own characteristic vegetation and patterns of rainfall and temperature (LIMA et al., 2000). In such situation, successful management of bovine babesiosis will be based on the knowledge of the interactions between *Babesia* parasites, their vector, and cattle host.

DIAGNOSIS

Several direct and indirect detection methods, including blood smears, in vitro cultures, DNA probes and serology have been used for the diagnosis of *B. bovis* and *B. bigemina* infections. Definitive diagnosis can be made by detecting infected erythrocytes in stained blood films. The technique is inexpensive and reasonably portable. The sensitivity of thin film examination therefore ranges between 10⁻⁵ to 10⁻⁶, one parasite per 10⁵-10⁶ erythrocytes. Blood smear examination is a practice under field conditions. However, the accuracy of diagnosis relies on the training and skill of the microscopist. In addition, the parasitemia in peripheral blood is often too low for this method to be used reliably for diagnostic purposes (GOFF, et al., 2006).

Polymerase chain reaction (PCR) assays for the diagnostic detection of *Babesia* parasites have the potential to provide rapidly qualitative results with high sensitivity. Among the molecular tools, PCR is the most commonly used, including other PCR-derived tools, such as the multiplex and nested PCR (FAHRIMAL et al., 1992; FIGUEROA et al., 1992, 1993; ABOULAILA et al., 2009). Recently, a PCR and reverse line blot hybridization assay for detection of *B. bigemina* based on the rap-1a

and 18S gene sequences, respectively, has been reported to give increased sensitivity. The one-step PCR assay is highly specific, with an estimated analytical sensitivity corresponding to 0.000002% parasitemia (PETRIGH et al., 2008). More recently, was described the successful development of a nested PCR method for the detection of *B. bovis*. The sensitivity of nPCR primers of the SBP2 gene was 10 times higher than that of *B. bovis* RAP-1 and permitted the detection of 2.7×10^{-2} infected RBC ($10^{-8}\%$ parasitemia) (ABOULAILA et al., 2009).

According to BÖSE et al. (1995) the high sensitivity and specificity of the PCR technique makes it useful for validating results from other diagnostic techniques and for applications such as export certification of livestock. However, these molecular tools require thermo cyclers and several other expensive operations, which make them unsuitable for routine diagnosis, especially in resource-poor countries.

For this reason, several serologic assays, including complement fixation (MAHONEY, 1962), indirect hemagglutination (GOODGER, 1971), rapid card agglutination (TODOROVIC & KUTTLER, 1974), latex bead agglutination (LOPEZ & TODOROVIC, 1978), and indirect immunofluorescence (IFI) (GOFF et al., 1982) have been used to detect antibody in infected cattle. Although these assays allow for the detection of persistently infected cattle, they have limitations in specificity and/or sensitivity. The IFI, OIE recommended test for bovine babesiosis, has been the most sensitive assay, but cross-reactivity among babesial species, subjective interpretation, and low throughput has limited its usefulness (GOFF et al., 2003).

Serological testing is used as a tool to determine the babesial infection statuses of individuals and herds in control programs, and in the absence of treatment, is the best indicator of persistently infected hosts (COMBRINK et al., 2010). Although a number of serologic assays have been developed and used for several years, they suffer to some extent in sensitivity, specificity, and objectivity. Furthermore, testing large numbers of samples is a cumbersome process.

In contrast, enzyme linked-immunosorbent assay (ELISA) is quite sensitive and may be easily used to test large numbers of samples. ELISA has previously been evaluated for the detection of antibodies to *B. bovis* (WALTISBUHL et al., 1987) and *B. bigemina* (ECHAIDE et al., 1993) by use of a native antigen. Its potential ability has been demonstrated to be a powerful tool for serological surveys (WALTISBUHL et al., 1987; BÖSE et al., 1990; DE ECHAIDE et al 1995; MACHADO

et al., 1997), but the poor quality of antigens and the cross-reaction have impeded its application.

According to GOFF et al. (2003) ELISAs have found wide application in infectious disease diagnosis. The assay is nonsubjective, can be automated for high throughput, and has the potential for improved specificity depending on the efforts made in antigen preparation and characterization.

A large number of ELISAs have been designed for the diagnosis of *Babesia* infection (ARAUJO et al., 1998; BARRY et al., 1982; MACHADO et al., 1997; MOLLOY et al., 1998; SILVA et al., 2009). Although these studies report an improvement in sensitivity over other assays, problems with specificity remain due to the fact that antigen preparations are at best only partially purified. The competitive ELISA (cELISA) format can provide an additional level of specificity since antibody against a single epitope known to be organism specific is detected. Several cELISA assays have been developed for diagnosis of hemoparasites, including *Theileria (Babesia) equi* (KNOWLES et al., 1991) and *Babesia caballi* (KAPPMAYER et al., 1999) in horses and *Anaplasma marginale* in cattle (DE ECHAIDE et al., 1998).

For these reasons a cELISA based on the ability of serum antibody to inhibit a monoclonal antibody (MAb) directed against *B. bovis* or *B. bigemina*-specific repetitive epitope within rhoptry-associated protein 1 (RAP-1) (SUAREZ et al., 1991, 1993, 1994; HÖTZEL et al., 1996, 1997; GOFF et al., 2003; BOONCHIT et al., 2006; GOFF et al., 2006, 2008) have been developed. The cELISA for detection of *B. bovis* infection was shown to possess high sensitivity (91.1%) and specificity (100%) when compared to IFAT (GOFF et al. 2006). In addition, this assay detected serological response in experimentally infected animals between 13 and 17 days post infection with *B. bovis* (GOFF et al. 2003). For detection of *B. bigemina* infection the cELISA based on a broadly conserved, species-specific, B-cell epitope within the C terminus of rhoptry-associated protein 1a was shown to possess 100% of specificity and 87.2% of sensitivity having the attributes to international application (GOFF et al., 2008).

One goal in developing improved diagnostics is to be able to identify the carrier animals. Another goal is differential diagnosis, a concern in countries where several hemoparasitic agents, particularly different babesial species, occur concomitantly. Since differences in pathogenicity, epidemiology, and treatment exist for each species, accurate diagnosis is important (GOFF et al., 2003). Hence, it is essential to be able to ascertain the serological status of cattle with regard to *Babesia*

1 *species* and have a rapid, inexpensive, and reliable test for the detection of the anti-
 2 specie-specific antibody. Such a test would have great benefits in large-scale
 3 epidemiological surveys and lead to eradication.

5 **CHEMOPROPHYLAXIS AND VACCINATION**

7 Chemoprophylaxis as a method of short-term protection against babesiosis is
 8 often used under particular instances such as the temporary residence of susceptible
 9 animals in an infected area (for example agricultural shows), when pregnant cows
 10 are at risk and during disease outbreaks (DE WAAL & COMBRIK, 2006). For
 11 treatment Imidocarb dipropionate is the only chemotherapeutic agent that provides
 12 any significant long-term protection from clinical disease. This drug at a dose rate of
 13 3 mg/kg provides protection from clinical disease for 4 weeks in the case of *B. bovis*,
 14 8 weeks in the case of *B. bigemina* (TAYLOR & MCHARDY, 1979).

15 A major problem associated with this approach is the concern about drug
 16 residues in meat and milk, which has led to the withdrawal of imidocarb (ZINTL et al.,
 17 2003). However, it is well established that protection against babesiosis can be
 18 achieved by vaccination of cattle, which thus prevents losses caused by the disease.
 19 Live vaccines based on attenuated strains are used to control bovine babesiosis in
 20 several affected countries, including Argentina and Brazil (ECHAIDE et al., 1993;
 21 GUGLIELMONE, 1994; SACCO et al., 2001; WILKOWSKY et al., 2008). In Argentina
 22 300,000 doses of culture-derived vaccines against *B. bigemina* and *B. bovis* are
 23 produced annually, and successfully applied in the field.

24 There is an increasing demand for highly valued cattle from the temperate
 25 region of Argentina in tropical and sub-tropical areas within Argentina and
 26 neighboring countries like Brazil and Uruguay. Such cattle are vaccinated before sale
 27 and veterinary practioners demand positive antibody diagnosis to confirm the efficacy
 28 of vaccination against these diseases. To reduce sampling costs antibody evaluation
 29 is made at about 60 days after vaccination (GUGLIELMONE et al., 1997).

30 Vaccination of the cattle with attenuated *Babesia* merozoites derived from
 31 either infected cattle or in vitro cultures results in protective immunity against
 32 challenge infection with both homologous and heterologous strains (DALRYMPLE,
 33 1992). Although these vaccines generally confer a long lasting protective immunity,
 34 they have a number of drawbacks that could be circumvented by a subunit vaccine

(BROWN & PALMER, 1999; JENKINS, 2001; NORIMINE et al., 2003). The main requirements for this type of vaccines are the inclusion of surface-exposed and functionally relevant antigens expressing B- and T-cell epitopes conserved among different strains, with the ability to generate a protective immune response in cattle (SANTANGELO et al., 2007; WILKOWSKY et al., 2008).

The feasibility of developing a successful subunit babesial vaccine is based on the observation that immunization with nonliving parasite extracts or highly purified proteins can induce partial protection against subsequent challenge (BROWN & RICE-FICHT, 1994; WRIGHT et al., 1992). The identification and characterization of such subunit vaccine candidates is an active field of research, and several recombinant *B. bovis* and *B. bigemina* antigens have been reported to confer at least partial protective immunity (BROWN & PALMER, 1999; JENKINS, 2001). In addition, conserved merozoite surface antigens, widely recognized by sera of *Babesia*-infected cattle, could be also used to develop sensitive and specific serological techniques such as ELISA.

Several attempts have been made to develop recombinant or subunit vaccines, against babesiosis. However, results to date suggest that vaccines based on neither single nor multiple antigen combinations can confer the cross-protection provided by attenuated vaccines (NORIMINE et al., 2003; WRIGHT et al., 1992). The search for vaccine candidate antigens has focused mainly on merozoite surface antigens that are functionally relevant and immunodominant in naturally immune cattle, as well as conserved among strains. Candidate antigens identified include *B. bovis* merozoite surface antigen 1 (MSA-1) (HINES et al. 1995), *B. bovis* merozoite surface antigen 2c (MSA-2c) (WILKOWSKY et al. 2003), and *B. bovis* and *B. bigemina* RAP-1 (WRIGHT et al. 1992; SUAREZ et al., 1991, 1993; NORIMINE et al. 2002).

Among the few *B. bovis* antigens that have been characterized, the rhoptry-associated protein, RAP1, is considered a good candidate for vaccine development. RAP1 of *B. bovis* is a 60-kDa protein localized to the apical surface and within the rhoptries of merozoites (GOFF et al., 1988; SUAREZ et al., 1991). RAP1 has T helper-lymphocyte epitopes in the aminoterminal region and B-cell epitopes in both the amino and carboxyterminal regions. Immunization of cattle with recombinant RAP1 was shown to significantly reduce parasitemia upon subsequent challenge (WRIGHT et al., 1992; BROWN et al., 1996).

1 The use of live recombinant bacteria, including BCG (SANTANGELO et al.,
2 2007), as a platform for vaccine development against parasitic diseases is highly
3 attractive given the proven ability of this strategy to induce robust immune responses
4 to numerous antigens from a variety of parasites (OHANA et al., 2001; VARALDO et
5 al., 2004). Recently, this antigen was expressed in the BCG strain of mycobacteria
6 and the recombinant strain elicited both humoral and cellular responses against
7 RAP1 in mice, demonstrating the promising aspect of this antigen (SANTANGELO et
8 al., 2007).

9 The members of the variable merozoite surface antigen (VMSA) gene family of
10 *B. bovis* were previously identified as candidates for the development of subunit
11 vaccines and new serological tests for improved control of bovine babesiosis (HINES
12 et al., 1989). According to WILKOWSKY et al. (2003) MSA-2c is a novel potential
13 vaccine candidate and diagnostic antigen. Immunization of cattle with recombinant
14 MSA-1 induced antibodies that were capable of neutralizing merozoite invasion of
15 erythrocytes, however the immunized cattle were not protected against virulent
16 challenge (HINES et al. 1995). MSA-2c has been identified as highly conserved
17 among *B. bovis* strains and bovine antibodies to recombinant MSA-2c were able to
18 neutralize the invasion of erythrocytes by merozoites indicating a functional role for
19 this antigen (WILKOWSKY et al. 2008). However, this antigen has not yet been
20 tested in cattle to determine its protective efficacy. Despite considerable effort
21 expended in the development of non-replicating babesiosis vaccines, no practical
22 candidates are yet forthcoming (BROWN et al., 2006; DE WAAL AND COMBRICK,
23 2006).

CONCLUSIONS

Bovine babesiosis is responsible for substantial morbidity and mortality in cattle throughout South America. However, the current epidemiologic situation persisting around the 32°S parallel are not known, because of lack of information concerning the potential for *R. (B.) microplus* ticks to become established. Thus, it is crucial to investigate the epidemiologic parameters in this region. Serological testing is used as a tool to determine the babesial infection statuses of individuals and herds in control programs. In the absence of treatment it is the best indicator of persistently infected hosts.

However, there is a need for a standardized diagnostic assay that can be used globally to detect *B. bovis* and *B. bigemina*-infected cattle. The cELISA reviewed by Goff et al. (2006; 2008) has the attributes necessary for worldwide application for the detection of specific antibody, including the use of a dried antigen plate format. However, in some cases, supplemental diagnostics such as PCR could be used for samples with inconclusive results. Furthermore, PCR has the potential to provide rapidly qualitative results with high sensitivity and make it useful for validating results from other diagnostic techniques and for applications such as export certification of livestock.

The protection against babesiosis can be achieved by vaccination of cattle, with live vaccines based on attenuated strains. Although these vaccines generally confer a long lasting protective immunity, they have a number of drawbacks that could be circumvented by a subunit vaccine. Despite considerable effort expended in the development of non-replicating babesiosis vaccines, no practical candidates are yet forthcoming. Further studies will be necessary to investigate such antigens. Finally, with the imminent completion of the *B. bovis* genome-sequencing, strategies using combined genomic and proteomic approaches to identify novel vaccine candidates will be necessary.

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2.2 . ARTIGO II

A FIELD VALIDATION OF A COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF ANTIBODIES AGAINST Babesia bovis

Artigo científico formatado segundo as normas da revista *Veterinary
Parasitology*

Este artigo constitui-se de uma validação a campo de um cELISA para diagnóstico da babesiose bovina causada por *Babesia bovis*. Esse trabalho foi realizado em parte no Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Rafaela, Santa Fe, Argentina, e com a contribuição dos pesquisadores Will Goff, Carlos Suarez e Donald Knowles, da Washington State University.

A FIELD EVALUATION OF A COMPETITIVE ENZYME-LINKED
IMMUNOSORBENT ASSAY FOR DETECTION OF ANTIBODIES
AGAINST *Babesia bovis*

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ABSTRACT

A competitive enzyme-linked immunosorbent assay (cELISA) was field validated for international use as a diagnostic test for *Babesia bovis*. True positives were identified using two previously established assays, a nested PCR (nPCR) and an indirect immunofluorescent test (IIF). Receiver operating characteristic (ROC) analysis revealed 18% inhibition as the threshold for a negative result, with an associated specificity of 98.8% and sensitivity of 94.8%. Increasing the threshold to 20% increased the specificity to 100% but modestly decreased the sensitivity to 91.6%. The increase in the inhibition threshold from 18% to 20% resulted in a reduction of the negative predictive value in an area with a prevalence of 25% from 98.2% to 97.2% but increased the positive predictive value from 96.3% to 100%. At 20% inhibition, the negative predictive value varied from 99.0% in an area with a prevalence of 10% to 38.5% in an area with a prevalence of 95%. The positive predictive values were 100% under all prevalence conditions. Based on these results, the cELISA has the attributes necessary for its use in Argentina and Brazil for diagnosis of bovine babesiosis caused by *B. bovis*.

1 INTRODUCTION

2 Babesiosis caused by *Babesia bovis* and *Babesia bigemina* is the most important
3 cattle disease transmitted by *Rhipicephalus (Boophilus) microplus* ticks in tropical
4 and subtropical areas of South America (Guglielmone, 1995). The cattle industry is
5 economically important in Southern Brazil and Argentina where this activity is
6 generally not integrated with agriculture practices. *Bos indicus* x *Bos taurus* crosses
7 are generally more resistant to *R. microplus* infestations than *B. taurus* breeds,
8 (Lemos et al., 1985; Sutherst et al., 1988; Guglielmone et al., 1992) which are
9 predominantly raised in these regions.

10 In the Brazilian territory, both species of *Babesia* and *Anaplasma marginale* the
11 rickettsial tick-borne disease agent are thought to be highly prevalent in most of this
12 region. However, some micro-regions of the Northeastern and Southern most regions
13 are exceptions. *R.B. microplus* ticks do not exist below parallel 30°S in Argentina and
14 32°S in Brazil.

15 Calves are protected by non-specific immunity until about seven months of age
16 (De Voz et al., 1987; Guglielmone et al., 1992; Goff et al., 2001). Infection during this
17 period induces a long-lasting immunity, whereas primary infection later in life can
18 produce severe illness (Mahoney et al., 1973, 1979). Surviving animals develop
19 persistent infections and serve as reservoirs for continued transmission. Therefore,
20 the likelihood of babesiosis outbreaks can be indirectly measured by detecting the
21 proportion of infected calves via determining babesial antibody prevalence (FAO,
22 1984).

23 Definitive diagnosis can be made by detecting infected erythrocytes in stained
24 blood smears; however the parasitemia in peripheral blood is often too low for this
25 method to be used reliably for diagnostic purposes (Böse, et al., 1995; Goff, et al.,
26 2006). For this reason, several serologic assays, including complement fixation
27 (Mahoney, 1962), indirect hemagglutination (Goodger, 1971), rapid card
28 agglutination (Todorovic et al., 1974), latex bead agglutination (Lopez et al., 1978)
29 and indirect immunofluorescence (IIF) (Goff et al., 1982) have been used to detect
30 antibody in infected cattle. Although these assays allow for the detection of
31 persistently infected cattle, they have limitations in specificity and/or sensitivity. The
32 IIF has been the most sensitive assay, but cross-reactivity among babesial species,

subjective interpretation, and low throughput has limited its usefulness (Goff et al., 2003).

Serological testing is used as a tool to determine the babesial infection statuses of individuals and herds in control programs, and in the absence of treatment, is the best indicator of persistently infected hosts. Although a number of serologic assays have been developed and used for several years, they suffer to some extent in sensitivity and specificity. ELISAs have found wide application in infectious disease diagnosis. The assay is nonsubjective, can be automated for high throughput, and has the potential for improved specificity depending on the efforts made in antigen preparation and characterization (Goff et al., 2003). The cELISA format can provide an additional level of specificity since antibody directed toward a single epitope known to be organism specific is detected. For these reasons a cELISA (Goff et al., 2006) based on the ability of serum antibody to inhibit a monoclonal antibody (MAb) directed against *Babesia bovis* or *Babesia bigemina*-specific repetitive epitope within rhoptry-associated protein 1 (RAP-1) (Suarez et al., 1991, 1993, 1994; Hötzel et al., 1996, 1997; Boonchit et al., 2006) has been developed.

Finally, we do not know the current epidemiologic situation persisting around the 30°S and 32°S parallel, because of lack of information concerning the potential for *R.B. microplus* ticks to become establish. Therefore, it is crucial to investigate the epidemiologic parameters in this region. In this study we compared the cELISA (Goff et al., 2006) with IIF and nPCR to demonstrate the sensitivity and specificity of the assays, under field conditions and to provide evidence for its utility in Argentina and Brazil.

MATERIALS AND METHODS

Sera. For the field validation, 461 known-positive and 481 known-negative sera were evaluated. The known-positive sera were from animals that were from regions of endemicity in Argentina (228 samples) and Brazil (233 samples) and were determined to be positive by a *B. bovis*-specific nested PCR. Known-negative samples were from below parallel 30°S in Argentina (252 samples) and 32°S in Brazil

(229 samples), where neither *B. bovis* and *B. bigemina* nor *R. B. microplus* tick vectors exist.

IIF. The indirect immunofluorescence (IIF) assay was performed as previously described (GOFF et al., 1982) using 10 µl of a 1/100 dilution of serum. A positive result was defined as fluorescence equal to or greater than that of a weak positive control sample.

PCR samples and reaction conditions. External and nested primers were previously described (FIGUEROA et al., 1994) and produced a nested amplification product of approximately 297 base pairs from within the gene coding for RAP-1. For the nPCR, the external-reaction primers were forward, 5'-CACGAGGAAGGAACTACCGATGTTGA-3'-, and reverse, 5'-CCAAGGAGCTTCAACGTACGAGGTCA-3'-, and the nested primers were forward, 5'-TCAACAAGGTACTCTATATGGCTACC-3'-, and reverse, 5'-CTACCGAGCAGAACCTTCTTCACCAT-3'-. The external reaction was performed in a 50 µl volume containing 2.0 µl of sample gDNA in Tris buffer, 1.5 µl of 50 mM MgCl₂, 5.0 µl 10X reaction buffer, 1.0 µl of 10 mM deoxynucleoside triphosphate mix, 1.0 µl of external primers (50 pmol/µl of both forward and reverse primers), 0.4 µl *Taq* polymerase (5 units/µl), and 39.1 µl of ultrapure water. The primary amplification was carried out with 25 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min, with a final extension time of 7 min at 72 °C. For the nested reaction, 1 or 2 µl from the primary reaction was used under the same conditions but increased to 35 cycles. The nPCR products were examined following 2% agarose gel electrophoresis.

cELISA. The format of the cELISA was as previously described using the same C terminus of RAP-1 antigen expressed as a histidine-tagged thioredoxin fusion protein purified on a ProBond resin column (Invitrogen, Carlsbad, Calif.) and dried on the wells of Immulon II plates (GOFF et al., 2003). Optimal concentrations of antigen and MAb were determined by block titration as previously described (GOFF et al., 2003). Prior to use, 200 µl of a blocking buffer (phosphate-buffered saline containing 0.2% Tween 20 and 20% nonfat dry milk) was added to each well, and the plates were incubated at room temperature for 1 h on a rotating platform. After the blocking buffer was aspirated off, 100 µl of undiluted serum was added to each well (principal test

sera and control sera), and the plates were incubated at room temperature for 30 min. After the serum from each well was aspirated off, 100 μ l (50 ng/well) of BABB75A4 MAb (GOFF et al., 1988) was added, and the plates were incubated at room temperature for 15 min. Each plate was then washed three times with 200 μ l of wash buffer (blocking buffer minus the nonfat dry milk), followed by the addition of 100 μ l of wash buffer containing an appropriate concentration of conjugate (horseradish peroxidase-labeled goat anti-mouse immunoglobulin G; KPL, Gaithersburg, MD). After incubation at room temperature for 15 min, each plate was washed three times, as before, and then allowed to set for 30 to 60 s in wash buffer before a final three washes. After equal volumes of 3,3',5,5'-tetramethylbenzidine and H₂O₂ were combined according to the manufacturer's instructions (KPL), 100 μ l of the substrate was added to each well and the plates were incubated at room temperature in the dark for 15 min, followed by the addition of 50 μ l of stop buffer (2 N H₂SO₄). The mean optical density (OD) at 450 nm was determined for all test wells and for duplicate wells of a positive control serum and negative control sera pooled from five known-negative animals using a microtiter plate reader (Multiskan MCC/340, Titertek Instruments). The percent inhibition for each test sample was determined using the mean of each duplicate well compared to the mean of duplicate control wells using the following formula: percent inhibition = [1 - (OD of sample – OD of buffer/OD of negative control – OD of buffer)] X 100.

Statistical analysis. To accurately assess the assay for diagnostic specificity, sensitivity, and predictive values, the results from the 461 known-positive and 481 known-negative samples were subjected to receiver operating characteristic (ROC) analysis performed using MedCalc statistical software (version 11.2.0.0), and a frequency distribution graph was generated.

RESULTS

Specificity, sensitivity, and predictive value. ROC analysis identified the threshold inhibition value for the definition of a negative result to be 18%. With this value, the assay had a specificity of 98.8% and a sensitivity of 94.8% (Fig. 1A). The area under the ROC curve was 0.981, indicating that the assay had an excellent ability to discriminate between positive and negative samples (Fig. 1B). To increase the

specificity to 100% without dramatically decreasing the sensitivity, we raised the threshold inhibition value to 20%. As a result, the sensitivity was reduced from 94.8% to 91.6%. Figure 1C shows the frequency distribution graph for the cELISA obtained with 461 known positive and 481 known negative samples and the associated threshold levels of 18% and 20%. The increase in the inhibition threshold from 18% to 20% resulted in a reduction of the negative predictive value in an area with a prevalence of 25% from 98.2% to 97.2% but increased the positive predictive value from 96.3% to 100%. At 20% inhibition, the negative predictive value varied from 99.0% in an area with a prevalence of 10% to 38.5% in an area with a prevalence of 95% (Fig. 2). The positive predictive values were 100% under all prevalence conditions.

DISCUSSION

A standardized diagnostic assay that can be used globally to detect *B. bovis*-infected cattle would be valuable. Other serological methods that have been developed and utilized in the past include an IIF assay (Goff et al., 1982) and a number of ELISAs (Araujo et al., 1998; Barry et al., 1982; Machado et al., 1997; Molloy et al., 1998; Waltisbuhl et al., 1987). Although these assays allow the detection of infected cattle, they all have limitations to some degree in specificity, sensitivity, reliability, or, in the case of IIF, subjective interpretation and low throughput.

Results indicate that the cELISA evaluated in this work is a valid and reliable test for diagnosing *B. bovis* infection. The test was evaluated under field conditions, where continuous natural *B. bovis* booster occurs and where herds are found with cattle infected solely by *B. bovis* or *B. Bigemina*.

The current cELISA protocol was used to test known-negative samples randomly selected for this report, along with the known positives ones. At a cutoff of 20% inhibition, the assay has a specificity of 100% and a sensitivity of 91.6%. This translates into a positive predictive value of 100% under any conditions. However, at 20% inhibition, the negative predictive value decreases as the prevalence increases and drops off dramatically as the prevalence increases from 80% to 90%. To our knowledge, 90% prevalence has not been reported for any region surveyed (data not shown). Based on this the cELISA has the attributes necessary for its use in

Argentina and Brazil for diagnostic application. The assay include purified recombinant antigen dried onto microtiter wells for ease of handling, distribution, and stability. This competitive ELISA exhibited performance comparable to other previously described assays using purified antigens.

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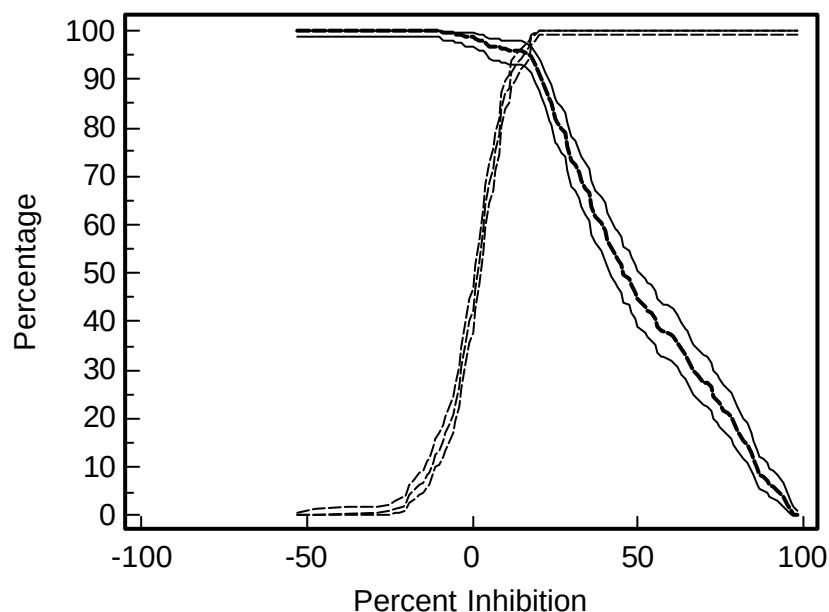
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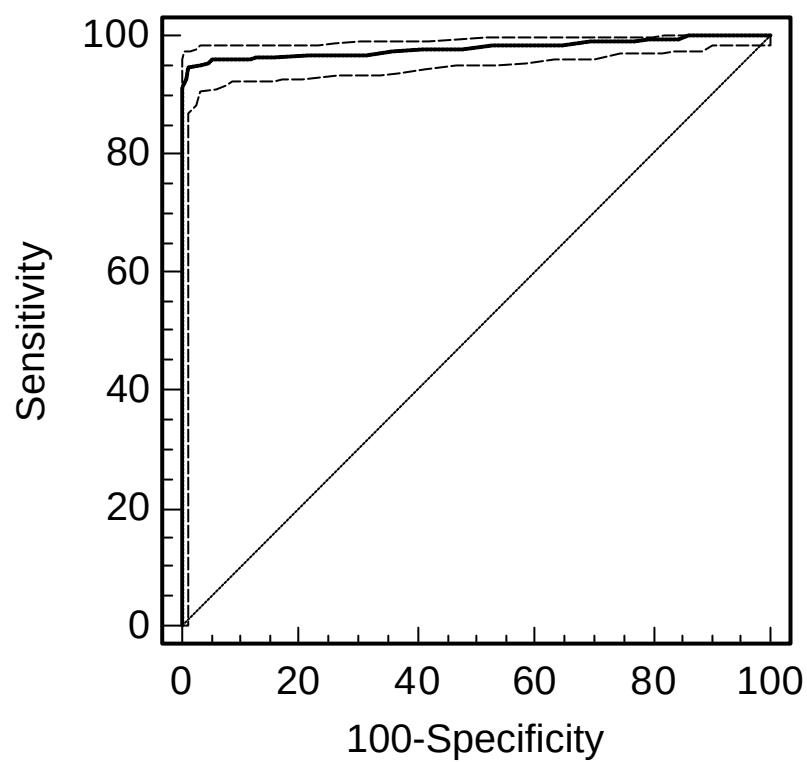
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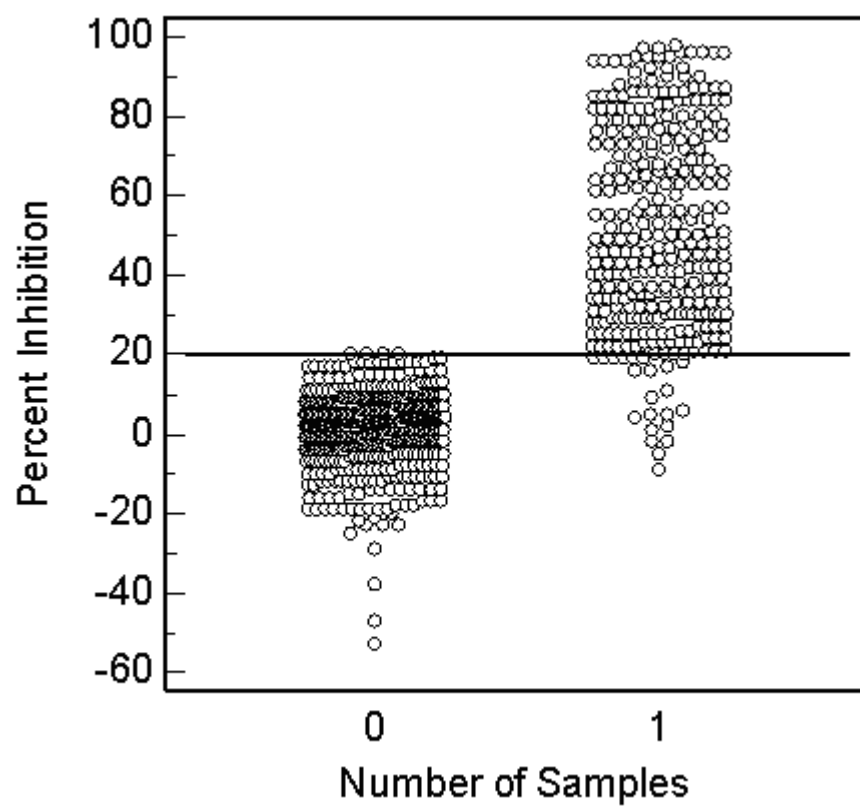
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3 **FIG. 1C**

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FIG. 1. ROC analysis of the cELISA with 461 known positive and 481 known negative serum samples. (A) Plot-versus-criterion graph identifying 18% inhibition as the suggested threshold, with a specificity (dashed line with 95% confidence intervals) of 98.8% and a sensitivity (solid line with 95% confidence intervals) of 94.8%; (B) ROC plot with an area under the curve of 0.981 (0.00584) and a 95% confidence interval between 0.969 and 0.989; (C) Distributions of the known negative (left cluster) and known positive (right cluster) serum samples by using 20% inhibition as threshold value.

FIG. 2

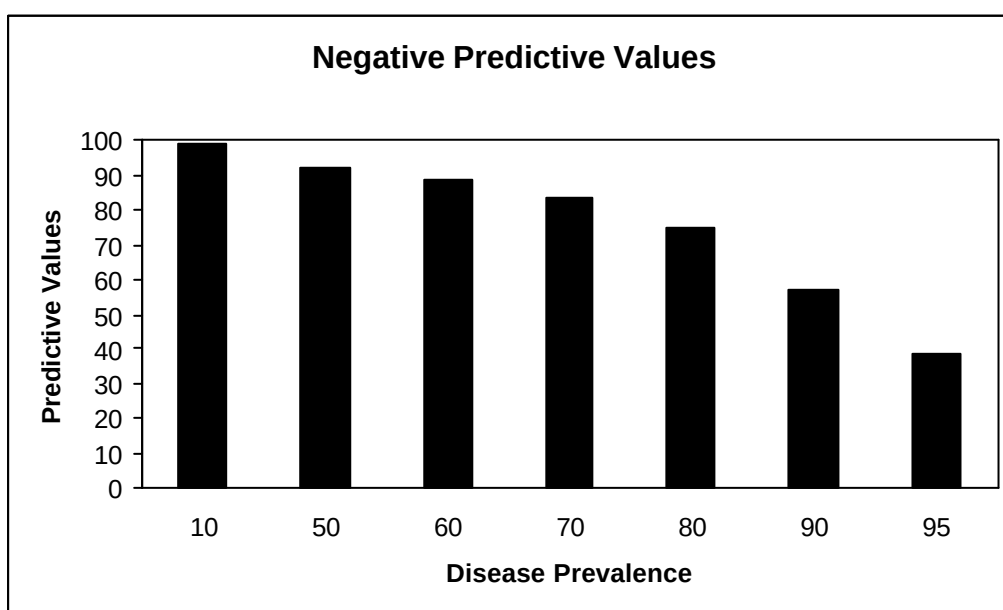


FIG. 2. Predictive value of a negative cELISA test result when applied to different levels of disease prevalence. The values were determined by ROC analysis based on a 20% cutoff.

3. CONCLUSÕES GERAIS

Levando-se em consideração os resultados obtidos neste trabalho, pode-se concluir que:

? O cELISA utilizado nesse trabalho, como meio de diagnóstico de *B. bovis*, apresentou excelente sensibilidade e especificidade. Sendo assim, o teste apresenta altos níveis de confiança frente a um diagnóstico positivo e diferencial de *B. bovis*.

? Esse teste é de fácil processamento, apresentando altos níveis de seguridade nos resultados, além da rapidez no diagnóstico de amostras em larga escala, sendo de grande utilidade para casos de surtos de babesiose bovina.

? O cELISA é um método de diagnóstico que pode ser utilizado em estudos de epidemiologia da babesiose bovina causada por *B. bovis*.

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