Development and use of an indirect ELISA in an outbreak of bovine besnoitiosis in Spain

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Summary

An indirect enzyme-linked immunosorbent assay (ELISA) based on a soluble extract from *Besnoitia besnoiti* tachyzoites has been developed and standardized. A set of positive and negative reference bovine sera was characterised by immunofluorescence antibody test (IFAT) and western blot (WB). A cut-off with a relative index percent (RIPC) of 8.1 was determined for equal sensitivity and specificity (100%) by two-graph receiver operating characteristic (TG-ROC) analysis. In addition, cross reactions with other closely related Apicomplexan parasites were discarded. The standardized ELISA was then used in an outbreak of bovine besnoitiosis in a mountainous area of central Spain. The outbreak occurred in 9 herds and 358 animals that share grazing lands during the summer season were affected. Clinical observation and blood sampling were performed on all animals, and skin biopsies were obtained from animals with skin lesions. The confirmatory diagnosis was carried out by means of the indirect ELISA, together with the identification of tissue cysts by microscopy. Most animals were seropositive (90.5%), whereas only 43% of seropositive cattle developed clinical signs compatible with besnoitiosis. Additionally, a significant increase in seroprevalence and in the presence of clinical signs was found with age, suggesting rapid horizontal transmission of the disease. This is the first description of bovine besnoitiosis in central Spain.

Introduction

Bovine besnoitiosis is a disease caused by the protozoan *Besnoitia besnoiti*. It has been previously described in Africa, the Middle East and Europe, and is deemed to be an emergent disease in European countries (Cortes et al., 2006c; Alzieu, 2007; Mehlhorn et al., 2009). In Spain, this disease was first described in the Pyrenees area (Basque Country and Navarra) (Juste et al., 1990), although during the last decade it has been also reported in several regions in Northeast Spain (Aragon, La Rioja, Valencia and Castilla and Leon) (Castillo J.A., personal communication).

Besnoitia besnoiti has a heteroxenous life cycle, with both domestic (cattle) and wild (antilopes) bovids as intermediate hosts (IH) (Pols, 1960; Basson et al., 1965). The definitive host (DH) has not been identified, although a role for cats has been suggested (Peteshev et al., 1974). Asexual stages of this parasite develop in the IH. Two infective stages have been described to date, the fast-replicating tachyzoites and the slow-dividing bradyzoites, which gather into macroscopic cysts located inside cells of the subcutaneous connective tissue (Bigalke et al., 1981; Diesing et al., 1988; Njagi et al., 1998).

Although many infected animals are asymptomatic, some animals may suffer the characteristic clinical signs and thus this disease can cause important economic losses. The acute phase of the disease starts with fever, weakness, swelling of the superficial lymph nodes and oedema. Respiratory disorders may also appear. The chronic disease phase is characterized by skin lesions, such as scleroderma, hyperkeratosis and alopecia, loss of body weight, sterility in males

(necrotising orchitis) and even death (Bigalke, 1968). Serological diagnosis will help to detect asymptomatic infected animals. Serology is also useful for investigating the prevalence of animals exposed to *Besnoitia* infection in an area and to establish follow-up control programmes. To date, serological techniques employed in the diagnosis of besnoitiosis include the indirect immunofluorescence antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA) (Janitschke et al., 1984; Shkap et al., 1984; Shkap et al., 2002; Cortes et al., 2006a) and the western blot (Cortes et al., 2006a; Fernández-García et al., manuscript accepted for publication). Regarding the seroprevalence of bovine besnoitiosis, diagnostic studies have mainly been carried out in South Africa and Israel using IFATs (Bigalke, 1968; Neuman, 1972; Frank et al., 1977; Goldman and Pipano, 1983). These studies reported higher seroprevalence rates in beef herds than in dairy herds, probably due to husbandry differences (beef and dairy cattle raised under extensive or intensive management conditions, respectively).

The objective of the current study was to develop and optimize a soluble extract-based indirect ELISA for the serological diagnosis of *B. besnoiti* infection in cattle, and then apply this assay to characterize an outbreak of bovine besnoitiosis in central Spain.

Materials and Methods

Clinical case, sampling and data collection

Several animals with skin lesions (scleroderma and hyperkeratosis), tissue cyst in scleral conjunctiva and vulvar region and loss of body weight were reported on several farms located in a mountainous area at 1300 metres above sea level in Central Spain, near Hayedo de Tejera Negra Natural Park. In addition the presence of cysts was confirmed in biopsied skin samples by direct microscopic examination in an affected animal where the Spanish bovine isolate Bb-Spain 1 was obtained (Fernández-García et al., 2009).

Of the first occurrence of clinical signs coincided with the arrival of a Charolais bull two years prior, which developed clinical signs (scleroderma, necrotising orchitis and tissue cysts in scleral conjunctiva) and finally died. The number of affected animals had gradually increased over time. This area was free of bovine besnoitiosis prior to the arrival of this bull. Ten cows with clinical signs indicative of besnoitiosis were observed in the first year and 20 more cows were detected in the second year; 3 of those 20 animals subsequently died. Moreover, the presence of clinical signs was linked to the summer season, when animals shared grazing fields. In addition, four bulls showed severe clinical signs (lower limb oedema, respiratory distress, severe lacerations to hooves, and orchitis that in one bull caused testicular atrophy) and died.

A thorough clinical examination of 358 animals from 9 farms was performed, and any sign compatible with acute (oedema) or chronic (scleroderma, alopecia, crusting on feet and udder, limping, weight loss, tissue-cysts in scleral conjunctiva, orchitis in males and tissue cysts in vulvar region in females)

111 infection was recorded. An animal was annotated with clinical signs if it showed
112 oedema (indicative of acute infection) or tissue cysts and/or skin lesions
113 (indicative of chronic infection). Furthermore, blood samples were collected from
114 all animals. Data collected from cattle and herds included breed, age and sex.
115
116 *Parasite production and antigen preparation*
117
118 The BbSpain-1 isolate of *B. besnoiti* was maintained *in vitro* by continuous
119 passage in MARC-145 cell cultures following previously described procedures
120 (Fernandez-Garcia et al., 2009). Tachyzoites were purified in cold sterile
121 phosphate-buffered saline (PBS; pH 7.2) using disposable PD-10 desalting
122 columns (GE Healthcare), and pelleted by centrifugation at 1,350 x*g* for 10 min at
123 4ºC. The pellet with tachyzoites was frozen at –80ºC until use in ELISA or WB,
124 or was resuspended in PBS and formalin-fixed for use in IFAT at a final
125 concentration of approximately 10^7 tachyzoites per ml.
126 Soluble antigen lysate for use in the ELISA was prepared as previously
127 described (Alvarez-Garcia et al., 2002). The supernatant was maintained at -80ºC
128 until use and proteins were quantified using a commercial kit (Micro BCA,
129 PIERCE).
130
131 *IFAT*
132

Sera were analysed by IFAT in double serial dilutions starting at 1:50, using a previously described method (Fernández-García et al., manuscript accepted for publication). Unbroken tachyzoite membrane fluorescence at titres equal to or higher than 1:100 was considered to be sero-positive. Membrane fluorescence titres equal to or lower than 1:50 were considered sero-negative.

Western blot

Samples containing $2x10^7$ *B. besnoiti* tachyzoites or bradyzoites were detergent disrupted at 95ºC for 5 min with Laemmli buffer (Laemmli, 1970), sonicated in an ultrasonic bath at 15ºC during 15 min and then heated for 5 min at 95ºC prior to being used. WB was carried out essentially as described by Fernández-García et al. (manuscript accepted for publication) and serum samples were used at a 1:20 dilution. Images from the WB membranes were obtained using a GS-800 Scanner (Bio-Rad Laboratories, CA, USA) and analysed with Quantity One® quantification software v. 4.0. (Bio-Rad Laboratories, CA, USA). Sera recognising a 37.1 kDa protein were considered positive.

ELISA development and optimization

The validation of a soluble extract-based indirect ELISA for the serological diagnosis of *B. besnoiti* infection was carried out following the recommendations

155 of several authors (Jacobson, 1998; Greiner and Gardner, 2000; Alvarez-Garcia et
156 al., 2003).

157 To discern between seropositive and seronegative cattle, cut-offs and
158 diagnostic characteristics of the tests were estimated using a panel of bovine sera
159 classified as 'positive or negative' based on the results obtained by IFAT and WB
160 as reference tests. The cut-off point was estimated with a total of 46 'positive'
161 bovine serum samples belonging to herds with a previous history of besnoitiosis
162 and 46 'negative' bovine serum samples belonging to herds without any prior
163 history of besnoitiosis.

164 In addition, 10 serum samples from cows naturally infected with either *N.*
165 *caninum* or *Sarcocystis* spp., and one serum sample from a *T. gondii*-
166 experimentally infected calf (Esteban-Redondo, 1999), were used to evaluate any
167 cross-reactivity with *B. besnoiti* antigens. *Sarcocystis* spp. infection was
168 evidenced by visualization of tissue cysts in the heart of affected animals by
169 means of histological examination, and *N. caninum* and *T. gondii* infections were
170 confirmed by serological tests.

171 In order to standardize the ELISA protocol checkerboard titrations of
172 antigen (0.05, 0.1 or 0.2 µg /well), sera (1:100 or 1:200) and conjugate (1:2000,
173 1:4000, 1:6000 or 1:8000) were performed. The combination offering the best
174 discrimination between positive and negative sera was considered the optimum
175 for the assay. The working conditions for the ELISA were determined using a
176 short panel of bovine sera. The optimised ELISA protocol is as follows. One
177 hundred microlitres of coating buffer (0.1 M carbonate-bicarbonate, pH 9.6)

containing *B. besnoiti* soluble extract (0.2 µg /well) was added to each well of a polystyrene microtitre plate (Immuno Plate Maxisorp, Nunc, Roskilde, Denmark) and incubated overnight at 4ºC. After three washes with phosphate-buffered saline containing 0.05% Tween 20 (PBST), blocking was performed with PBST containing 10% horse serum for 2 hours at room temperature. Wells were then washed again and incubated with 100 µl of bovine sera diluted in blocking solution, for 1 hour at 37ºC. After three washes, 100 µl of a monoclonal anti bovine IgG1/IgG2 peroxidase-conjugated (LSI laboratories, Lissieu, France) at 1/6000 dilution in PBST were added and incubated for 1 hour at 37ºC. Wells were rinsed three times with PBST and bound antibodies were detected by incubation with 100 µl of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid substrate (Sigma) at room temperature in the dark. After 20 minutes, the reaction was stopped by adding 100 µl of 0.3 M oxalic acid. Absorbance was measured as optical density values (O.D.) at 405 nm using a microplate reader (Multiscan RC 6.0, Labsystems). Positive control serum was a pool of three positive sera with an IFAT titre of 1:1,600. As a negative control we used a pool of three sera determined to be negative by IFAT. Serum samples were analyzed in duplicate and the mean value of the O.D. was converted into a relative index percent (RIPC) by employing the following formula: RIPC = ($O.D._{405}$ sample – $O.D._{405}$ negative control)/ ($O.D._{405}$ positive control – $O.D._{405}$ negative control) x100. Samples with a RIPC equal to, or higher than, 8.1 were considered positive.

Analysis of data

A non-parametric two-graph receiver operating characteristic (TG-ROC) analysis using the TG-ROC CMDT software was applied for the selection of cut-off values and diagnostic performances in diagnostic tests (Greiner, 1995). The TG-ROC CMDT software determines a cut-off point (d_o) for equal sensitivity and specificity values. The precision of the ELISA assay was also measured by its repeatability including inter-assay, intra-assay and operator-to-operator variations (Jacobson, 1998), which were calculated in triplicate for seven positive samples and eight negative samples determined by IFAT.

Individual seroprevalence was estimated as the percentage of seropositive animals over all tested animals. Four age groups were established (6 month to 1 year old, 1-3 years old, 4-7 years old and older than 7 years old). Determination of the number of animals with clinical signs was conducted by considering as positive those animals that showed at least one clinical sign characteristic of this disease. Differences in both the percentage of seropositive animals and animals with clinical signs among the different age groups were evaluated by means of the Chi-square test (χ^2) followed by Fisher's exact test whenever significant differences were found between age groups. Statistical analysis was carried out using GraphPad Prism 4 v.4.03 (San Diego, CA, USA).

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Results

Diagnostic performances and precision of B. besnoiti soluble extract-based ELISA

TG-ROC analysis displayed 100% sensitivity (Se) and specificity (Sp) for the soluble extract-based ELISA, corresponding to a cut-off RIPC value equal to or higher than 8.1 (Fig. 1).

When cross reactivity with closely related Apicomplexan parasites was also investigated, serum samples from bovines infected with *Sarcocystis* spp., *N. caninum,* or *T. gondii* proved to be seronegative, using the *B. besnoiti* soluble extract antigen-based ELISA.

Finally, most of the variation coefficients for the ELISA were lower than 10%. The overall variation coefficients were 3.35, 5.05 and 9.93% for intra-assay, inter-assay and operator-to-operator precision, respectively.

Besnoitiosis outbreak

Clinical signs observed in animals included oedema in the jowls and limbs (25.4%; 91/ 358), skin lesions such us scleroderma and hyperkeratosis (13.4%; 48/ 358), alopecia (5.6%; 20/ 358) and crusting on feet and udder (20.7%; 74/ 358), as well as limping and weight loss in severe cases (1.4%; 5/ 358). One male also developed orchitis (1/ 7). Tissue cysts were observed in scleral and palpebral conjunctiva (31.6%; 113/ 358) and in vulvar region (18.4%; 66/ 358). Most animals that exhibited oedemas also had cysts in their scleral and palpebral

conjunctiva and/ or in vulvar region (84.6%; 77/ 91). Of the animals, 6.14% (22/ 358), including 1 male, had cysts with no *Besnoitia*-related clinical signs. The prevalence results are summarized in Table 1. 90.5% animals were seropositive two years after the disease was first described. Of the cattle, 90.8% (319/351) of females and 71.4% (5/7) of males were seropositive. Nevertheless, only 43.2% (154/358) of examined animals showed at least one clinical sign; 43% of females (151/351) and 42.8% of males (3/7). In addition, only 5 out of 358 animals that showed clinical signs were seronegative by ELISA. In these animals, clinical signs consisted of mild oedema and skin lesions in eyes, on the udder and on the feet. Sera from these animals were also tested by WB and only one animal was positive.

With regard to breed, 93% (333/358) animals were crossbred and 7% (25/358) of animals were either Charolais, Limousin, Avileña-Black Iberian, Brown Swiss, Holstein or Asturian Valley breed. No analysis regarding breed-infection association was carried out. When considering age, the population was divided into 4 groups: 6 month to 1 year old (n=6), 1 to 3 years old (n=47), 4 to 7 years old (n=117) and older than 7 years old (n=188). The percentage of ELISA-positive animals with clinical signs significantly increased with age (χ^2= 80,7; χ^2= 31,7; P< 0.01) (Fig. 2).

Discussion

Reports of cattle affected by besnoitiosis have progressively increased in European countries in the last decade, and the need for a serological tool for the

early diagnosis of this disease is becoming critical. In the present work, we developed an indirect ELISA employing soluble extracts of *B. besnoiti* tachyzoites, in order to improve the serological diagnosis of bovine besnoitiosis. The use of this assay confirmed the existence of besnoitiosis for the first time in central Spain and allowed for the detection of seropositive cattle. The existence of seropositive animals may be indicative of subclinical cases, where the number of cyst on cattle may be scarce on a histological skin slide and which could play a role as carriers of the infection (Cortes et al., 2006a), or exposed animals to the infection that do not develop clinical signs. In addition, once the first clinical cases appear in a herd infection spreads rapidly with the subsequent increment of seropositivity. Traditional diagnosis of the disease has been primarily based on the macroscopic detection of cysts in the scleral conjunctiva, or the observation of clinical signs such as skin lesions. Besnoitiosis is usually confirmed by the histological examination of skin biopsies. Nevertheless, while cysts can be seen in typical clinical cases, they are rarely found in sub-clinical cases (Bigalke, 1981). Serological tools, therefore, are very useful for the early detection of possible asymptomatic carriers. To date, the IFAT has been the primary tool used to carry out seroprevalence studies (Bigalke, 1968; Neuman, 1972; Frank et al., 1977; Goldman and Pipano, 1983), although a few ELISAs have been developed for this purpose (Janitschke et al., 1984; Shkap et al., 1984; Cortes et al., 2006a). In this context, the ELISA technique presented in this work offers both improved diagnostic performance to detect *B. besnoiti* infection in cattle and concordance with both the IFAT and WB assays.

Although previous studies using ELISA tests have been reported, the results have been controversial. Janitsche et al. (1984) developed an ELISA that showed less sensitivity than the IFAT, and both tests were less sensitive when compared to clinical findings primarily based on visual inspection of macroscopic cysts in sclera. On the other hand, Shkap et al. (1984) reported a more sensitive ELISA when compared with an IFAT. The most reasonable explanation for these differences may be the different procedures and the antigen solubilisation protocols employed. Our results also showed that a low number of clinical cases are not detectable by serological methods (less than 1%). The rapid development of clinical signs without time for seroconversion after the first contact with the parasite although unlikely might happen and could explain the existence of false negative results. In practical use, this limitation in individual diagnoses could be solved in a particular herd by combining clinical inspection with serology. However, the low number of false negative results may not represent a great inconvenience in prevalence studies. Cortes et al. (2006a) have recently reported an indirect ELISA also based on soluble *B. besnoiti* tachyzoite antigen, which showed high specificities ranging between 96.4% and 98%, whereas only 87% sensitivity was obtained. For this reason, it was reasonably suggested that the combined use of both ELISA and WB could reliably detect animals that have been exposed to *B. besnoiti* infection. However, another advantageous option would be the use of the ELISA developed in this study, since it offers higher sensitivity values. In addition, unlike the WB protocol previously described (manuscript accepted for publication), the use of the soluble extract of *B. besnoiti*

316 did not adversely affect specificity, based on the lack of cross-reactions with other
317 parasites of the phylum Apicomplexa at the serum dilution used in the ELISA.
318 Moreover, very good reproducibility and repeatability have been demonstrated for
319 this technique.

320 One of the problems faced during ELISA standardization and
321 interpretation is the determination of an optimal cut-off point and the criteria used
322 to better discriminate between positive and negative control values. In this work,
323 we used TG-ROC analysis to establish an optimal cut-off value for maximum
324 sensitivity and specificity. Moreover, an interesting feature of this test is that most
325 results were not close to the cut-off. Therefore, it is unlikely that sensitivity
326 and/or specificity will be affected if future validation studies require cut-off
327 recalculation. This feature is an important advantage of our test over other tests
328 for differentiating between infection with *B. besnoiti* and closely related
329 protozoan infections. In particular, in the diagnosis of bovine neosporosis, an
330 opposite situation is found when serological tests are validated, and the major
331 challenge for serodiagnosis is to accurately detect chronically- and subclinically-
332 infected animals with serological results below the cut-off. To solve this problem
333 and in order to compare results among different tests, a range of intermediate
334 results is usually recommended, together with the employment of an *a posteriori*
335 test, such as the WB (Alvarez-Garcia et al., 2003; von Blumröder et al., 2004).

336 The standardised ELISA developed here was employed in the study of the
337 first besnoitiosis outbreak described in the central region of Spain. This initial
338 epidemiological study has shown that bovine besnoitiosis is a disease that spreads

easily once established in an area, regardless of the sex of the animals. The results obtained showed a notably increased seroprevalence over time after the first appearance of the disease three years before. Our results agree with a recent study of a beef cattle farm in Portugal, wherein a 36% seroprevalence rate increased to 70% over the course of 18 months (Cortes et al., 2006b). Another interesting finding of our study was that a higher seroprevalence rate was observed than suggested by the presence of clinical disease, revealing the existence of numerous subclinical cases, as other authors have previously described (Bigalke, 1968). The detection of subclinical cases by the improved ELISA may be helpful in implementing additional control measures, in order to minimize propagation of the infection and to avoid the entry of infected animals onto a farm. Moreover, horizontal transmission was responsible for the spread of the disease, as a significant association was found between the prevalence of the disease and the age of the animal. Accordingly, Bigalke (1981) reported that there is a relationship between the age of the animal and the epidemiology of the disease. In that study, a statistically significant increase in seroprevalence and morbidity, evidenced by typical clinical signs, was associated with age, since the highest incidence of infection was detected in the 18-month to 6-year-old animals on a farm where the disease was present but was rarely encountered in calves under 6 months of age. In addition, animals that contracted the infection developed antibodies for life. It is likely that horizontal transmission mainly occurs as a consequence of direct contact among animals with wounds or lacerations given the fact that subcutaneous tissue cysts can be located very superficially (Bourdeau

et al., 2004). Indeed, in the present outbreak, it was observed that the emergence of clinical signs coincides with the summer, when mixed herds shared pastures. However, some authors have associated this phenomenon with the period when blood-sucking arthropods such as horseflies become active, and may play a role as a mechanical vector (Bigalke et al., 1968).

The existence of vectors could also be a risk factor for the rapid spread of the disease in our study, since the sampling area is located in a mountainous area where horsefly populations can be found. Thus, our epidemiological data also support the idea of vector-borne horizontal transmission, which might assist in the rapid propagation of the parasite. However, this hypothesis does not completely solve the transmission riddle, since only clinically-unapparent cases have been produced experimentally (Bigalke, 1968). As a consequence, the intervention of wildlife reservoirs of disease, e.g. rodents, has also been suggested (Alzieu, 2007; Mehlhorn et al., 2009).

In summary, our results indicate that the ELISA developed here is a highly sensitive and specific diagnostic tool for detecting *B. besnoiti* infection in cattle, and may be very valuable in the establishment and maintenance of control programs and for studying the seroprevalence status in one herd or region, at least in Spain, where bovine besnoitiosis is present.

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514 **Tables**

515 **Table 1:** Herd distribution of seropositivity by ELISA and the presence of clinical

516 signs.

517
518

Herd	Clinical signs				ELISA				Total
	w/signs*	%	wo/signs#	%	Negative	%	Positive	%	
A	18	75.0	6	25.0	2	8.3	22	91.7	24
B	5	35.7	9	64.3	2	14.3	12	85.7	14
C	32	62.7	19	37.3	3	5.9	48	94.1	51
D	32	40.3	45	59.7	16	20.8	61	79.2	77
E	7	29.2	17	70.8	-	0.0	24	100.0	24
F	13	33.3	26	66.7	1	2.6	38	97.4	39
G	17	51.5	16	48.5	1	3	32	96.9	33
H	16	36.4	28	63.6	2	4.5	42	95.5	44
I	14	26.9	38	73.1	7	13.5	45	86.5	52
Total	**154**	**43.02**	**204**	**56.98**	**34**	**9.50**	**324**	**90.50**	**358**

519 * with clinical signs
520 # without clinical signs
521

522

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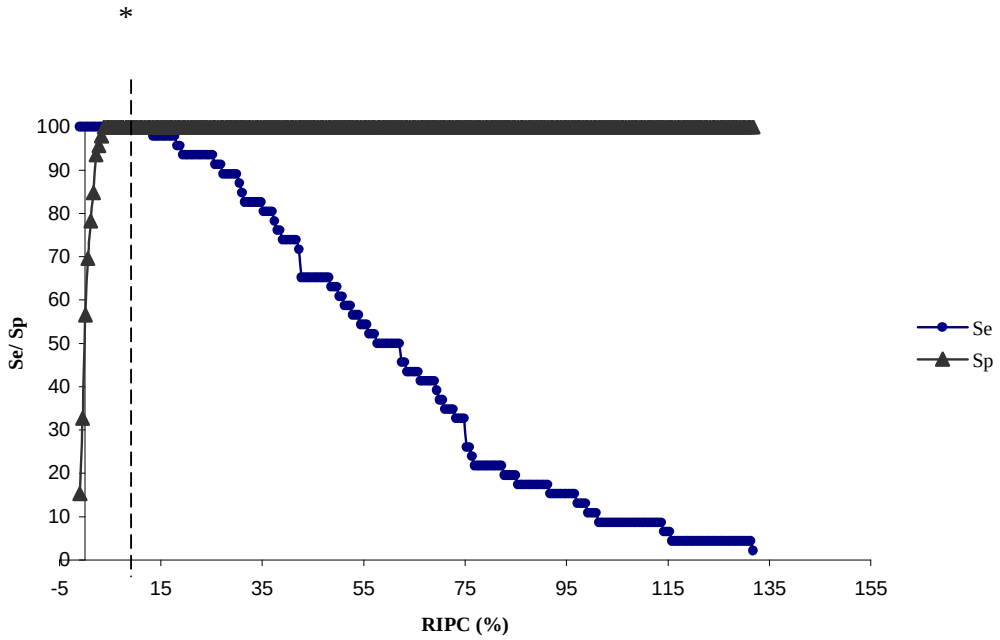
Figure captions

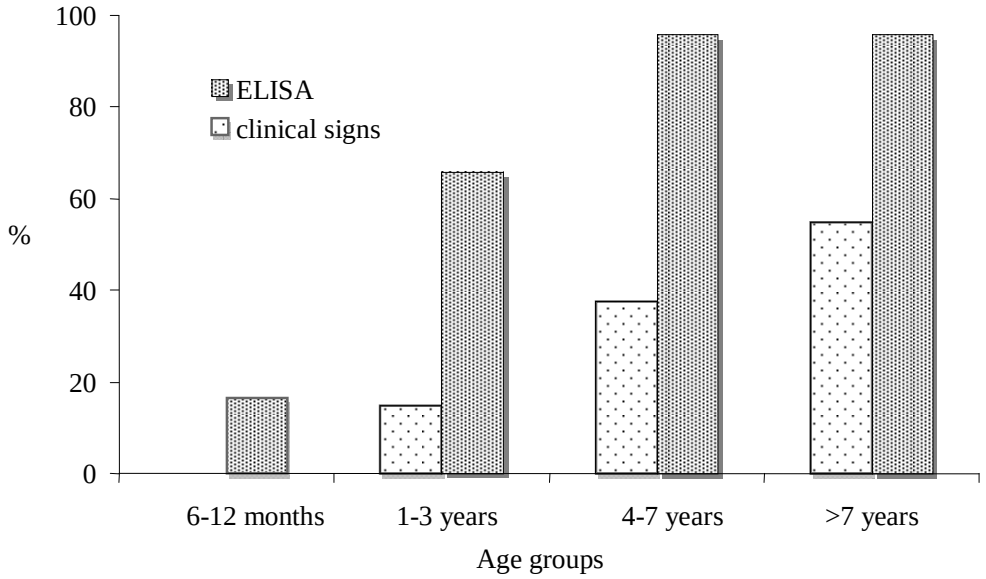
Figure 1: Two-graph receiver operating characteristic (TG-ROC) of *B. besnoiti* soluble extract-based ELISA. * The line represents the cut-off points selected for equal sensitivity (Se) and specificity (Sp) values.

Figure 2: Age distribution regarding the presence of clinical signs and seropositivity by ELISA.


*
100
90
80
70
60
50
40
30
20
10
0
Se/ Sp
-5
15
35
55
75
95
115
135
155
RIPC (%)
Se
Sp

1

2


100
80
60
40
20
0
%
ELISA
clinical signs
6-12 months
1-3 years
4-7 years
>7 years
Age groups