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Abstract:

Improved immunodiagnosis of *Besnoitia besnoiti*-infection in cattle by the use of ELISA and Westernblot.

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Abstract

Besnoitia besnoiti, an obligate intracellular apicomplexan protozoan parasite, is the causative agent of bovine besnoitiosis. This infection may dramatically affect body condition, be responsible for abortion and, in males, lead to irreversible infertility, thus overall resulting in important economical losses in livestock production. Identification of infected animals for subsequent culling is one of the only presently appropriate measures of control. While identification of clinical cases is relatively easy to carry out, the finding of subclinical forms of infection is more difficult, thus serology may be an appropriate diagnostic tool. In view to improve and validate immunodiagnosis, we evaluated an enzyme-linked immunosorbent assay (ELISA), complemented with a Westernblot (both using a somatic *B. besnoiti*-tachyzoite antigen) to detect anti-*Besnoitia besnoiti* antibodies in bovine sera. The comparative evaluation of the two methods, using 13 sera from animals affected by the chronic phase of besnoitiosis and 10 asymptomatic carriers, yielded a diagnostic sensitivity of 87% for ELISA and 91% for Westernblot analyses. Specificity was tested with sera from animals with confirmed *Toxoplasma gondii* (n=5) and *Neospora caninum* (n=12) infection, and with 64 negative sera from either an endemic or a non-endemic area. The ELISA specificity ranged between 97.4% - 98%, the Westernblot specificity between 94.9% - 100%. The present study demonstrated that ELISA and Westernblot, using in vitro generated somatic *B. besnoiti* antigen, is a useful tool combination to reliably detect animals that have been exposed to *Besnoitia besnoiti* infection, including both asymptomatic and symptomatic courses of disease.

Key words:

Besnoitia, cattle, immunodiagnosis, ELISA, Westernblot

1. Introduction

Bovine besnoitiosis is caused by infection with *Besnoitia besnoiti* (Marotel 1912). This tissue cyst-forming coccidian protozoan parasite is classified in the family of Sarcocystidae within the Phylum Apicomplexa (Fayer, 1981; Tadros & Laarman, 1982). All cattle breeds, both sexes, and animals of all ages can be affected, except that clinical disease occurs rarely in calves less than 6 months of age (Bigalke, 1968).

Bovine besnoitiosis is ubiquitously distributed in Sub-Saharan Africa (Bigalke *et al.*, 1967; Shkap *et al.*, 1994) and Asia (Peteshev *et al.*, 1974; Krasov *et al.,* 1975). In Europe, it has been reported in France (Besnoit & Robin, 1912; Bourdeau *et al.*, 2004), Spain (Juste *et al.*, 1990; Irigoien *et al.*, 2000) and Portugal (Franco & Borges, 1915; Leitão, 1949; Malta & Silva, 1991; Cortes *et al.*, 2003; Cortes *et al.*, 2005). It is interesting to notice that, in these countries, after the first reports in the early 20th century (Besnoit & Robin, 1912; Franco & Borges, 1915) the disease received very little attention until the end of the 20th century, since when its prevalence seemed to be increasing (Cortes *et al.*, 2005). At the acute stage of infection, tachyzoites proliferate predominantly in endothelial cells of the blood vessel walls, macrophages and fibroblasts. The result is vasculitis and thrombosis, especially in capillaries and small veins of the dermis, subcutis, fascia, testes and upper respiratory mucosae (Basson *et al.*, 1970). Clinical signs consist of raised body temperature, increased heart and respiratory rates, serous nasal and ocular discharges, anorexia, weight loss, generalized weakness, swelling of superficial lymph nodes, generalized edema of the skin, acute orchitis with swollen, painful testes and, in some cases, anasarca (Schulz, 1960). Inspiratory dyspnea may result from inflammation of the upper respiratory mucosae (McCully *et al.*, 1966). Diarrhoea and abortion are other more rare manifestations of the disease (Pols, 1960; Juste *et al.,* 1990).

The subsequent, chronic stage of disease is characterized by formation of large numbers of tissue micro-cysts, up to 0.4 mm in diameter, containing bradyzoites. A low-grade, intermittent febrile reaction may be observed, reluctance to move, anorexia continues, and the loss of body condition can be severe. The characteristic cysts are formed in the same

tissues in which the tachyzoites were present during acute disease, especially in cutaneous and subcutaneous tissues, and in intermuscular fascia (McCully *et al.*, 1966). The skeletal muscles, tendons, tendon sheaths and periosteum of the limbs, the testicular parenchyma, and the upper respiratory mucosae may also be extensively involved (Basson *et al.*, 1970). Tiny cysts are usually apparent upon close visual inspection of the scleral conjunctiva, a feature that is of considerable value in clinical diagnosis (Sannusi, 1991).

Dermal lesions are always present during chronic disease. These consist of rather dramatic thickening, hardening and folding or wrinkling of the skin, especially around the neck, shoulders and rump, always accompanied by hyperkeratosis, hyperpigmentation and alopecia (Pols, 1960). The thickening of the skin is caused by scleroderma, (Basson *et al.*, 1970). Scleroderma and alopecia are permanent disfigurements in surviving animals (Bigalke, 1960).

There may also be pronounced thickening of the limbs, and locomotion may be difficult and painful (Pols, 1960). A mucopurulent nasal discharge may be accompanied by inspiratory dyspnea (McCully *et al.*, 1966). Severely affected bulls often develop irreversible intratesticular lesions of vasculitis, focal necrosis, sclerosis and atrophy, which usually result in permanent infertility (Ferreira *et al.*, 1982; Cortes *et al.*, 2005).

Few animals die during the acute stages of disease. The case fatality rate during the chronic stage is usually on the order of 10% (Pols, 1960).

In the presence of disease on a herd level or by the time the option is on buying animals for reproduction purposes, it is important to avoid acquirement of or to eliminate the presence of infected animals. Clinically manifest cases, due to the occurrence of typical signs, are easily detectable. Subclinically infected animals, however – more difficult to be diagnosed – play an important role, as the parasite may be inapparently transmitted either iatrogenically or by insect vectors (Bigalke, 1968). Histopathology, due to the very high number of cyst on the skin of sick animals is a good method to diagnose acute disease, but not for the detection of chronic or subclinical infection, where the number of cyst on an overall cattle may still be high, but scarce on a histological skin slide (Cortes *et al.*, 2004).

Previous studies addressing operating characteristics of the IFAT demonstrated the absence of cross-reactions with sera from animals infected with *N. caninum*, conversely no reactivity occurred when using *N. caninum* IFAT-antigen probed with *B. besnoiti* antiserum (Shkap *et al., 2002).* The large variety of antigen preparations used e.g. in bovine neosporosis (Bjorkmann et al., 1994, 1997; Lally et al. 1996; Williams et al., 1997; Gottstein et al., 1998) reflects concern about the potential problem of antigenic cross-reactivity with other closely related parasites. Whilst the use of highly purified antigen fractions or proteins may minimize the risk of cross-reactivity with other parasite species, a limited repertoire of antigenic epitopes may restrict individual recognition by sera from different animals, thus causing putative problems of diagnostic sensitivity. The use of somatic antigens in ELISA may exhibit a greater diagnostic sensitivity (Pare *et al.*, 1995), but may include some problems of specificity, thus requiring an additional test to solve this problem. We approached this by adding a Western blot that can reliably distinguish between specific reaction and non-specific or cross-reactions. Our experimental strategy was based upon an primary ELISA as proposed by Shkap (Shkap *et al.*, 1984), but complemented with an additional Westernblot test that can elucidate the problems of specificity.

2. Material and methods

2.1. Tissue culture and parasite purification

Vero cell cultures were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 100 U of penicillin/ml, and 100 μg of streptomycin/ml at 37ºC with 5% CO_2 in tissue culture flasks. Cultures were trypsinized at least once a week. *B. besnoiti* (Bb1Evora03) tachyzoites were grown in Vero cell monolayer with DMEM supplemented with 10% fetal calf serum, previously inactivated at 56º C for 30 min. Tachyzoites were harvested by scraping the monolayer and collection of the media with free parasites and infected Vero cells, followed by repeated passages through a 25-gauge needle at 4°C and, separation on Whatman CF-11 column as described by Shkap (Shkap *et al.*, 1990), followed by a final sedimentation at 770x g for 15 minutes at 4ºC. The parasites were subsequently washed and centrifuged twice in phosphate buffered saline (PBS), pH 7.2 and stored frozen at –80°C.

2.2. Bovine Sera

A total of 104 sera were used in this study. Serum from 23 infected Portuguese cattle with *B. besnoiti* (13 with clinical signs of besnoitiosis and 10 without any clinical sign of disease, besides the presence of cysts, as determined upon histopathology (Fig. 1). Twelve sera were from Swiss cattle having experienced a *Neospora caninum*-PCR-positive abortion, and 5 sera were from Swiss cows/heifers exhibiting a *Toxoplasma gondii*-positive PCR with either cerebral or placental/fetal tissues and being serologically positive in the SAG1(P30)-ELISA. Additional (negative) control sera were collected from 39 healthy animals that were negative by histopathology and seronegative by IFAT, they were obtained from a Portuguese endemic area. Sera from 25 healthy cows were collected in a Swiss non-endemic area.

152 **2.3. ELISA**

153 The *Besnoitia*-ELISA was basically carried out as described for *N. caninum* (Gottstein et al.,
154 1999), and included the following steps: Frozen *B. besnoiti* tachyzoite pellets were
155 resuspended in PBS containing 0.01% NaN_3. Subsequent treatment by three freezing-
156 thawing cycles (- 50º/+ 37ºC) and final processing by ultrasonication (3 x 20 s at 65 W) at
157 1°C yielded a soluble *B. besnoiti*-extract (somatic antigen). The extract was sedimented at
158 10,000g at 4°C for 30 min. The soluble supernatant was used as somatic ELISA-antigen
159 (hereinafter referred to as *Besnoitia*-SA-ELISA). Coating Dynatech polystyrene plates was
160 done at a concentration of 0.4 μg protein per ml carbonate buffer (pH 9.6) at 4°C for 12 h.
161 All protein concentrations were assessed by the Bio-Rad protein assay using bovine
162 albumin as the standard. *Besnoitia*-ELISA plates were subsequently processed as
163 described elsewhere for *N. caninum* (Gottstein *et al.*, 1997). In brief, the washing, blocking
164 and serum dilution solution was PBS containing 0.3% Tween 20 (PBS-Tween). The test
165 and control sera were diluted 1: 200 in PBS-Tween with 1% horse serum. Serum
166 incubations were for 90 min at 37°C. The second antibody was an alkaline phosphatase-
167 conjugated rabbit anti-bovine antibody (Sigma Immmunochemicals, cat. No. A 0705) diluted
168 at 1:5000 in PBS-Tween. The conjugate was incubated for 1 h at 37°C. The substrate used
169 was 4-nitrophenylphosphate. The enzyme reaction was stopped after 15 min with 3N
170 NaOH, and absorbance values were determined at A_{405nm} using a Dynax technologies MRX
171 II reader coupled to a computer with the corresponding Biocalc@ software (Dynatech).
172 Positive and negative control sera used for the *Besnoitia*-SA-ELISA were the same as used
173 for the Western blot.
174 The basic test parameters for the *Besnoitia*-SA-ELISA were determined by investigating 39
175 sera from animals (no history of clinical disease; anamnestic lack of clinical or
176 epidemiological signs indicative for besnoitiosis) originating from Portugal and 25 sera from
177 Swiss cattle. For both geographically independent negative control groups, the respective
178 ELISA ROC analysis was determined and subsequently used as the cut-off value. Any
179 value higher than this cut-off value was considered to be “positive”; lower values were
180 considered as “negative”. Reproducibility of ELISA results was monitored by including a low

reactive bovine control serum in triplicate, this in addition to the negative and positive standard sera, both also tested in triplicate.

2.4. Western blot

Thawed samples containing 10^8 pelleted *B. besnoiti* tachyzoites were solubilized for 10 min at 94ºC in sample buffer (2%[w/v] sodium dodecyl sulfate (SDS), 7%[v/v] glycerol, 0,05% bromophenol blue, 0,75% tris(hydroxymethyl)aminomethane, pH 6.8). The samples were subsequently electrophoresed by SDS-PAGE (12.5%[w/v]) and electrophoretically transferred to PVDF (Schares *et al.*, 1998) membranes (Immobilon-P, Millipore). Strips were blocked with PBS+T+G (PBS, 0.05% [v/v] Tween 20, 2%[w/v] Fish gelatine 1%), and subsequently incubated for 60 min at 37°C with bovine sera diluted 1:100 in PBS+T+G. After washing the strips with PBS+T, antibody reactions were visualized with an anti-bovine IgG (H+L) peroxidase conjugate (Dianova, Cat. No 101-035-003)and 4-chloro-1-naphthol as a substrate. Relative molecular masses were determined by comparing with respective molecular weight standards (Precision Plus Protein™ Standards Catalogue 161-0374 BIO-RAD.

3. Results

3.1. ELISA cut-off point determination

The operative absorbance value of each serum was expressed as the percentual absorbance value (AU%), which was calculated on the basis of the negative control serum in each microplate, in order to minimize interplate variation. Thus, the AU% was calculated according to the formula: AU% = (A_{404nm} test serum / A_{404nm} negative control serum) x 100. The cut-off points were calculated by ROC analyses using the 39 and 25 negative control sera (originating from Portugal and Switzerland, respectively) and the above mentioned positive besnoitiosis sera (Fig. 2). Although the cut-off points for each geographical area were individually determined and showed some differences (see below), the differences between both was not relevant in discriminating between positive and negative cases, thus only one threshold value could be used for the sensitivity and specificity analyses (see Fig. 3).

3.2. Reactivity by ELISA

ELISA-results are presented in Table 1 and Fig. 3. Although most heterologous- or non-infection sera were clearly negative in ELISA, there was one out of five *T. gondii*-sera that showed an absorbance value even higher than those obtained with sera from animals infected with *B. besnoiti* (Fig. 3). No sera from animals infected with *N. caninum* showed reactivities above the cut-off value. The difference found between the two negative control groups was statistically significant (p-value = 0.031). The correlation between ELISA-findings versus histopathological and IFAT-findings – concerning the besnoitiosis sera – is shown in Table 2. These data were used to calculate diagnostic sensitivity, specificity and other operating characteristics as shown in Table 3.

3.3. Sensitivity and specificity of ELISA

The overall sensitivity of ELISA using sera from individuals in chronic symptomatic and asymptomatic phases of besnoitiosis was 87.0%. Specificity was calculated as 97.4% when

including only Portuguese negative controls and 98% when including only Swiss negative controls (Table 3). Positive predictive values, based upon an assumed prevalences of 10% for Portugal and 0.001% for Switzerland, were 78.8% and <0.001%, respectively, negative predictive values were 99% and 100%, respectively.

3.4. Western blot

Westernblot performance was assessed with the same besnoitiosis and control sera as used for ELISA (Figures 4 and 5). Results showed that some negative control sera that had exhibited slightly elevated ELISA-values also recognized a few bands in the somatic parasite extract, but the pattern exhibited by infected versus non-infected animals was clearly different and thus allowed reliable discrimination. Detailed Westernblot findings with regard to negative/positive discrimination of besnoitiosis sera are presented in Table 1. The typical besnoitiosis profile on the Westernblot included 3 major antigenic areas of reactivity (Figs. 4 and 5). One localized in the range between 15 and 20 kDa; a second one between 22 and 37 kDa and a third one between 47 and 100 kDa. Eventual ghost bands appearing in negative control lanes were subtracted from the whole banding pattern for interpretation of findings. Sera were considered to be positive when reactivity was present against at least two out of these 3 antigenic areas. In this study, two infected individuals (number 8 and 12) out of 23 animals with *B. besnoiti* showed a negative Westernblot (exemplified by no. 14 in Fig. 5, after subtraction of ghost bands in the upper area between 47 – 100 kDa). From all sera from animals selected out of a non-endemic area, no animal showed Westernblot-positive results. In the Portuguese group of presumably negative animals, 2 sera showed a positive Westernblot pattern (data not shown).

Sera from animals with *Toxoplasma* and *Neospora* infection (two phylogenetically closely related parasites) did not recognize any of the antigens characteristic for *B. besnoiti*-infection (data not shown).

3.5. Sensitivity and specificity of Westernblot

The sensitivity of Westernblot using sera of individuals exhibiting chronically symptomatic and asymptomatic phases of besnoitiosis was 91.3% (Table 4). Specificity was calculated as 94.9% and 100% for Portuguese and Swiss animals, respectively. As for ELISA, predictive test values of Westernblots were calculated on the basis of an assumed prevalence of 10% for Portugal and 0.001% for Switzerland. Positive predictive value was thus 67% for the Portuguese endemic and 100% for the Swiss non-endemic area, negative predictive values were 99% and 100%, respectively.

3.6. Comparison of ELISA and Westernblot

The percent agreement between ELISA and the Westernblot in infected animals was 100% ((21+2)/23) and in non-infected animals 95.1% ((77)/81), respectively (Table 5). The overall percent agreement combining infected and non-infected animals was 96.2% ((21+2+77)/104). By using the data from Tables 2, 3 and 4, respectively, the Kappa values for ELISA and Westernblot provided the following features: $K = (P_o-P_e)/(1-P_e)$, where $P_o =$ a/n+d/n and $P_e = ((a+c)/n)\ ((a+b)/n)\ ((b+d)/n)\ ((c+d)/n)$.

The ELISA P_o was 0.76 and $P_e = 0.028$, from which a Kappa value of K = 0.97 resulted. The data for the Westernblot were $P_o = 0,72$, $P_e = 0.029$, and the resulting Kappa value was 0.97.

4. Discussion

We describe the development of an ELISA and Westernblot to detect *Besnoitia*-specific antibodies in the serum of bovine hosts. A somatic in vitro cultured tachyzoite antigen was used, basically easy to produce and yielding consistent results in both assay systems. The ELISA provided a good diagnostic sensitivity including all the different clinical courses of bovine besnoitiosis, cross-reactivity was not apparent with any of the neosporosis sera, but there was some degree of crossreactivity with toxoplasmosis sera. Such real or putative cross-reactions were the main reason to elaborate a complementary Westernblot. The Westernblot aimed not only at the elucidation of doubtful ELISA-findings, but also to detect potential double infections (besnoitiosis and toxoplasmosis or besnoitiosis and neosporosis) in a single host. For ELISA, we determined the cut-off absorbance value to distinguish *Besnoitia*-infected and uninfected animals by using bovine sera collected from 23 animals infected with *B. besnoiti* with different levels of infection: either with, or without clinical signs; but all infections were evidenced by histopathology, as a direct diagnostic technique, allowing to identify parasite cysts and bradyzoites. Addressing other infections with however very closely related parasites, we employed 12 sera from cattle infected with *N. caninum* and 5 sera from animals infected with *T. gondii* to elucidate specificity. Finally, serum samples obtained from 64 healthy and uninfected animals from Portugal and similar animals from Switzerland were used to elaborate the reaction range of negative bovines, thus providing background data to calculate negative/positive threshold values. Not infectiologically related non-specific reactions have been referred to bovine immunoglobulins that harbor a non-specific component that may bind to *N. caninum* tachyzoites (Williams et al., 1997) as well as to *T. gondii* tachyzoites. Horse serum was, therefore, used as a blocking agent, together with 0.3% Tween 20. This solution was selected for its highest signal/noise ratio. Consequently, by applying all these technological approaches for ELISA and combining them with Westernblot, we were able to solve most of the problems that can be faced while carrying out serodiagnosis of bovine besnoitiosis.

The epidemiological information available so far concerning bovine besnoitiosis documents its existence in the South of Portugal, but the true prevalence of the disease and of the infection extensity remains unknown. Some studies, conducted in spatially defined areas, reported high seropositive percentages in analyzed herds, i.e., 36% in herds followed-up for 3 years (Cortes *et al.,* 2004) and 93% in herds followed-up for longer time periods (data not published).

The now presented ELISA and Westernblot exhibits a sensitivity and specificity that will allowed to reliably serodiagnose chronic symptomatic animals as well as asymptomatic cases in endemic areas. ELISA, with its sensitivity of 87% and specificity of 97.4%, appears suitable for a first mass-screening approach. Westernblot, with its sensitivity of 91.3% and specificity of 94.9%, appears suitable as a secondary test to detect putative false positive ELISA results. For areas of very low (or absent) endemicity, however, screening of populations by ELISA, due to the very low positive predictive value of the test, will unconditionally requires specificity elucidation by Westernblot. As a consequence, the test combination will now be practically investigated in sero-epidemiological studies, which are important to address the real distribution of bovine besnoitiosis not only in Portugal, but also in other countries of Europe, where the disease may be present but has not yet been detected.

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References

Basson, P. A., McCully, R. M., & Bigalke, R. D., 1970. Observations on the pathogenesis of bovine and antelope strains of *Besnoitia besnoiti* (Marotel, 1912) infection in cattle and rabbits. Onderstepoort J. Vet. Res. 37, 105-126.

Besnoit, C. & Robin, V., 1912. Sarcosporidioses cutanée chez une vache. Rec. Vet. 37, 649.

Bigalke, R. D., 1960. Preliminary observation on the mechanical transmission of cyst organisms of *Besnoitia besnoiti* (Marotel, 1912) from a chronically infected bull to rabbits by *Glossina brevipalpis* Newstead, 1910. J. S. Afr. Vet. Assoc. 31, 37-44.

Bigalke, R. D., van Niekerk, J. W., Basson, P. A., & McCully, R. M., 1967. Studies on the relationship between *Besnoitia* of blue wildebeest and impala, and *Besnoitia besnoiti* of cattle. Onderstepoort J. Vet. Res. 34, 7-28.

Bigalke, R. D., 1968. New concepts on the epidemiological features of bovine besnoitiosis as determined by laboratory and field investigations. Onderstepoort J. Vet. Res. 35, 3-138.

Bjorkman, C., Holmdahl, O. J., & Uggla, A., 1997. An indirect enzyme-linked immunoassay (ELISA) for demonstration of antibodies to *Neospora caninum* in serum and milk of cattle. Vet. Parasitol. 68, 251-260.

Bjorkman, C., Lunden, A., Holmdahl, J., Barber, J., Trees, A. J., & Uggla, A., 1994. *Neospora caninum* in dogs: detection of antibodies by ELISA using an iscom antigen. Parasite Immunol. 16, 643-648.

Bourdeau, P. J., Cesbron, N., Alexandre, F., Marchand, A. M., Desvaux, J. P., & Douart, A., 2004. Outbreak of bovine besnoitiosis (*Besnoitia besnoiti*) in the west of France and its diagnosis by immunofluorescence assay. IX European Multicolloquium of Parasitology, 18-23 July, Valencia, Spain 459-460.

348 Cortes, H., Ferreira, M. L., Silva, J. F., Vidal, R., Serra, P., & Caeiro, V., 2003. Contribuição
349 para o estudo da besnoitiose bovina em Portugal. Revista Portuguesa de Ciências
350 Veterinárias 98, nº 545, 43-46.

351 Cortes, H., Leitão, A., Vidal, R., Reis, Y., Waap, H., Pereira da Fonseca, I., Fazendeiro, I.,
352 Ferreira, M. L., & Caeiro, V., 2004. Bovine besnoitiosis, one approach for a better
353 understanding of its importance in Portugal Proceedings of the 23rd World Buiatrics
354 Congress, pp. 35-36.

355 Cortes, H., Leitão, A., Vidal, R., Vila-Viçosa, M. J., Ferreira, M. L., Caeiro, V., & Hjerpe, C.
356 A., 2005. Bovine Besnoitiosis in Portugal. Vet. Rec. "in Press".

357 Fayer, R., 1981. Coccidian Taxonomy and Nomeclature. J. Protozool. 28, 266-270.

358 Ferreira, M. L., Nunes Petisca, J. L., & Dias, O. H., 1982. Alterações testiculares em touros
359 de Moçambique assintomáticos e com sintomas clínicos de besnoitiose. Rep. Trab. I. N. V.
360 XIV, 97-108.

361 Franco, E. E. & Borges, I., 1915. Nota sobre a sarcosporidiose bovina. Revista de Medicina
362 Veterinária Ano XIV, 255-299.

363 Gottstein, B., Pozio, E., Connolly, B., Gamble, H. R., Eckert, J., & Jakob, H. P., 1997.
364 Epidemiological investigation of trichinellosis in Switzerland. Vet. Parasitol. 72, 201-207.

365 Gottstein, B., Hentrich, B., Wyss, R., Thur, B., Busato, A., Stark, K. D., & Muller, N., 1998.
366 Molecular and immunodiagnostic investigations on bovine neosporosis in Switzerland. Int.
367 J. Parasitol. 28, 679-691.
368
369 Gottstein, B., Hentrich, B., Wyss, R., Thur, B., Bruckner, L., Muller, N., Kaufmann, H., &
370 Waldvogel, A., 1999. [Molecular and immunodiagnostic studies of bovine neosporosis in
371 Switzerland]. Schweiz. Arch. Tierheilkd. 141, 59-68.
372

373 Irigoien, M., Del Cacho, E., Gallego, M., Lopez-Bernad, F., Quilez, J., & Sanchez-Acedo,
374 C., 2000. Immunohistochemical study of the cyst of *Besnoitia besnoiti*. Vet. Parasitol. 91, 1-
375 6.

376 Juste, R. A., Cuervo, L. A., Marco, J. C., & Oregui, L. M., 1990. La besnoitiosis bovina:
377 desconocida en España? Medicina Veterinária 7, 613-618.

378 Krasov, V. M., Omarov, Z. K., Uvaliev, I. U., Khvan, M. V., 1975. Besnoitiosis in animals.
379 Veterinariya, 2, 65-70.
380
381 Lally, N. C., Jenkins, M. C., & Dubey, J. P., 1996. Development of a polymerase chain
382 reaction assay for the diagnosis of neosporosis using the *Neospora caninum* 14-3-3 gene.
383 Mol. Biochem. Parasitol. 75, 169-178.

384 Leitão, J. L. S., 1949. Globidiose bovina por *Globidium besnoiti* (Marotel 1912). Revista de
385 Medicina Veterinária 330, 152-158.

386 Malta, M. & Silva, M., 1991. Besnoitiose no Alentejo. XIV Jornadas de Medicina Veterinária
387 "Bovinos de Carne" Faculdade de Medicina Veterinária de Lisboa, Portugal, January, 15th,
388 1991 (not published).

389 McCully, R. M., Basson, P. A., van Niekerk, J. W., & Bigalke, R. D., 1966. Observations on
390 *Besnoitia* cysts in the cardiovascular system of some wild antelopes and domestic cattle.
391 Onderstepoort J. Vet. Res. 33, 245-276.

392 Pare, J., Hietala, S. K., & Thurmond, M. C., 1995. An enzyme-linked immunosorbent assay
393 (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. J. Vet. Diagn. Invest 7,
394 352-359.

395 Peteshev, V. M., Galuzo, I. G., & Polomoshov, A. P., 1974. Cats - definitive hosts *Besnoitia*
396 (*Besnoitia besnoiti*) (in Russian. Izvestiae Akademii Nauk Kazakheskan SSR B, 33-38.

Pols, J. W., 1960. Studies on bovine besnoitiosis with special reference to the aetiology. Onderstepoort J. Vet. Res. 28, 265-356.

Sannusi, A., 1991. A simple field diagnostic smear test for bovine besnoitiosis. Vet. Parasitol. 39, 185-188.

Schares, G., Peters, M., Wurm, R., Barwald, A., & Conraths, F. J., 1998. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. Vet. Parasitol. 80, 87-98.

Schulz, K. C. A., 1960. A report on naturally acquired besnoitiosis in bovines with special reference to its pathology. J. S. Afr. Vet. Med. Ass. 31, 21-35.

Shkap, V., Ungar-Waron, H., Pipano, E., & Greenblatt, C., 1984. Enzyme linked immunosorbent assay for detection of antibodies against *Besnoitia besnoiti* in cattle. Trop. Anim Health Prod. 16, 233-238.

Shkap, V., Ungar-Waron, H., & Pipano, E., 1990. Identification and partial purification of soluble antigens from culture-grown *Besnoitia besnoiti* endozoites. Rev. Elev. Med. Vet. Pays Trop. 43, 63-68.

Shkap, V., Pipano, E., Marcus, S., & Krigel, Y., 1994. Bovine besnoitiosis: transfer of colostral antibodies with observations possibly relating to natural transmission of the infection. Onderstepoort J. Vet. Res. 61, 273-275.

Skhap, V., Reske, A., Pipano, E., Fish, L., Baszler, T., 2002. Immunological relationship between *Neospora caninum* and *Besnoitia besnoiti.* Vet. Parasitol. 106 35-43.

Tadros, W. & Laarman, J. J., 1982. Current concepts on the biology, evolution and taxonomy of tissue cyst- forming eimeriid coccidia. Adv. Parasitol. 20, 293-468.

Williams, D. J. L., McGarry, J., Guy, F., & Trees, A. J., 1997. Novel ELISA for detection of *Neospora* specific antibodies in cattle. Vet. Rec. 140, 328-331.

Legends to Figures and Tables

Fig. 1

Characteristic tissue cyst of *B. besnoiti*, presented in a skin biopsy specimen from an infected bovine with no other clinical sign of disease other then the presence of a small number of cyst. Hematoxylin-Eosin-stain. Bar = 200µm.

Fig. 2

ELISA cut-off determination by ROC analysis, using the 64 negative control sera from both geographical origins together versus the positive controls.

Fig. 3

ELISA operating characteristic analyses (sensitivity and specificity)*:*
23 sera from animals infected with *Besnoitia besnoiti*; 6 sera seropositive to *Toxoplasma gondii*; 12 sera from animals with *Neospora caninum* infection, and sera from uninfected cattle (negative controls). The cut-off points were independently determined by ROC analyses for the Swiss and Portuguese situation, but finally one discriminating threshold value could be used for both (·–·–·–).

Fig. 4

Comparative Westernblot analysis with animals suffering from besnoitiosis. 1: conjugate control; 2: negative control; 3: positive control; 4 and 5: cysts on the skin without other sign of disease; 6 and 7: with disease for two months; 8, 9 and 1:) presented disease, but improved; 11 to 16: persistently sick animals

Fig 5

Comparative Westernblot analysis with animals infected with *B. besnoiti* and negative control animals. 1: conjugate control; 2: negative control; 3: Positive control; 4 to 7:

uninfected animals; 8 to 16: cysts on the skin without other sign of disease.

Table 1

Classification of cattle with besnoitiosis and corresponding ELISA and Westernblot findings. Cattle were clustered in groups with (a) clinical findings and histopathological evidence of chronic besnoitiosis, (b) presence of *B. besnoiti* cyst on histopathology, PCR-positivity of biopsy specimen, Westernblot-seropositivity but absence of clinical signs. The IFAT positive cut-off titer was ≥1:256; the ELISA positive cut-off value was $\geq A_{404nm} = 0.39$.

Table 2

Overall comparison between ELISA-findings and diagnosis carried out by IFAT and histology (gold standard) on animals with suspected clinical besnoitiosis (Portuguese and Swiss negative controls included together).

Table 3

Comparison of operating characteristics (sensitivity and specificity, positive and negative predictive values) of ELISA and Westernblot, based upon data derived from Tables 1 and 2 and Fig. 2.The predictive values of positive and negative results were calculated for a hypothetical prevalence of 10% for Portugal and of 0.001% for Switzerland.

Table 4

Overall comparison between Westernblot-findings and diagnosis carried out by IFAT and histology (gold standard) on animals with suspected clinical besnoitiosis (Portuguese and Swiss negative controls included together).

Table 5

Performance agreement data between the ELISA and Westernblot in diseased (+) and non-diseased (-) animals.

Table

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Table 1

Animal ID	*Besnoitia* serology			Parasitological characterization
	IFAT	ELISA	western blot	
1	1/1024	0.79	+	(a)
2	1/1024	0.65	+	(b)
3	1/1024	0.45	+	(b)
4	1/512	0.50	+	(b)
5	1/1024	0.71	+	(b)
6	1/1024	0.71	+	(b)
7	1/512	0.47	+	(b)
8	1/512	0.09	-	(b)
9	1/1024	0.53	+	(b)
10	1/1024	0.58	+	(b)
11	1/1024	0.53	+	(b)
12	1/1024	0.18	-	(a)
13	1/1024	0.39	+	(a)
14	1/1024	0.44	+	(a)
15	1/1024	0.74	+	(a)
16	1/1024	0.41	+	(a)
17	1/1024	0.62	+	(a)
18	1/1024	0.60	+	(a)
19	1/1024	0.73	+	(a)
20	1/1024	0.60	+	(a)
22	1/1024	0.62	+	(a)
23	1/1024	0.63	+	(a)

Table

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Table 2

ELISA anti-*B. besnoiti*	Histopathology and/or IFAT		
	Positive	Negative	Total
Positive	20(a)	2(b)	22
Negative	3(c)	79(d)	82
Total	23	81	104 (n)

Table

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Table 3

	ELISA		Westernblot	
	Portugal	Switzerland	Portugal	Switzerland
Sensitivity	87.0%	87.0%	91.3%	91.3%
Specificity	97.4%	98.0%	94.9%	100%
Positive predictive value	78.8%	<0.001%	67.0%	100%
Negative predictive value	99.0%	100.0%	99.0%	100%

Table

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Table 4

Wester blot anti-*B. besnoiti*	Histopathology and/or IFAT		
	Positive	Negative	Total
Positive	21(a)	2(b)	23
Negative	2(c)	79(d)	81
Total	23	81	104

Table

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Table 5

	Disease +	Disease -	Total
ELISA + WB+	21	0	21
ELISA + WB-	0	2	2
ELISA – WB+	0	2	2
ELISA- WB-	2	77	79
Total	23	81	104

Figure

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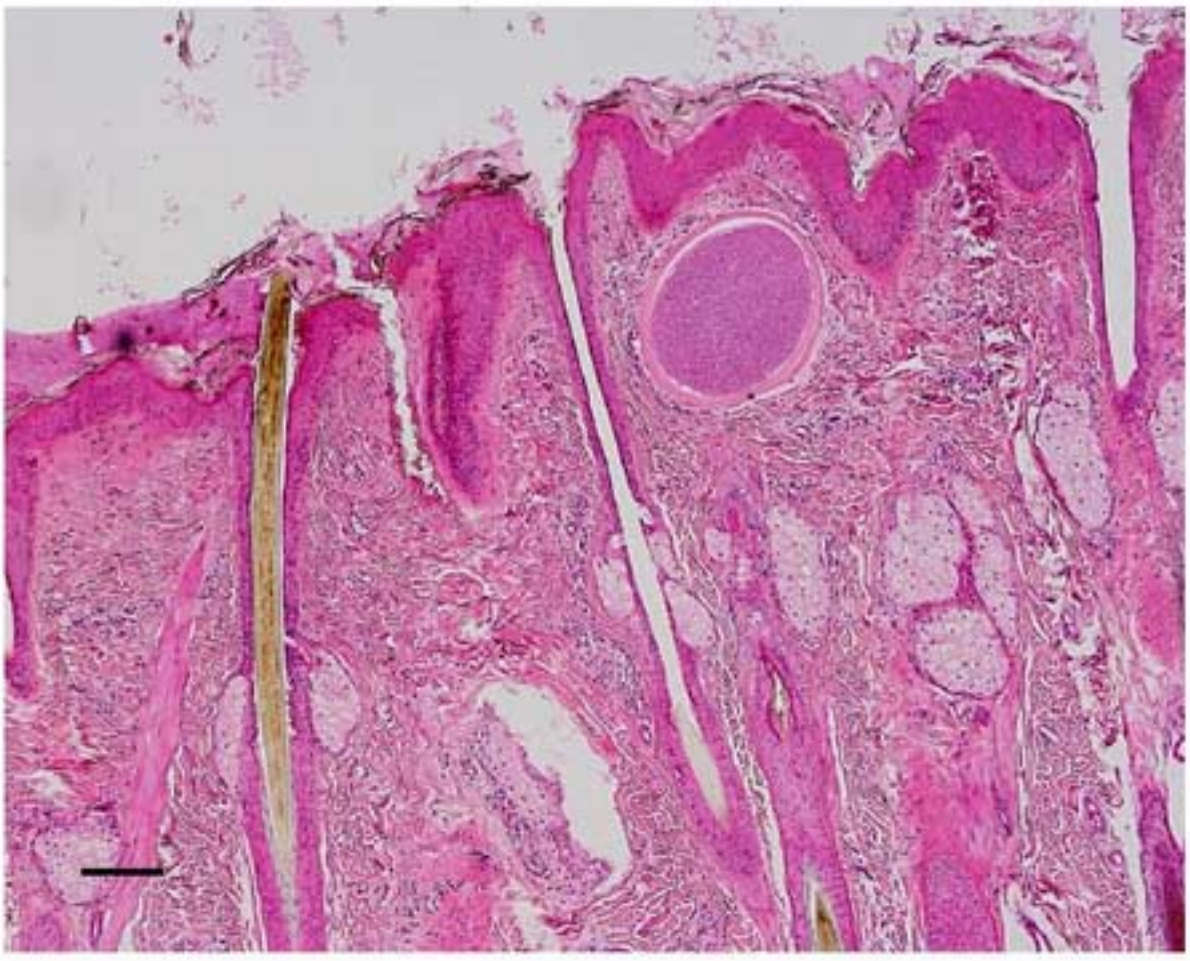
Fig. 1



Figure
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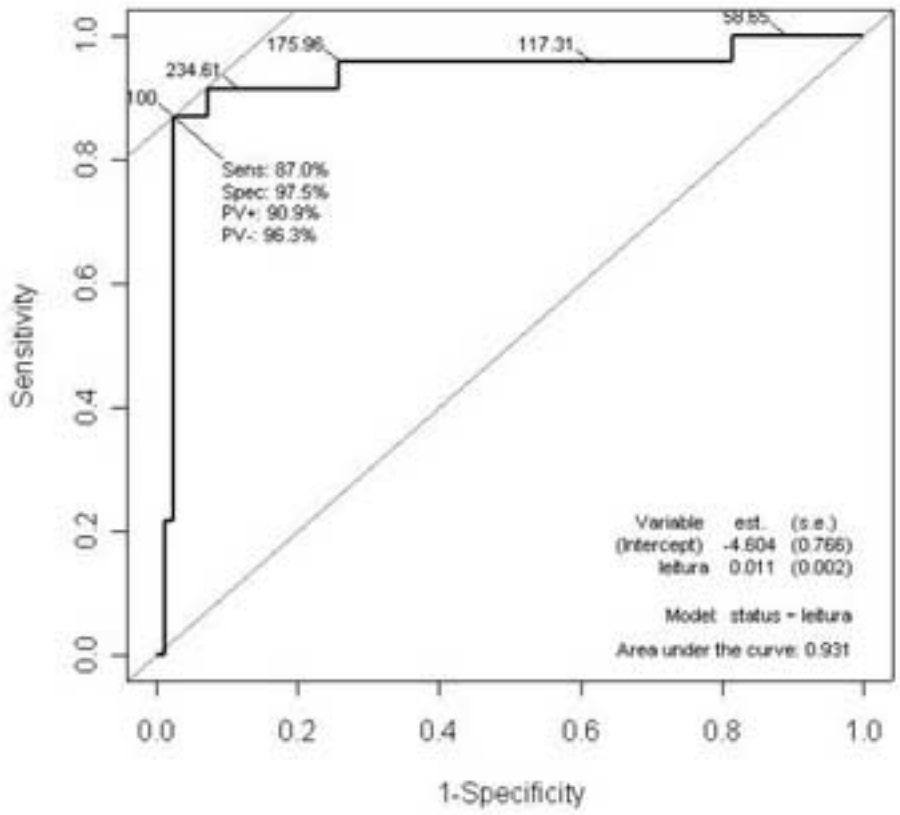
Fig. 2



Figure
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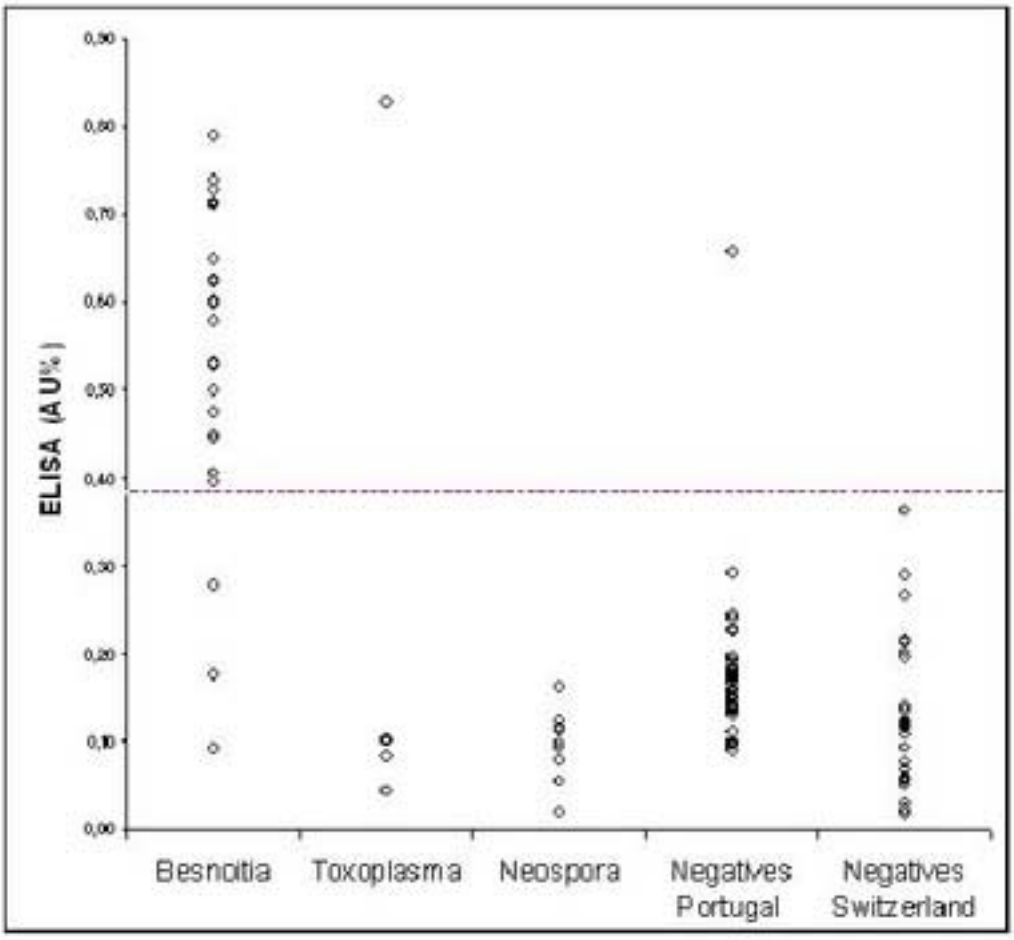
Fig. 3



Figure

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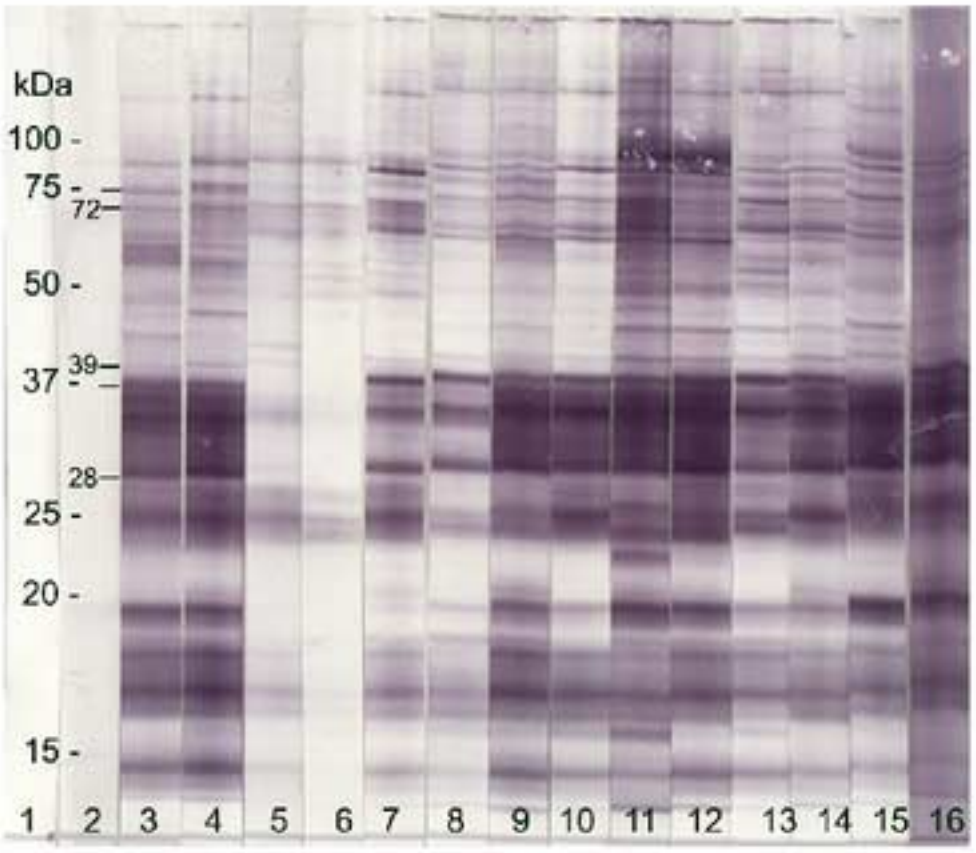
Fig. 4



Figure

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Fig. 5