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DIAGNOSIS OF *BABESIA CABALLI* INFECTIONS IN HORSES BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) AND WESTERN BLOT

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Abstract—BÖSE R. and PEYMANN B. 1994. Diagnosis of *Babesia caballi* infections in horses by enzyme-linked immunosorbent assay (ELISA) and Western blot. *International Journal for Parasitology* **24**: 341–346. From *Babesia caballi in vitro* cultures a preparation of 100% infected erythrocytes was obtained. From this, *B. caballi* antigens were extracted with the detergent 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) and used as ELISA antigens. A control antigen of normal erythrocytes from the same donor horse was prepared in an identical manner. The ELISA and Western blot were validated by testing of sera from horses experimentally infected with *B. caballi* or *B. equi* or not infected with *Babesia* spp. ELISA and Western blot results were compared with those obtained by the immunofluorescence antibody test (IFAT) and complement fixation test (CFT). The sensitivity of the ELISA of 98.3% obtained for sera from day 14 after infection was superior to the Western blot (94.9%), the IFAT (96.6%) and the CFT (28.8%). No positive results were obtained in the ELISA and Western blot with 106 sera from horses not infected with *Babesia* spp. resulting in a calculated specificity of 100% for both tests. Cross reactions of *B. equi*-positive sera did occur to a larger extent in the ELISA (20%) than in the IFAT (4%). No cross reactions were observed with the Western blot and the CFT. The higher sensitivity of the ELISA was also demonstrated by testing of 132 field sera: more positive results were obtained by ELISA (112) as compared to IFAT (92) or CFT (41). The validity of these results was confirmed by testing of sera by Western blot. The ELISA as the most sensitive test provides the best method for the identification of carrier horses to prevent the introduction into non-endemic areas (export testing). Positive ELISA results can be confirmed by Western blot, if a species-specific diagnosis is required.

INDEX KEY WORDS: *Babesia caballi*, sero diagnosis; ELISA; Western blot; IFAT; CFT.

INTRODUCTION

Babesia caballi and *B. equi* are obligate intraerythrocytic parasites of equines. They are the causative agents of equine babesioses which are endemic in most tropical and subtropical areas of the world (Friedhoff, 1982; Friedhoff, Tenter & Müller, 1990). Whereas *B. caballi* only invades erythrocytes *B. equi* is also capable of infecting lymphocytes (Schein, Rehbein, Voigt & Zweygarth, 1981). Both parasites are transmitted by tick vectors with almost worldwide distribution (Friedhoff, 1982). Consequently, it is important to prevent the introduction of carrier animals into non-endemic areas, particularly where the diseases could be spread by vector ticks. Horses to be exported into the U.S.A., Japan, Australia or other countries have to be tested negative for babesioses by the complement fixation test (CFT) or the immunofluorescence antibody test (IFAT). The CFT yields a considerable number of false-negative results (Weiland, 1986; Tenter & Friedhoff, 1986). The IFAT is laborious and not amenable to standardization. Thus, there is a need for improved serological tests for the diagnosis of equine babesioses. A first step was made by developing a Western blot for the diagnosis of *B. caballi* infections. This test can be used in case of contradicting CFT and IFAT results and provides a species-specific diagnosis (Böse & Daemen, 1992). For routine diagnosis, however, the test is too laborious. In general, the advantages of the ELISA in contrast to CFT or IFAT are its high sensitivity and the possibility of standardization and computer evaluation. We here report the development of a sensitive ELISA and the application of the Western blot for the diagnosis of *B. caballi* infections.

MATERIALS AND METHODS

Antigen preparation. Parasites were cultured using the microaerophilous stationary phase culture technique (Levy & Ristic, 1980) essentially as described previously (Böse & Daemen, 1992). Parasitized erythrocytes were enriched to

about 100% infected cells with density gradient centrifugation on a two-step Percoll gradient (Bhushan, Müller & Friedhoff, 1991), washed three times in RPMI-1640 tissue culture medium and stored in aliquots at −80°C. A control antigen of non-infected erythrocytes of the same doner horse used for the cultures was prepared in the same manner. For the ELISA both antigens were extracted with urea or with detergents. Urea (Gibco/BRL, Eggenheim, Germany, no. 540-5505UV) was used in a final concentration of 4.5 M-Sodium dodecyl sulfate (SDS) (Gibco/BRL, no. 5525UA), Triton X-100 (Boehringer, Mannheim, Germany, no. 1332481), Nonidet P-40 (Boehringer, no. 1332473) and 3-[3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) (Boehringer, no. 810118) were used in final concns of 1%. Antigen and reagent were mixed and incubated for 20 min at 37°C (urea, Triton X-100, Nonidet P-40, CHAPS) or for 5 min at 95°C (SDS) and centrifuged (22,000 *g*, 10 min, 4°C). The supernatant was used as antigen in the ELISA.

ELISA protocol. The ELISA was performed as a heterogeneous, indirect, non-competitive test for the detection of antibodies. Extracted antigens were diluted in 0.1 M-carbonate-bicarbonate buffer, pH 9.6, 200 μl transferred to each well of a microtitre plate and incubated for 1 h at 37°C. The following microtitre plates were tried: 2 plates from Dynatech, Denkendorf, Germany, nos. MA 1501 and M 129 A; 1 plate from Becton Dickinson, Heidelberg, Germany, Falcon no. 3912; 4 plates from Nunc, Wiesbaden, Germany, nos. 44 24 04, 47 50 94, 26 96 20 and 26 97 87; 4 plates from Greiner, Nürtingen, Germany, nos. 65 50 61, 65 50 01, 65 51 80 and 65 01 80; 3 plates from Flow Laboratories, Meckenheim, Germany, nos. 76-307-05, 76-381-04 and 76-181-04; 1 plate from Renner, Darmstadt, Germany, Costar no. 3590. Criteria for the selection of a suitable microtitre plate were high reactions with the high-titred *B. caballi* serum pool and a good discrimination between positive and negative sera. Plates were washed 3 times for 5 min with PBS (5 mM-phosphate buffer 147 mM-NaCl, pH 7.2) containing 0.05% Tween 20R (PBS–T). Assays were performed without a blocking step or plates were blocked for 30 min at 37°C. Gelatin (Sigma, Deisenhofen, Germany, no. G-6144), BSA (Sigma, no. A-2153), ovalbumin (Sigma, no A-5253), rabbit serum, a 'blocking reagent' (Boehringer, Mannheim, Germany, no. 1142372) and low-fat milk (Uelzena Milchwerke, Uelzen, Germany) were used in concns of 1%. A chemically modified BSA (BSAc) (Aurion, Wageningen, Netherlands) was tested in a concn of 0.2%. Sera were diluted 1/100 in PBS with 1% BSA and plates incubated for 1 h at 37°C followed by a set of washes and a 30 min incubation for the conjugate (rabbit anti-horse IgG (H+L) HRP, Dianova GmbH, Hamburg, Germany, no. 308-035-003) diluted 1/3000 in PBS with 1% BSA. When assays were performed without a blocking step, different dilution buffers were tested. Sera and conjugate were diluted in PBS with 1% BSA, PBS with 0.05% Tween 20 or PBS with 1% BSA and 0.05% Tween 20. After a further set of washes substrate (1 mg ml^{-1} of 5-aminosalicylic acid (Ellens & Gielkens, 1980) in 0.1 M-sodiumphosphate buffer, pH 6.0 containing 0.1 mM-EDTA and 6 mM-H_2O_2) was dispensed and plates were read.

Western blot. The Western blot was carried out as described previously (Böse & Daemen, 1992), except that TBS with 1% gelatin was substituted for TBS with 0.05% Tween 20. Sera reacting with antigens of mol. wts of 48 and 50 kDa and one or more of the 70, 112 or 141 kDa antigens were regarded positive. Sera reacting strongly only with the 48 and 50 kDa antigens were also considered positive.

Sera. Sera tested were as follows: (1) sera from 106 horses not infected with *Babesia* spp., i.e. horses from Germany tested negative in preliminary studies by IFAT at a serum dilution of $< 1/40$ and by CFT at a serum dilution of $< 1/5$; the results for these sera were used to calculate the diagnostic specificity of the ELISA and Western blot; (2) 71 sera from 15 horses experimentally infected with *B. caballi* or with *B. caballi* and *B. equi*; these results were used to calculate the diagnostic sensitivity of the ELISA, Western blot, IFAT and CFT; (3) 76 sera from 13 horses experimentally infected with *B. equi*; these results were used to evaluate the extent of cross reactions; (4) 132 field sera, i.e. 20 sera from Corsica, 35 sera from different European countries and 77 sera from 18 horses from Brazil. For the ELISA the following standards were used: (1) a high-titred *B. caballi* serum pool; (2) a low-titred *B. caballi* serum pool; (3) a pool of sera negative for *B. caballi* and *B. equi*. The CFT was performed following the instructions of the United States Department of Agriculture (1979) and the IFAT according to Tenter & Friedhoff (1986).

Data generation and evaluation. To allow a comparison between different microtitre plates and different runs at each microtitre plate the three standards were run on each plate in duplicates. The absorbance values were measured approx. 2, 4 and 6 min after substrate addition with an ELISA reader (Titertek Multiskan plus MK II, Flow Laboratories, Meckenheim, Germany) interfaced with an IBM compatible personal computer. The computer based kinetics linked immunosorbent assay (KELA) programme was used to calculate slope values as the relationship between the rate of substrate conversion by enzyme and time (Jacobson, Downing & Lynch, 1982; Barlough, Jacobson, Downing, Marcella, Lynch & Scott, 1983; Böse, Jacobson, Gale, Waltisbuhl & Wright, 1990). Further, the KELA programme was used to calculate 'delta values' by subtraction of the KELA slope values for control antigen from those for *B. caballi* antigen. Data generated by the KELA programme provided a basis for maintaining quality control in the assays. The KELA programme included assessment of the: (1) linearity of the reaction rate (OD vs time) in each well of the microtitre plate to ascertain whether the enzyme-substrate-chromogen reactants were performing properly, (2) mean, S.D. and coefficient of variation for the slopes of sample replicates to determine within-run variation, (3) degree of correlation of observed slopes for standard sera compared with their expected values by linear regression analysis and (4) normalization of mean slopes for each sample to the expected values for controls thus allowing direct comparison of results on a day-to-day basis. All of these data were generated automatically at the end of each run by the KELA programme and presented in printed reports for easy evaluation. The threshold was determined arbitrarily under

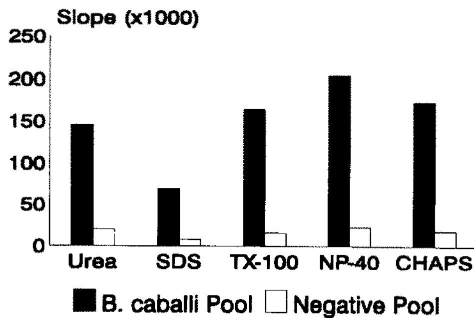

FIG. 1. Reactivity of different *Babesia caballi* antigen preparations in the ELISA. A preparation of 100% infected erythrocytes was extracted with urea, sodium dodecyl sulfate (SDS), Triton X-100 (TX-100), Nonidet P-40 (NP-40) or with 3-[(3/Cholamidoproyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), centrifuged and the supernatant used as an antigen in a heterogenous, non-competitive ELISA. Antigen coated wells were incubated with a high-titred *B. caballi* serum pool or a serum pool from horses not infected with *Babesia* spp. ELISA reactions are given as slope values of the curve describing the rate of substrate conversion by enzyme vs times.

consideration of the results of 71 sera from 15 horses experimentally infected with *B. caballi* and 106 horses not infected with *B. caballi* obtained in all 4 tests. For validation of the assay, sensitivity and specificity (Tyler & Cullor, 1989) were calculated.

RESULTS

Optimization of the ELISA protocol

Antigen preparation. When extracted with urea, Triton X-100, Nonidet P-40 or CHAPS, a high specific activity of the antigen was observed. In contrast the use of SDS led to a low reaction between antigen and the high-titred *B. caballi* serum pool (Fig. 1.). The best discrimination between positive and negative sera was obtained with the antigen extracted with CHAPS that was selected for further studies. Checkerboard titrations with *B. caballi* antigen, the control antigen and serum pools revealed suitable dilutions of 1/4000 to 1/8000 for the *B. caballi* antigen. For the control antigen the reaction with the high-titre *B. caballi* serum pool and the negative serum pool remained at an almost constant level for dilutions from 1/1000 to 1/64,000. Thus, we used the same working dilution of 1/4000 for both, the *B. caballi* antigen and the control antigen.

Selection of the microtitre plate. ELISAs were performed using 15 different microtitre plates. Considerable differences between the performance of the plates tested were observed and the selection of a suitable plate was crucial to obtain a good discrimination in the ELISA. Best results were obtained with the plates no. 26 97 87, Nunc GmbH and no. 76-181-04, Flow Laboratories GmbH, and the latter was chosen for the further studies.

Blocking of plates and diluents for sera. Several blocking reagents were investigated regarding their ability to reduce the non-specific binding of sera and thereby improve the discrimination. Results were compared with those obtained without a blocking step. No improvement in the discrimination could be achieved by introducing a blocking step with any of the 7 blocking reagents tried.

When different dilution buffers for sera and conjugate were tested, best results were obtained with PBS–T with 1% BSA.

Optimized ELISA protocol. B. caballi antigen and control antigen were extracted with CHAPS and used in a final dilution of 1/4000. The microtitre plate no. 76-181-04, Flow Laboratories, was used; a blocking step was omitted. Sera were diluted 1/100 and conjugate 1/3000 in PBS–T with 1% BSA. As the problem of non-specific binding of test sera could not be solved by introducing a blocking step the control antigen was used. Delta values were calculated by subtraction of slope values of control antigen from slope values for *B. caballi* antigen.

Assay evaluation under optimized conditions

Determination of the threshold. Under consideration of the results in all four tests the threshold was set at 13.669 for the delta values ($\times 10^3$). The average of all delta values ($\times 10^3$) of sera from horses not infected with *Babesia* spp. was 4.617 (range −6.19 to 13.41) with a S.D. of 3.748.

Validation of the assay. Calculations of the sensitivity of the tests were carried out either with the results of sera from day 1 or from day 14 after experimental infection with *B. caballi* (Table 1). In both cases the sensitivity of the ELISA was superior to all other tests; the CFT revealed the lowest sensitivity (Table 2). All sera from horses not infected did not react in the ELISA and in the Western blot leading to a specificity of 100% (Table 2). The specificity was not calculated for the IFAT and CFT as a negative result was required for the sera to be considered as originating from horses not infected.

Cross reactions. In the ELISA for *B. caballi* 15/76 (20%) sera from horses experimentally infected with *B. equi* reacted, the highest delta value ($\times 10^3$) being 31.125. None of these sera was positive for *B. caballi* in the Western blot. Of the sera cross reacting in the ELISA 14/15 had a titre of $< 1{:}160$ in the IFAT for *B.*

TABLE 1—VALIDATION OF THE ELISA AND WESTERN BLOT FOR *Babesia caballi* AND COMPARISON WITH IFAT AND CFT

	Sera from horses experimentally infected with *B. caballi**				Sera from horses not infected with *Babesia* spp.†	
	day 1($n=71$)		day 14 ($n=59$)			
	positive	negative	positive	negative	positive	negative
ELISA	64	7	58	1	0	106
Western blot	61	10	56	3	0	106
IFAT	61	10	57	2	0	106
CFT	17	54	17	42	0	106

*Sera from horses taken from day 1 or day 14 after experimental infection.
†Sera from horses originating from Germany, tested negative for *B. caballi* and *B. equi* by IFAT and CFT.

TABLE 2—CALCULATION OF THE DIAGNOSTIC SENSITIVITY AND SPECIFICITY OF THE ELISA, WESTERN BLOT, IFAT AND CFT FOR *Babesia caballi*

	Sensitivity*		Specificity†
	day 1	day 14	
ELISA	90.1%	98.3%	100%
Western blot	85.9%	94.9%	100%
IFAT	85.9%	96.6%	n.c.
CFT	23.9%	28.8%	n.c.

*Data are based on the results of sera from horses experimentally infected with *B. caballi*. Sera were tested from day 1 after infection ($n=71$) or from day 14 after infection ($n = 59$).
†Data are based on the results of sera from horses not infected with *Babesia* spp., i.e. horses originating from Germany and tested negative for *B. caballi* and *B. equi* by IFAT and CFT. Consequently, the specificity was not calculated (n.c.) for the IFAT and CFT.

TABLE 3—SEROLOGICAL DIAGNOSIS OF *B. caballi*. COMPARISON OF CFT AND IFAT RESULTS FOR 132 FIELD SERA WITH THOSE OBTAINED BY ELISA AND WESTERN BLOT

	ELISA		Western blot	
	positive	negative	positive	negative
35 sera CFT positive IFAT positive	35	0	35	0
6 sera CFT positive IFAT negative	5	1	5	1
57 sera CFT negative IFAT positive	55	2	49	8
34 sera CFT negative IFAT negative	17	17	10	24

equi. The IFAT revealed 3/76 cross reactions (4%); with the Western blot and the CFT no cross reactions were observed.

Comparison of ELISA, Western blot, IFAT and CFT as diagnostic tests for field sera. Tested were 132 field sera, i.e. 20 sera from Corsica, 35 sera from different European countries, and 77 sera from Brazil. Whereas ELISA, Western blot and IFAT showed similar results, the CFT revealed significantly fewer reactions; the agreement between CFT results and those of the other tests was not calculated. In 96/132 cases (73%) identical results were obtained with ELISA, Western blot and IFAT. The results of ELISA and Western blot agreed to 83% (109/132), the results of ELISA and IFAT agreed to 82% (108/132) and the results of Western blot and IFAT agreed to 83% (109/132). All 35/132 sera positive by CFT and IFAT were also positive by ELISA and Western blot (Table 3). 6/132 sera reacted in the CFT and not in the IFAT; 5 of these sera were positive in the ELISA and in the Western blot suggesting false negative results in IFAT, whereas the remaining serum tested probably false positive by CFT. 57/132 sera reacted not in the CFT but in the IFAT. Two of these sera were negative in the ELISA and Western blot suggesting false positive results of the IFAT. From 34 sera negative by CFT and IFAT, 17 sera, all from Brazilian horses, reacted in the ELISA. Seven of these sera tested positive and 10 negative with the Western blot; 8 of the sera negative in the Western blot originated from foals younger than 3 months born to sero positive mares.

DISCUSSION

One of the problems developing ELISAs for babesial infections is the contamination of antigen preparations with host proteins, namely from erythrocytes. For *B. bovis* a simple method for the enrichment of infected erythrocytes by selective lysis of non-infected erythrocytes is available (Mahoney, 1967) and preparations of 100% infected erythrocytes have been used for the development of sensitive and specific ELISAs (Waltisbuhl, Goodger, Wright, Commins & Mahoney, 1987; Böse *et al.*, 1990). For *B. caballi* only small amounts of 100% infected erythrocytes can be

obtained using Percoll gradients (Bhushan *et al.*, 1991). Attempts to develop an ELISA for *B. caballi* with crude antigen made from blood with low parasitaemias have led to limited improvements in the sero diagnosis of *B. caballi* (Weiland, 1986). In preliminary expts we used antigen with a percentage of parasitized erythrocytes (PPE) of 3–4% from *in vitro* cultures and found this preparation not suitable as antigen in the ELISA. Reactions with positive sera were low and a poor discrimination was obtained (data not shown). Only antigens extracted from a preparation of 100% infected erythrocytes were suitable as ELISA antigens.

A particular problem in preliminary studies was the high background staining caused by negative sera. Background staining could not be reduced by the introduction of a blocking step after antigen coating without a loss of sensitivity. However, when different methods of antigen extraction (Fig. 1), different microtitre plates and different dilution buffers were tried simultaneously, conditions could be defined which reduced the background staining and improved the discrimination significantly. The reason for the high background staining caused by equine sera is not known. For *B. bovis* there is evidence, that serum components other than IgG are involved (Böse *et al.*, 1990). These serum components, possibly IgM by nature, are recognised by conventional conjugates and thus cause the background staining. For *B. caballi* we found that IgG itself is probably causing most of the background staining in the ELISA. Background staining was not reduced when an IgG Fc-specific conjugate was used, but was almost completely absent after equine sera were passed over a protein G affinity column (data not shown). It appears that immunoglobulins from horse sera exhibit a high tendency to adhere to the surface of polystyrene microtitre plates. Thus, to date the only practical solution for the problem is the use of an appropriate control antigen.

Apart from the high non-specific binding of horse sera a strong reaction of some negative sera with *B. caballi* antigens or erythrocyte antigens extracted from the preparation of 100% infected erythrocytes was observed. We did not attempt to solve this problem by adsorption with normal erythrocytes as described for *B. bovis* (Waltisbuhl *et al.*, 1987) as this would have introduced another time-consuming step into the ELISA protocol. With the use of the control antigen the reaction of negative sera with the antigen preparation and the background staining were not eliminated, but did no longer lead to false positive results after calculation of delta values.

Defined sera were used to validate the ELISA and Western blot (Table 1). The ELISA proved to be the test with the highest sensitivity (Table 2). Western blot and IFAT provided comparable results, while the CFT must be regarded as obsolete due to a low sensitivity which does not meet the requirements for export testing or epidemiological studies. The consideration of sera taken from day 14 of the infection for the calculation of the sensitivity of a diagnostic test seems to be sufficient for the requirements of the export testing as no serological test is capable to detect fresh infections.

The main disadvantage of the ELISA is the high percentage of cross reactions with sera from horses infected with *B. equi*. Thus a species-specific diagnosis is not always possible by ELISA, i.e. positive reactions up to delta values ($\times 10^3$) of approx. 32 can be due to a *B. equi* infection. For export testing the cross reactions are of little significance as horses infected with *B. equi* are excluded from export as well.

The disadvantage of cross reactions in the ELISA can be overcome by testing of ELISA-positive sera by Western blot. Sera were regarded positive, when a clearly visible reaction with the 48 and 50 kDa antigens was present and one or more of the 70, 112, and 141 kDa antigens was also recognized. A number of *B. caballi* sera, however, particularly shortly after infection, recognized only the 50 and 48 kDa antigens, albeit strongly. Thus strong reactions with the 50 and 48 kDa antigens were also regarded positive. With these criteria applied no false positive reactions were obtained with *B. equi* sera. Most *B. equi* sera did not react with any of the 5 diagnostic antigens of *B. caballi*. Some sera showed a faint reaction with the 50 and 48 kDa antigens which was clearly distinguishable from the strong reactions of *B. caballi* sera. Thus, the Western blot can serve as a confirmative test which provides a species-specific diagnosis.

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