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RESEARCH NOTE

IDENTIFICATION OF DIAGNOSTIC ANTIGENS FOR SOUTH AMERICAN *BABESIA CABALLI* INFECTIONS

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Abstract—BÖSE R., PEYMANN B. and PFEIFER BARBOSA I. 1994. Identification of diagnostic antigens for South American *Babesia caballi* infections. *International Journal for Parasitology* **24**: 255–258. Sera from 60 horses held in breeding herd in Brazil were examined monthly by ELISA, immunofluorescence antibody test (IFAT) and Western blot. All foals had maternal antibodies detectable by ELISA and IFAT, and sero-conversion took place between the 2nd and 5th month of age. The 48 and 50 kDa antigens were recognized first in the course of infection. Of 79 sera taken after sero-conversion 78 reacted with the 48 kDa antigen, 76 with the 50 kDa, 50 with the 70 kDa, 54 with the 112 kDa, 72 with the 141 kDa antigen. In general, sera from horses older than 1 year reacted with all 5 diagnostic antigen bands, but sera from horses older than 3 years only weakly. The antigens of 48 and 50 kDa appear to be conserved among all strains of *B. caballi* examined so far and are consistently recognized by all infected horses. They are the target antigens for a serological test based on anitigens produced by recombinant DNA techniques.

INDEX KEY WORDS: *Babesia caballi*; Western blot; ELISA; IFAT.

BABESIA CABALLI and *B. equi* are the causative agents of equine babesioses. They are endemic in most tropical and subtropical areas of the world (Friedhoff, 1982; Friedhoff, Tenter & Müller, 1990). The transmitting tick vectors are distributed almost worldwide. In the Old World *Dermacentor* and *Hyalomma* spp. are vectors for both *Babesia* spp.; *B. equi* is transmitted also by *Rhipicephalus* spp. (see Friedhoff, 1988). On the American continent the epidemiological situation is largely unknown. For *B. equi* which is highly endemic in South America no tick vector was found (Dennig F., unpublished thesis, Hannover School of Veterinary Medicine, 1988; Pfeifer Barbosa I., unpublished thesis, Hannover School of Veterinary Medicine, 1993) demonstrating differences in the biology of Old and New World strains of this parasite. This observation was supported by the analysis of *B. equi* antigens by two-dimensional electrophoresis. Most antigens present in European strains of *B. equi* were not recognized by South American sera (Böse & Hentrich, in press). For *B. caballi*, *Anocentor nitens* was demonstrated as a vector on the American continent (Roby & Anthony, 1963; Friedhoff, 1988). By Western blot diagnostics antigens with a relative molecular mass of 141, 112, 70, 50 and 48 kDa were identified with sera from different European countries (Böse & Daemen, 1992). However, no information is available on the conservation of these antigens among other than European strains of *B. caballi*. This study was undertaken to answer the question whether *B. caballi* antigens diagnostic for Old and New World strains can be identified.

Antigen for the ELISA, Western blot and immunofluorescence antibody test (IFAT) was obtained from microaerophilous stationary phase cultures initiated from the blood of a horse infected with the USDA strain (Tenter & Friedhoff, 1986) as described previously (Böse & Daemen, 1992). Parasitized erythrocytes were enriched to about 100% infected cells with density gradient centrifugation on a two-step Percoll^{R} gradient (Bhushan, Müller & Friedhoff, 1991). ELISA antigen was prepared by extraction with the detergent 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) (Boehringer, Mannheim, Germany) in a final concn of 1%. Antigen and the detergent were mixed and incubated for 20 min

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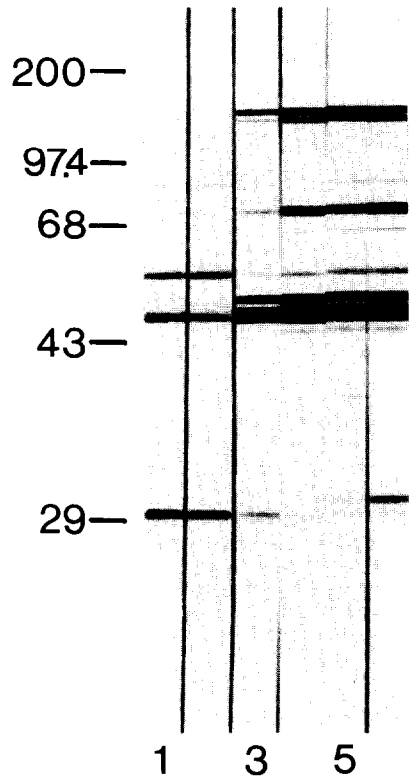

FIG. 1. Demonstration of maternal antibodies and antibodies after field-infection with *Babesia caballi* on Western blots. Maternal antibodies and sero-conversion were demonstrated in 31 foals by ELISA, IFAT and Western blot. A representative Western blot pattern obtained with sera from a foal which sero-converted in the third month of life is shown. Strips were incubated with a serum taken in the 1st month of life (lane 1), the 2nd (lane 2), the 3rd (lane 3), the 4th (lane 4), the 5th (lane 5) and 6th month of life (lane 6). Bars on the left indicate position of molecular weight markers (figures in kDa).

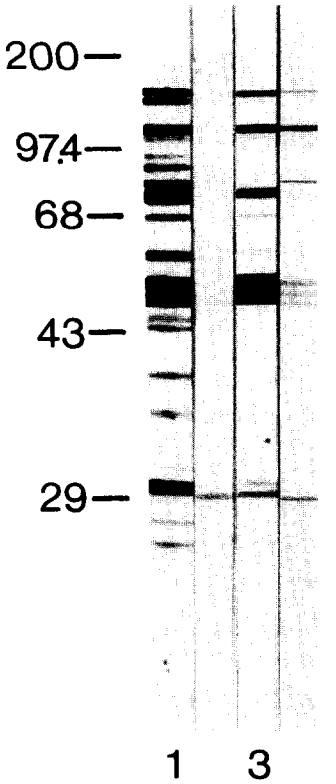

FIG. 2. Reactivity of equine sera with *Babesia caballi* antigens on Western blots. From 246 sera from 60 Brazilian horses tested by Western blot representative patterns are shown. Strips shown were reacted with a high-titred *B. caballi* serum pool (USDA-strain) (lane 1) and a pool of sera from horses not infected with *Babesia* spp. (lane 2) as controls, a serum from a horse field-infected in Brazil showing strong reactions with all 5 diagnostic antigen bands (lane 3) and a serum of a four year old horse field-infected in Brazil showing weak reactions with the diagnostic antigen bands (lane 4). Bars on the left indicate position of molecular weight markers (figures in kDa).

at 37°C and centrifuged (22,000 *g*, 10 min, 4°C). The supernatant was diluted 1/1000 in 0.1 M-carbonate-bicarbonate buffer, pH 9.6 resulting in a working dilution of 1/4000. A control antigen of non-infected erythrocytes from the same donor horse used for the cultures was prepared in an identical manner. The ELISA was performed essentially as described previously (Böse, Jacobson, Gale, Waltisbuhl & Wright, 1990) except that a blocking step was omitted. Sera were diluted 1/100 and conjugate (rabbit anti-horse IgG (H+L) HRP, Dianova GmbH, Hamburg, Germany) 1/3000 in PBS (5 mM-phosphate buffer 147 mM-NaCl, pH 7.2) with 0.05% Tween 20^{R} and 1% BSA (Sigma, Deisenhofen, Germany). Plates were incubated at 37°C for 1 h for the antigen and serum incubation steps and for 30 min for the conjugate incubation. Approximately 2, 4 and 6 min after substrate addition (1 mg ml^{-1} of 5-aminosalicylic acid (Ellens & Gielkens, 1980) in 0.1 M-phosphate buffer, pH 6.0 containing 0.01 mM-EDTA and 6 mM-H_2O_2) the absorbance values were measured with an ELISA reader (Titertek Multiskan plus MK II, Flow Laboratories, Meckenheim, Germany) interfaced with an IBM compatible personal computer. The kinetics-based enzyme-linked immunosorbent assay (KELA) programme was used to calculate the slope values as the relationship between the rate of substrate conversion by enzyme and time (Jacobson, Downing

TABLE 1.—KINETICS OF THE ANTIBODY RESPONSE OF HORSES IN THE COURSE OF A *B. caballi* INFECTION DEMONSTRATED BY WESTERN BLOTTING

Age (months)	Horses tested* Number	Reactive‡	*M*, of antigen bands recognized (kDa)† 48	50	70	112	141
<1	11	5	2	1	3	3	2
1	15	9	6	4	2	5	2
2	15	14	14	11	6	2	9
3	18	17	17	13	9	2	12
4	19	16	16	13	14	4	12
5	14	14	14	13	11	6	11
6	13	12	12	11	7	5	10
7	11	11	11	11	8	6	10
8	7	7	7	7	6	4	7
9	8	8	8	8	3	7	8
10	9	9	9	9	5	9	9
11	9	9	9	9	6	9	9
12	8	8	8	8	4	8	8
Total	157	139	133	118	84	70	109

*Sera from foals born to *Babesia caballi* sero-positive mares were tested. Maternal antibodies were detected in all foals by ELISA and IFAT. Sero-conversion was demonstrated between the 2nd and 5th month by ELISA or between the 3rd and 7th month of age if IFAT.

†Given is the number of sera recognizing the particular antigen band.

‡Number of sera recognizing one or more *B. caballi* antigen bands on Western blots.

& Lynch, 1982; Barlough, Jacobson, Downing, Marcella, Lynch & Scott, 1983; Böse *et al.*, 1990). Further the KELA programme was used to calculate "delta values" by subtraction of the slope values for control antigen from those for *B. caballi* antigen. The IFAT was carried out according to Tenter and Friedhoff (1986). The Western blot was performed as described previously (Böse & Daemen, 1992), except that TBS with 0.5% Tween 20 was used instead of TBS with 1% gelatin. Sera from horses held in a breeding herd in Brazil were examined monthly by ELISA and IFAT to demonstrate maternal antibodies and sero-conversion for *B. caballi*. These semi-defined sera were also tested by Western blot to identify diagnostic antigens. A total of 246 sera from 60 horses were tested, i.e. 157 sera from 31 foals examined monthly for antibodies against *B. caballi* by ELISA, IFAT and Western blot and 89 sera from 29 horses older than 12 months and examined for antibodies against *B. caballi* by ELISA, IFAT and Western blot. All horses, 5 months or older were positive for *B. equi* by IFAT with a titre of 1/40 or higher. The following standard sera were used for the ELISA and Western blot: (1) a high-titred *B. caballi* serum pool; (2) a low-titred *B. caballi* serum pool; (3) a pool of sera from horses not infected with *Babesia* spp. Sera considered positive for *B. caballi* by ELISA reacted with a delta value ($\times 10^3$) larger than 13.669. Sera considered positive for *B. caballi* by IFAT reacted with a titre of 1/80 or higher.

Sera from foals taken in the first month after birth contained high levels of maternal antibodies against *B. caballi* as indicated by positive ELISA and IFAT reactions. High titres in the first month of life were followed by an interval with decreased reactions. None of the *B. caballi* antigens was detected consistently by maternal antibodies on Western blots. If detected, maternal antibodies were reactive with the 48 kDa antigen (Fig. 1 lanes 1 and 2) or with one or two of the other diagnostic antigens (Table 1). Sero-conversion was demonstrated by both ELISA and Western blot (Fig. 1, Table 1) in all foals between the 2nd and 5th month of age. By IFAT sero-conversion was detectable between the 3rd and 7th month. Positive Western blot reactions showed the typical *B. caballi* banding pattern as described previously (Böse & Daemen, 1992). Major babesial antigens recognized by Brazilian sera were the same as those recognized by European sera, i.e. antigens of 48 and 50 kDa, 70, 112 and 141 kDa (Fig. 2, lane 3). In the course of infection the antigens of 48 and 50 kDa appeared first and remained throughout the examination period (Table 1). The 48 kDa antigen was consistently recognized already from the 2nd month, the 50 kDa antigen from the 5th month of age (Table 1). From the 79 sera taken from the 5th month of age, when all foals were infected, 78 reacted with the 48 kDa antigen, 76 with the 50 kDa, 50 with the 70 kDa, 54 with the 112 kDa, and 72 with 141 kDa antigen. The kinetics of the antibody response of all sera from foals tested is given in Table 1. All sera from the 29 horses older than 1 year reacted in the ELISA and the sera from 14 horses were also positive by IFAT. By Western blot all sera from 7

horses between 1 and 3 years of age showed strong reactions with the 48, 50 and 141 kDa antigen bands and usually also with the 70 and 112 kDa antigens (data not illustrated). Sera from 7/22 horses older than 3 years reacted strongly, whereas sera from 15/22 horses showed a weak albeit specific banding pattern (Fig. 2, lane 4).

We demonstrated, that at least the 5 diagnostic antigens described previously (Böse & Daemen, 1992) are present not only in European, but also in South American strains of *B. caballi*. This indicates that some antigens are probably highly conserved among all strains of the parasite. From the conserved antigens known, the 48 and 50 kDa antigens are particularly useful as these antigens are recognized early and continuously throughout the infection. Unfortunately maternal antibodies were not regularly detected by any of the *B. caballi* antigens on Western blots, although both ELISA and IFAT were positive. Thus a test based on one of these *B. caballi* antigens would probably not detect maternal antibodies reliably. Maternal antibodies however, are of little significance for the identification of carrier horses (export testing). It is interesting to note that sero-conversion against the 48 kDa antigen was demonstrated early in the infection before most other antigens were detected (Table 1). This indicates that this antigen is expressed in the early phase of the infection and is readily recognized by the immune system. Other antigens particulary the 112 and 141 kDa were usually not recognized until several months after infection. By the age of 1 year all horses strongly recognized most or all diagnostic antigens. With most sera from older horses however, weak reactions were observed by Western blotting. As most antigens including the 48, 50, and 141 kDa antigens are still recognized, a test employing these antigens should be able to detect older carrier horses as well.

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