SEROLOGIC DIAGNOSIS OF BOVINE BESNOITIOSIS BY ELISA AND IMMUNOFLUORESCENCE TESTS*

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ABSTRACT

JANITSCHKE, K., DE VOS, A. J. & BIGALKE, R. D., 1984. Serodiagnosis of bovine besnoitiosis by ELISA and immunofluorescence tests, Onderstepoort Journal of Veterinary Research, 51, 239–243 (1984) Sera from non-infected cattle and cattle infected with Anaplasm a, Babesia, Theileria and Sarcocystis were tested for antibodies to Besnoitia in ELISA and immunofluorescence tests (IFT) with Besnoitia besnoiti of blue wildebeest origin as antigen. Only 2 out of 86 sera gave false positive reactions in ELISA and none in the IFT, indicating a high specificity for the tests.

Three-hundred-and-three bovine sera from 3 farms in an area endemic for besnoitiosis were similarly tested and the results were correlated with clinical findings based on visual inspection for typical symptoms and the presence of cysts in the scleral conjunctiva. Most of the positive tests were observed in cattle older than 1 year. Of the positive in the ELISA and 81.74% in the IFT. However, 45.74% (ELISA) and 49.47% (IFT) of the clinically negative cattle were clinically positive, indicating a high incidence of clinically inapparent infection. These results indicate a relatively low sensitivity for these serological tests.

An unexpected finding was that the ELISA remained negative for at least 60 days after experimental infection of the cattle, the maximum period for which tests were done, whereas the IFT became positive. No antibodies against B. besnoiti could be found in human sera.

Besnoitia jellisoni antigen gave positive results with B. besnoiti antibodies in ELISA, but not in the IFT.

INTRODUCTION

The clinical diagnosis of bovine besnoitiosis is based on the presence of anasarca, scleroderma, alopecia and seborrhea in affected animals. In addition, Pols (1960) and Bigalke & Naudé (1962) reported the diagnostic value of macroscopically visible cysts in the scleral conjunctiva. Bigalke & Naudé (1962) and Bigalke (1968) concluded that many more cases of bovine besnoitiosis may exist than could be detected by clinical and histological examinations. The need for a more reliable diagnostic method was therefore apparent.

The first serological test for bovine besnoitiosis was performed by Frenkel (1953). He applied the Sabin-Feldman dye test (SFT), using Besnoitia jellisoni as antigen, but failed to obtain positive reactions. More recently, Kravos & Omovar (1975) used Besnoitia besnoiti parasites as antigen for haemagglutination, immunodiffusion and complement fixation tests. These authors obtained positive results with sera from cattle, but observed cross-reactions with Toxoplasma. A high prevalence of Besnoitia infections in cattle was recorded in Israel by immunofluorescence tests (IFT) (Frank, Klinger & Pipano, 1970; Neuman, 1972). To improve the serological diagnosis of besnoitiosis, Kagawa (1977) compared different techniques, especially the enzyme-linked immunosorbent assay (ELISA) on rabbits and mice, infected with B. besnoiti and B. jellisoni.

The first aim of the present study was to compare the ELISA and the IFT as tools for diagnostic and epidemiological studies on bovine besnoitiosis. Secondly, we followed the rise in the IFT- and ELISA-titres in experimentally infected calves. Thirdly, human sera were examined by the IFT for possible cross-reactions between Besnoitia and Toxoplasma. Finally, B. jellisoni was tested as antigen for the IFT and ELISA.

MATERIALS AND METHODS

Sera

Three farms with cattle known to be infected with besnoitiosis were selected as the source of test sera. A total of 303 animals were bled for serum on these farms and the animals were also examined macroscopically for the presence of cysts in the scleral conjunctiva and for skin lesions. A further 86 animals, born and kept on concrete under tick-free conditions at the Veterinary Research Institute Onderstepoort and assumed not to be infected with any systemic protozoan parasites, while the remaining 36 were previously infected artificially with Babesia spp., Theileria spp., Anaplasm a spp. or Sarcocystis spp., as outlined in Table I. All the sera were inactivated for 30 min at 56°C and tested by the ELISA test and the IFT after storing at −20°C, as outlined below.

Serological tests

ELISA: The ELISA test was performed using the technique described by Janitschke, Werner & Bode (1982). For the production of antigen, B. besnoiti (blue wildebeest strain) was cultured in vitro in monkey kidney cells as described by Bigalke (1968). Parasites obtained from the infected cultures were washed 3 times by centrifugation for 15 min at 500 × g in carbonate buffer (pH 9.6), frozen and thawed 4 times and then sonicated for 1 min at 50 Hz. After further centrifugation (500 × g for 15 min) of the suspension the supernatant was collected and centrifuged further at 100 000 × g for 1 h at 4°C. The final supernatant was divided into 1 ml aliquots and stored at −20°C.

Before use, the antigen was thawed and diluted 1:800, the optimum dilution as determined by the method of Janitschke et al. (1982). Rabbit anti-bovine IgG (H + L chain) labelled with peroxidase* was used as conjugate at a working dilution of 1:1000. Orthophenylendiamine** and peroxide were used as substrates. The optimum serum dilution was 1:320 and, based on a standard curve, titres of 1:160 and higher were considered to be positive.

IFT: The IFT was conducted, using standard procedures with culture-derived parasites of the blue wildebeest strain as antigen. For antigen production, the supernatant of an infected culture was collected and washed 3 times in PBS (pH 7.4) by centrifugation at 500 × g for 15 min. The concentration of parasites was determined and adjusted with the addition of PBS to approximately 100 parasites/25 μL. The parasite suspension was dispensed in 25 μL volumes on printed microscope slides, air dried and stored at −20°C.

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Before use the antigen slides were thawed, air-dried and fixed in methanol for 5 min. Rabbit anti-bovine globulin conjugated with FITC* was used as conjugate and the test read with a Leitz orthoplan microscope fitted with a ×50 water immersion lens (total magnification ×500), H2 filter and 50 W mercury vapour lamp. Bright fluorescence of more than 50% of the parasites at a minimum serum dilution of 1:20 was considered to be a positive reaction. A strong non-specific polar staining of the parasites, reminiscent of Toxoplasma (Renterghem & Nimmen, 1976), was observed in most sera without Besnoitia antibodies at dilutions of less than 1:20.

**Experimental infections**

Two 3-month-old calves, born and kept under isolated conditions at this institute, were used to monitor the serological response after artificial infection. Prior to infection, both calves and their mothers were free from antibodies to B. besnoiti, as determined by the IFT.

Calf 1 was inoculated intravenously with ± 3 × 10⁶ culture-derived B. besnoiti parasites of a bovine strain, while Calf 2 was inoculated subcutaneously with 1 dose (2 ml) of elephant skin disease vaccine containing ± 1 × 10⁶ culture-derived parasites of the blue wildebeest strain. After infection, serum samples were collected twice a week from each calf and tested by the ELISA and IFT.

**Serological cross-reactions between B besnoiti and T. gondii in humans**

A total of 164 human serum samples were obtained from the rural population in the same region as the bovine sera were collected. These people are known to eat beef and game meat, usually in a cooked form, but the possibility of ingestion of live Besnoitia parasites could not be excluded. The sera were tested for B. besnoiti by the IFT, using sheep anti-human immunoglobulin conjugated with FITC, and for T. gondii by the SFT (Anon., 1980). B. besnoiti (blue wildebeest strain) and T. gondii (BK strain) were respectively used as antigens in the 2 tests.

**B. jellisoni**

Trophozoites of this parasite were collected from artificially infected mice and the antigens for the IFT and ELISA were prepared as for B. besnoiti.

**RESULTS**

**Specificity of ELISA and IFT**

The results of serological tests done on 50 cattle, assumed to be free of B. besnoiti and known not to have been exposed to other systemic protozoal or rickettsial infections, as well as on 36 animals, known to be infected with Anaplasma, Babesia, Theileria or Sarcocystis but assumed to be free of B. besnoiti, are recorded in Table 1. Only sera from 1 uninfected animal and 1 animal infected with A. marginale gave positive reactions by ELISA, while all the sera were negative by the IFT.

### Table 2 Results of clinical and serological examination of 303 cattle in an area endemic for besnoitiosis

<table>
<thead>
<tr>
<th>Farm</th>
<th>No. of cattle</th>
<th>Group 1: Positive for scleral cysts</th>
<th>Group 2: Negative for scleral cysts</th>
<th>Serological tests (total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ELISA</td>
<td>IFT</td>
<td>ELISA</td>
</tr>
<tr>
<td>1</td>
<td>51</td>
<td>29 56.9%</td>
<td>27 93.1%</td>
<td>2 6.9%</td>
</tr>
<tr>
<td>2</td>
<td>92</td>
<td>33 35.9%</td>
<td>25 75.8%</td>
<td>8 24.2%</td>
</tr>
<tr>
<td>3</td>
<td>160</td>
<td>53 33.1%</td>
<td>27 50.9%</td>
<td>26 49.1%</td>
</tr>
<tr>
<td>Total</td>
<td>303</td>
<td>115 38.0%</td>
<td>79 68.2%</td>
<td>36 31.3%</td>
</tr>
</tbody>
</table>

*Wellcome Reagents Ltd, Beckenham, England*
TABLE 3 Agreement between the ELISA and IFT

<table>
<thead>
<tr>
<th>Total No. of sera*</th>
<th>Total No. (1 or both tests)</th>
<th>ELISA + IFT+</th>
<th>ELISA + IFT-</th>
<th>ELISA - IFT+</th>
</tr>
</thead>
<tbody>
<tr>
<td>303</td>
<td>219</td>
<td>133 (60.7%)</td>
<td>32 (14.6%)</td>
<td>54 (24.7%)</td>
</tr>
</tbody>
</table>

* Sera collected from cattle in an area endemic for besnoitiosis.

Effect of age on antibody titres

Antibody titres of 303 naturally exposed animals as determined by ELISA and the IFT are recorded in Table 5. Only 21 of the animals tested were less than 12 months old, and of these, 3 were positive by ELISA and 4 by the IFT. Measured by ELISA, the number of positive reactions increased from 42.2% in animals under 24 months of age to 61.5% in the older animals. The corresponding figures for the IFT were 37.7% and 70.2%.

Antibody response in artificially infected calves

The IFf antibody response of 2 calves inoculated with the bovine and blue wildebeest strains of B. besnoiti respectively is represented in Fig. 1. Antibodies were first detected 10 days after intravenous inoculation of the bovine strain and reached a maximum titre of 1:2560 30 days later. In the calf inoculated subcutaneously with the wildebeest strain, antibodies were detected after 22 days and reached a peak titre of 1:640 45 days after inoculation.

FIG. 1 IFf titre of 2 experimentally infected calves. Calf 1 received ± 3 X 10^6 trophozoites of B. besnoiti (bovine strain) intravenously and Calf 2 received ± 1 X 10^6 trophozoites (blue wildebeest strain) subcutaneously.

TABLE 4 Correlation between clinical severity and test results in 303 cattle in an area endemic for besnoitiosis

<table>
<thead>
<tr>
<th>Clinical severity*</th>
<th>ELISA titre</th>
<th>IFT titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;1:160</td>
<td>1:160</td>
</tr>
<tr>
<td></td>
<td>&lt;1:20</td>
<td>1:20</td>
</tr>
<tr>
<td>-</td>
<td>102</td>
<td>29</td>
</tr>
<tr>
<td>+</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>++</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>+++</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>++++</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>138</td>
<td>41</td>
</tr>
</tbody>
</table>

* Based on number of scleral cysts:
- = no cysts
+ = less than 5 cysts in 1 or both eyes
++ = 5–20 cysts per eye
+++ = >20 cysts per eye
++++ = many cysts and scleroderma

TABLE 5 Distribution of ELISA and IFT titres according to age groups of cattle

<table>
<thead>
<tr>
<th>Age of cattle</th>
<th>No. in age group</th>
<th>ELISA titre</th>
<th>IFT titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;1:160</td>
<td>1:160</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;1:20</td>
<td>1:20</td>
</tr>
<tr>
<td>1 month</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>1–6 months</td>
<td>9</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>6–12 months</td>
<td>7</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>12–24 months</td>
<td>64</td>
<td>37</td>
<td>7</td>
</tr>
<tr>
<td>24 months</td>
<td>218</td>
<td>84</td>
<td>34</td>
</tr>
<tr>
<td>Total</td>
<td>303</td>
<td>138</td>
<td>41</td>
</tr>
</tbody>
</table>

TABLE 6 Serological tests on 176 human sera for Besnoitia and Toxoplasma antibodies

<table>
<thead>
<tr>
<th>IFT Besnoitia*</th>
<th>Negative</th>
<th>Positive</th>
<th>SFT Toxoplasma**</th>
<th>Negative</th>
<th>1:4</th>
<th>1:16</th>
<th>1:64</th>
<th>1:256</th>
<th>1:1000</th>
<th>≥1:4000</th>
<th>Total positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>0</td>
<td>139</td>
<td>3</td>
<td>11</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* B. besnoiti (blue wildebeest strain)
** T. gondii (BK strain)
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B. jellisoni antigen

Eight sera from cattle which were positive in the IFT with B. besnoiti as antigen reacted negatively in the IFT and with B. jellisoni as antigen. Nine out of 10 cattle sera, positive in the IFT and ELISA (B. besnoiti antigen), reacted positively in ELISA with B. jellisoni antigen.

Discussion

The reliability of serological tests can be evaluated in terms of their specificity and sensitivity. For specificity studies, sera originating from animals with known pure infections should be used. Because of the conditions under which certain cattle at this institute were kept, we were able to obtain sera from animals the infection history of which was known. Only 2 false positive out of 86 ELISA reactions were observed. The reason for this is not known, since all other sera tested were completely negative. In accordance with Neuman (1972), we could not find cross-reactions in the IFT between B. besnoiti antigen and Anaplasma, Babesia, Theileria and also Sarcocystis antibodies. The specificity of both techniques is therefore very high.

Extensive tests for sensitivity could only be done on cattle sera originating from the field. Therefore, scleral cysts and scleroderma were used as parameters of infection. It is known from the experiments of Bigalke (1968) that half of the animals infected artificially will develop scleral cysts. It is therefore not surprising that we could demonstrate Besnoitia antibodies not only in clinically positive cases (ELISA 68.7%; IFT 81.7%) but also in a fairly high percentage (45.7 and 49.5, respectively) of clinically negative cases.

The data in Table 2 show big differences between the ELISA and the IFT results obtained on the 3 farms. The first 2 farms belonged to 1 owner and the herds had been established many years before, only a few animals having been bought in recently. The owner of the 3rd farm started cattle farming 2 years before and the animals had been bought in from different parts of Transvaal. One can assume therefore that both Besnoitia-infected and non-infected cattle came into a well-known endemic area and that they got newly acquired infections had occurred. In the artificial infection studies, we could not detect antibodies by ELISA up to 2 months after infection. Why this is so is not clear. There is a slight possibility that it takes more than 2 months after infection for this test to become positive. This would offer an explanation for the many false negative ELISA reactions on Farm 3. Kagwa (1977) found the ELISA not very reliable in acute and inefficient in latent infections in rabbits and mice.

The problem of false negative reactions in ELISA and also in the IFT needs further research, especially on experimentally infected animals.

This investigation indicates that serological tests can be of use for the detection of infection with B. besnoiti in mass surveys. In the case of the individual animal, however, these results suggest that the diagnosis should primarily be based on eye inspection. In supposed cases where scleral cysts are absent, ELISA and/or the IFT should be conducted because of their ability to detect clinically negative cases and their high specificity. The examination of eyes for cysts under field conditions needs experience. It is also laborious and has a reliability of probably not more than about 50%, as was indicated previously.

A serious disadvantage of these tests is their low sensitivity for reasons not yet clearly understood. We would suggest their application for field surveys with the proviso that the data be interpreted very carefully. Despite its lower specificity, the ELISA has some useful advantages over the IFT for mass surveys. This test requires relatively small quantities of reagents, hundreds of sera can be tested in 1 day and the test can be automated. Further research is justified to iron out the shortcomings.

We could detect many clinically negative but serologically positive cattle and, on the other hand, some with many cysts which were serologically negative. Skin lesions are visible for many years in affected animals and the possibility exists that titres could have dropped. The serological tests support the observation (Bigalke, 1968) that animals rarely get infected before they are weaned. The only serologically positive animal at the age of 1 month could be explained by passive transfer of antibodies from the dam.

In the 2 experimentally infected calves, the IFT-antibody titres started rising 10 days after intravenous and 22 days after subcutaneous injection respectively. This difference can probably be attributed to differences in infection routes, doses and strains. Maximum IFT titres of 1:2560 and 1:640 were reached respectively after 40 and 45 days. It therefore seems as if naturally infected cattle cannot be distinguished from vaccinated ones by the IFT. The constant negativity of ELISA for at least 60 days is surprising. Unfortunately, we were not able to test the calves for a longer period.

Because of the close relationship between Besnoitia and Toxoplasma and possible influences on the serodiagnostic results for toxoplasmosis, we examined human sera from an area where bovine besnoitiosis exists for Besnoitia and Toxoplasma antibodies. There was no evidence of either Besnoitia antibodies or a cross-reaction between Besnoitia antigen and Toxoplasma antibodies, which is in accordance with the observations of Goldman, Carver & Sulzer (1957) and Tadros & Laarmann (1976). However, Lunde & Jacobs (1965) and Suggs, Walls & Kagan (1968) described some cross-reactions in immunodiffusion, complement fixation, hemagglutination and immunofluorescence tests. Our negative results suggest that man is not a final host for B. besnoiti. Furthermore, we could demonstrate experimentally that the jackal (Canis mesomelas, Schreber, 1975), hyaena (Crocuta crocuta, Erxleben, 1777) and fox (Vulpes chama, A. Smith, 1833) are not final hosts for this parasite from cattle (K. Janitschke, unpublished data, 1982).

The results obtained by ELISA with B. jellisoni as antigen are more promising than those of Kagwa (1977). It seems that only in ELISA could B. jellisoni find some practical application for the serological diagnosis of B. besnoiti infections.

Our serological results indicate a high prevalence (72,30%) of bovine besnoitiosis in some areas in Transvaal and are comparable with the 76% prevalence found in Israel by Neuman et al. (1972) with the IFT.

Serological techniques can improve the diagnosis of bovine besnoitiosis and can be used for sero-epidemiological studies which could help to discover the life cycle of Besnoitia besnoiti.

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REFERENCES


