Onderstepoort J. vet. Res., 51, 239–243 (1984)

SERODIAGNOSIS OF BOVINE BESNOITIOSIS BY ELISA AND IMMUNOFLUORESCENCE TESTS*

K. JANITSCHKE[1], A. J. DE VOS[2] and R. D. BIGALKE[3]

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 *Anaplasma*, *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 cases with scleral cysts, 68,7 % were 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, Krasov & Omarov (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, Kaggwa (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 *B. besnoiti*, were bled as negative controls. Of the 86 sera, 50 originated from animals not known to be infected with any systemic protozoan parasites, while the remaining 36 were previously infected artificially with *Babesia* spp., *Theileria* spp., *Anaplasma* spp. or *Sarcocystis* spp., as outlined in Table 1. 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°. 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. Orthophenylenediamine** 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.

[1] Present address: Robert Koch-Institut, Berlin 65, Nordufer 20, D-1000 Berlin 65, Federal Republic of Germany

[2] Present address: Tick Fever Research Centre, Grindle Road, Wacol, Queensland 4076, Australia

[3] Veterinary Research Institute, P.O. Onderstepoort, Republic of South Africa 0110

* This study was supported in part by funds obtained from the Kleine Trust

Received 26 June 1984—Editor

* Nordic Immunological Laboratories, Tilburg, Netherlands

** Sigma Chemie GMBH, Taufkirchen, West Germany

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), H_2 filter and 50 W mercuray 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 $\pm 3 \times 10^6$ 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 $\pm 1 \times 10^6$ 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 1 ELISA and IFT results with *B. besnoiti* antigen on 86 sera from cattle not infected with this parasite

Infection status of cattle	No. of cattle	No. positive on ELISA	No. positive on IFT
Not infected	50	1	0
Anaplasma centrale	8	0	0
A. marginale	2	1	0
Babesia bigemina + *B. bovis*	10	0	0
Theileria parva	1	0	0
T. lawrencei	2	0	0
T. mutans	4	0	0
T. taurotragi	3	0	0
Sarcocystis bovicanis	3	0	0
S. bovihominis	3	0	0
TOTAL	86	2	0

Sensitivity of ELISA and IFT

The results of tests done on 303 cattle from 3 known besnoitiosis-infected farms are summarized in Table 2. The animals were divided into 2 groups, Group 1 consisting of 115 animals (38,0 %) showing detectable cysts of *Besnoitia* in the scleral conjunctiva and Group 2 comprising the remaining 188 animals with no detectable scleral cysts.

Table 2 illustrates the great variation in the results obtained from the 3 farms. Of the animals in Group 1, 68,2 % gave positive titres by ELISA compared with 81,7 % by the IFT, while, in Group 2, 45,7 % of the sera gave positive reactions by ELISA and 49,5 % by the IFT.

Agreement between ELISA and IFT

The number of positive cases detected serologically in the 303 animals on known infected farms are tabulated in Table 3. Substantially more positive cases were detected when both techniques were used (219) than when ELISA alone (165) or the IFT alone (187) was used.

Clinical severity and test results

The clinical severity of besnoitiosis in 303 naturally exposed animals is tabulated in Table 4 against the results of the ELISA and IFT. The severity of the infections was judged by the number of cysts in the scleral conjunctiva. Some cattle which were negative on scleral examination had detectable antibody levels at all serum dilutions in both the ELISA and the IFT. Conversely, some heavily infected cattle were serologically negative.

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

Farm	No. of cattle	Group 1: Positive for scleral cysts					Group 2: Negative for scleral cysts					Serological tests (total)			
		Total	ELISA		IFT		Total	ELISA		IFT		ELISA		IFT	
			Pos.	Neg.	Pos.	Neg.		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
1	51	29 56,9%	27 93,1%	2 6,9%	26 89,7%	3 10,3%	22 43,1%	15 68,2%	7 31,8%	13 59,1%	9 40,9%	42 82,4%	9 17,6%	39 76,5%	12 23,5%
2	92	33 35,9%	25 75,8%	8 24,2%	30 90,9%	3 9,1%	59 63,1%	37 62,7%	22 37,3%	41 69,5%	18 40,5%	62 67,4%	30 32,6%	71 77,2%	21 22,8%
3	160	53 33,1%	27 50,9%	26 49,1%	38 71,7%	15 28,3%	107 66,9%	34 31,8%	73 68,2%	39 36,5%	68 63,5%	61 38,1%	99 61,9%	77 48,1%	83 51,9%
Total	303	115 38,0%	79 68,2%	36 31,3%	94 81,7%	21 18,3%	188 62,0%	86 45,7%	102 54,3%	93 49,5%	95 50,5%	165 54,5%	138 45,5%	187 61,7%	116 38,3%

* Wellcome Reagents Ltd, Beckenham, England

TABLE 3 Agreement between the ELISA and IFT

Total No. of sera*	Total No. + (1 or both tests)	ELISA + IFT +	ELISA + IFT −	ELISA − IFT +
303	219	133 (60,7%)	32 (14,6%)	54 (24,7%)

* 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 IFT 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.

The ELISA remained negative in both calves for at least 2 months.

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

All human sera tested for possible cross-reactions between *B. besnoiti* and *T. gondii* (Table 6) gave negative results in the IFT with *B. besnoiti* antigen. In the SFT, with *T. gondii* trophozoites, 15,2 % of the sera gave positive reactions. Therefore, no cross-reactions were observed between the 2 parasites.

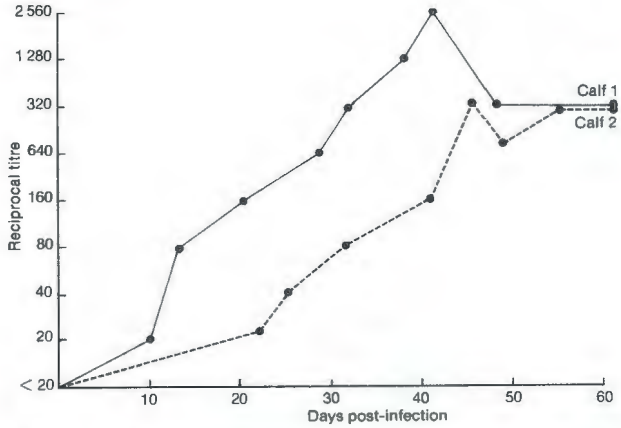

FIG. 1 IFT titre of 2 experimentally infected calves. Calf 1 received ± 3 × 10^6 trophozoites of *B. besnoiti* (bovine strain) intravenously and Calf 2 received ± 1 × 10^6 trophozoites (blue wildebeest strain) subcutaneously

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

Clinical severity*	ELISA titre						IFT titre					
	<1:160	1:160	1:320	1:1640	1:1280	≧1:2560	<1:20	1:20	1:40	1:80	1:160	≧1:320
−	102	29	18	12	8	19	95	7	22	23	22	19
+	17	7	11	7	1	11	11	1	6	12	13	9
++	11	5	4	6	5	5	5	0	4	7	10	12
+++	7	0	0	2	2	6	4	0	0	1	7	7
++++	1	0	3	0	3	1	1	0	0	0	3	2
Total	138	41	36	27	19	42	116	8	32	43	55	49

* 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

Age of cattle	No. in age group	ELISA titre						IFT titre					
		<1:160	1:160	1:320	1:640	1:1280	1:2560	<1:20	1:20	1:40	1:80	1:160	1:320
1 month	5	4	0	0	0	0	1	4	0	0	0	0	1
1–6 months	9	8	0	1	0	0	0	9	0	0	0	0	0
6–12 months	7	5	0	1	1	0	0	4	0	1	0	1	1
12–24 months	64	37	7	3	4	3	10	34	2	9	7	5	7
24 months	218	84	34	31	22	17	30	65	6	22	36	49	40
Total	303	138	41	36	27	20	41	116	88	32	43	55	49

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

IFT *Besnoitia**		SFT *Toxoplasma***							
Negative	Positive	Negative	1:4	1:16	1:64	1:256	1:1000	≥1:4000	Total positive
164	0	139	3	11	7	2	2	0	25

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

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 some 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. Kaggwa (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 $\leqq$ 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 & Laarman (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, 1977) 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 Kaggwa (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*.

Acknowledgements

The authors wish to thank Dr C. F. Hansford, Prof. D. A. van Staden, Dr G. Weiland, Dr I. Reiter and the State Veterinarian, Louis Trichardt, for sera, Dr D. W. Verwoerd for the preparation of antigen, Dr A. Verster for her co-operation in the life cycle studies, and Messrs N. Berg, M. Combrink and C. G. Fourie for technical assistance.

REFERENCES

ANON., 1980. Empfehlungen für die Durchführung der *Toxoplasma*-Seroreaktionen mittels Mikromethode. *Bundesgesundheidsblatt*, 20, 108–112.

BIGALKE, R. D. & NAUDÉ, T. W., 1962. The diagnostic value of cysts in the scleral conjunctiva in bovine besnoitiosis. *Journal of the South African Veterinary Medical Association*, 33, 21–27.

BIGALKE, R. D., 1968. New concepts on the epidemiological features of bovine besnoitiosis as determined by laboratory and field investigations. *Onderstepoort Journal of Veterinary Research*, 35, 1–138.

FRANK, M., KLINGER, J. & PIPANO, E., 1970. The presence of antibody against *Besnoitia besnoiti* in dairy and beef cattle. *Journal of Protozoology*, Supplement 17, 31.

FRENKEL, J. K., 1953. Infections with organisms resembling *Toxoplasma*, together with the description of a new organism: *Besnoitia jellisoni*. *Atti VI Congress Microbiology, Rome*, 426–434.

GOLDMAN, M., CARVER, R. K. & SULZER, A. J., 1957. Similar internal morphology of *Toxoplasma gondii* and *Besnoiti jellisoni* stained with silver protein. *Journal of Parasitology*, 43, 490–491.

JANITSCHKE, K., WERNER, H. & BODE, L., 1982. Applicability of an automated ELISA for the serological diagnosis of echinococcosis. *Zentralblatt für Bakteriologie*, I. *Abt. Referate*, 277, 117.

KAGGWA, E., 1977. The evaluation of various serological methods for the diagnosis of *Besnoitia besnoiti* and *B. jellisoni* infections in rabbits and mice. M.Sc. thesis, Makerere University, Uganda.

KRASOV, V. M. & OMAROV, Z. K., 1975. Koprosy besnoitiza zhivotnykh, *Veterinarya, Moscov*, No. 2, 65–67.

LUNDE, M. N. & JACOBS, L., 1965. Antigenic relationship of *Toxoplasma gondii* and *Besnoitia jellisoni*. *Journal of Parasitology*, 51, 273–276.

NEUMAN, M., 1972. Serological survey of *Besnoitia besnoiti* (Marotel, 1912) infection in Israel by immunofluorescence. *Zentralblatt für Veterinärmedizin, Reihe B*, 19, 391–396.

POLS, J. W., 1960. Studies on bovine besnoitiosis with special reference to the aetiology. *Onderstepoort Journal of Veterinary Research*, 28, 265–356.

RENTERGHEM, L. VAN & NIMMEN, L. VAN, 1976. Indirect immunofluorescence in toxoplasmosis: Frequency, nature and specificity of polar staining. *Zentralblatt für Bakteriologie I. Abteilung Originale A.*, 235, 559–565.

SUGGS, M., WALLS, K. W. & KAGAN, I. G., 1968. Comparative antigenic study of *Besnoitia jellisoni*, *B. panamanensis* and five *Toxoplasma gondii* isolates. *Journal of Immunology*, 101, 166–175.

TADROS, W. & LAARMAN, J. J., 1976. *Sarcocystis* and related coccidian parasites: a brief general review, together with a discussion on some biological aspects of their life cycles and a new proposal for their classification. *Acta Leidensia*, 44, 1–137.