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Evaluation of enzyme immunoassays in the diagnosis of camel (*Camelus dromedarius*) trypanosomiasis: a preliminary investigation

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SUMMARY

Three enzyme immunoassays were used for the serodiagnosis of *Trypanosoma evansi* in camels in the Sudan in order to evaluate their ability to discriminate between infected and non-infected animals. Two assays were used for the detection of trypanosomal antibodies, one using specific anti-camel IgG conjugate and another using a non-specific Protein A conjugate. The third assay detected the presence of trypanosomal antigens using anti-*T. evansi* antibodies in a double antibody sandwich assay. Inspection of the frequency distribution of assay results suggested that the ELISA for circulating trypanosomal antibodies using specific antisera and the ELISA for circulating antigens can distinguish between non-infected camels and infected camels exhibiting patent infections or not. The ELISA using Protein A conjugate to bind non-specifically to camel immunoglobulin did not appear to discriminate between infected and non-infected animals.

INTRODUCTION

Infections of livestock with *Trypanosoma evansi* occur in many countries in Asia, northern Africa and Latin America. Trypanosomiasis in the camel (*Camelus dromedarius*) has been recognized as an important disease of livestock in the Sudan since the beginning of this century. According to a recent animal census (Boid, Jones & Luckins, 1986), the camel population in Sudan was approximately 2·5 million, the majority of animals being in the eastern and western regions. Dromedary camels are important to the nomadic peoples of Northern Sudan because, in addition to providing food, transport and clothing, they also represent personal wealth within the community. The province of Kassala supports a large population of camels, and veterinarians there still rank trypanosomiasis as a major threat to the camel industry.

Control of the disease by drug treatment relies on effective diagnosis primarily on parasitological techniques (Godfrey & Killick-Kendrick, 1962). In order to improve diagnostic efficiency, several serological tests have been used to detect *T. evansi* antibodies in the camel. These include the complement fixation test (Schoening, 1924), indirect fluorescent antibody assays (Luckins *et al.* 1979) and indirect haemagglutination tests (Wilson *et al.* 1983).

More recently, the enzyme-linked immunosorbent assay (ELISA) has been developed for the detection of *Trypanosoma* spp. antibodies and antigens in animal sera (Luckins & Mehlitz, 1978; Luckins *et al.* 1979; Rae & Luckins, 1984). The ELISA is a test that is both analytically and diagnostically sensitive and diagnostically specific. Interpretation of results can be quantitative and objective, and the assay may be fully automated, so that large numbers of sera can be screened (Ruitenberg *et al.* 1977).

The purpose of the present study was to evaluate the ability of three enzyme immunoassays to discriminate between infected and non-infected camels, using blood samples collected from camels in the Sudan over a 2-year period.

MATERIALS AND METHODS
Serum samples

A total of 944 serum samples was collected from camels (*Camelus dromedarius*) in Kassala Province, Eastern Sudan, during the period January 1985 to December 1986. Eighteen serum samples were collected from police camels submitted for their annual veterinary inspection during June and July 1985; 29 samples were obtained from camels at a slaughterhouse in June 1985; the remaining 897 samples were collected from sick camels, possibly with trypanosomiasis, presented by their owners at Kassala Veterinary Clinic. All sera were heat inactivated at 56 °C for 30 min before shipment to the Centre for Tropical Veterinary Medicine for ELISA testing. 'Negative' control sera were collected from four camels kept at Edinburgh Zoological Park, Scotland. These were also heat inactivated as described.

Parasitological diagnosis

Venous blood samples were taken from the jugular vein and collected into ethylenediaminetetra-acetic acid (EDTA) for parasitological examination. The microhaematocrit centrifuge technique (Woo, 1970) was performed on all blood samples for the identification of *T. evansi* infection.

Trypanosomes

Two stocks of *Trypanosoma evansi* were used in the preparation of antigens for ELISA:

(1) *T. brucei evansi*, isolated from a naturally infected camel at Kassala Province, Eastern Sudan, in 1977, and passaged in rats and mice before cryopreservation as TREU 1418. This antigen was used in Tests 1 and 2;

(2) *T. brucei evansi*, isolated from a naturally infected ox at Bogor, Indonesia, in 1984, and passaged in mice before being cloned and cryopreserved as TREU 1942. This antigen was used for the production of rabbit anti-*T. evansi* immune serum.

Ten mice were infected with *T. evansi* and, after a fulminating parasitaemia had developed, blood was collected. Blood was passed through a column of diethylaminoethyl cellulose (Whatman, DE52) as described by Lanham & Godfrey (1970), in order to separate parasites from blood elements. The trypanosome suspension was centrifuged at 3200 g for 15 min, the supernate discarded, and the resulting pellet washed three times in 100 mM phosphate-buffered saline, pH 7·2 (PBS) by centrifugation at 2500 g for 15 min. The trypanosomes were resuspended to 1·0 ml in PBS and the antigens prepared using the method described by Luckins (1977). The protein content of each antigen preparation was determined using BCA protein assay reagent (Pierce Chemical Company). Antigens were subsequently stored at −79 °C until required.

Reagents for use in ELISA

Horseradish peroxidase-conjugated rabbit anti-camel IgG

Rabbit anti-camel IgG antiserum was prepared as described elsewhere (Boid *et al.* 1980). Horseradish peroxidase (Type VI, Sigma) was conjugated to the specific antiserum using the technique described by Wilson & Nakane (1978). Conjugates in PBS containing 0·15 mM bovine serum albumin were sterilized by filtration through a 0·22 μm filter (Millipore) and stored at −20 °C until used.

Horseradish peroxidase-labelled staphylococcal Protein A

Conjugated protein A was obtained commercially (Sigma) in a lyophilized form. After reconstitution according to the manufacturer's instructions, the conjugate was divided into 50 μl amounts and stored at −20 °C.

Production of anti-T. evansi antiserum and conjugate

Adult male New Zealand White rabbits were used for the production of serum against a sonicated *T. evansi* (TREU 1942) antigen preparation. The method described by Thalhamer & Freund (1984) was applied. Briefly, 2 mg soluble trypanosome protein (Luckins, 1977) were injected subcutaneously at day 0 and again intramuscularly at day 7 and day 21. Following this, 5 mg of protein were given subcutaneously at day 22 and intravenously at day 23. The first dose was given with complete Freund's adjuvant (Difco), subsequent injections were given in 140 mM sodium chloride. Antisera were collected 7 days after the last injection and stored at −40 °C. The activity of the hyperimmune serum was analysed against the soluble proteins obtained from TREU 1942 by SDS–PAGE and standard immunoblotting techniques. At least 250 antigens were detected using this antiserum (R. Boid, personal communication). The specific antibody content of the serum was tested by ELISA (Luckins, 1986) using the same antigen preparation.

An immunoglobulin fraction of the antiserum was obtained by precipitation using ammonium sulphate, reconstitution in PBS and then extensive dialysis against the same buffer. The protein concentration of the immunoglobulin fraction was determined using BCA protein assay reagent (Pierce Chemical Company) before conjugation with horseradish peroxidase (Rae & Luckins, 1984).

Serological tests

For each of the enzyme immunoassays, optimal antigen, antibody and conjugate dilutions were established by chequerboard titrations using 'negative' control sera from uninfected camels at Edinburgh Zoo and 'positive' control sera selected from camels with parasitologically proven *T. evansi* infections.

The three test assays were:

(1) a conventional ELISA test for detecting trypanosomal antibodies as described by Luckins (1986) (Test 1);

(2) an assay using commercially available staphylococcal Protein A labelled with horseradish peroxidase to detect primarily IgG class trypanosomal antibodies (Zweygarth, Sabwa & Röttcher, 1986) (Test 2);

(3) a double antibody sandwich ELISA technique for detecting circulating trypanosomal antigens (Luckins, 1986) (Test 3).

All three enzyme immunoassays were conducted using the substrate 3,3′5,5′-tetramethylbenzidine (Sigma) as described by Luckins (1986). Plates were set to zero absorbance using the PBS-T control wells.

Data recording

For each camel, data were recorded on:

(1) the presence or absence of patent infection, as determined by microscopic examination of blood;

(2) the optical density values of the three ELISA tests;

(3) the month in which the camel was sampled.

Data were initially recorded on the 'PANACEA' database management system (Pan Livestock Services, Department of Agriculture, University of Reading) on an AMSTRAD PC1640 microcomputer, and were then moved to an AMDAHL mainframe computer at the University of Edinburgh's Computer Service, where they were inspected using the 'MINITAB' statistical package (Ryan, Joiner & Ryan, 1985).

RESULTS

The results of the parasitological investigation showed that of the 944 camels considered, 751 did not have patent infection and 193 had patent infection. Histograms, illustrating various features of the distributions of the ELISA results, are given in the figures. (Note that in a histogram the area, not the height, of a particular rectangle is proportional to the number, or frequency, of observations lying in the interval covered by the base of the rectangle. For the special case where the rectangles all have equal base widths, that is, when all the class intervals are of equal widths, the heights of the rectangles are proportional to the areas and thus to the frequencies. For the more general case, as in this paper, where the rectangles may have different base lengths, the height is represented as a 'frequency density', that is, as a frequency per unit measurement on the base line. For example, in Fig. 1b the unit measurement is taken to be $0.1 /A_{450\,nm}$; other figures have different unit measurements. This procedure ensures that the areas of the rectangles are proportional to the appropriate class frequencies.) The effect on the histograms of the removal of the results for those camels with ELISA values
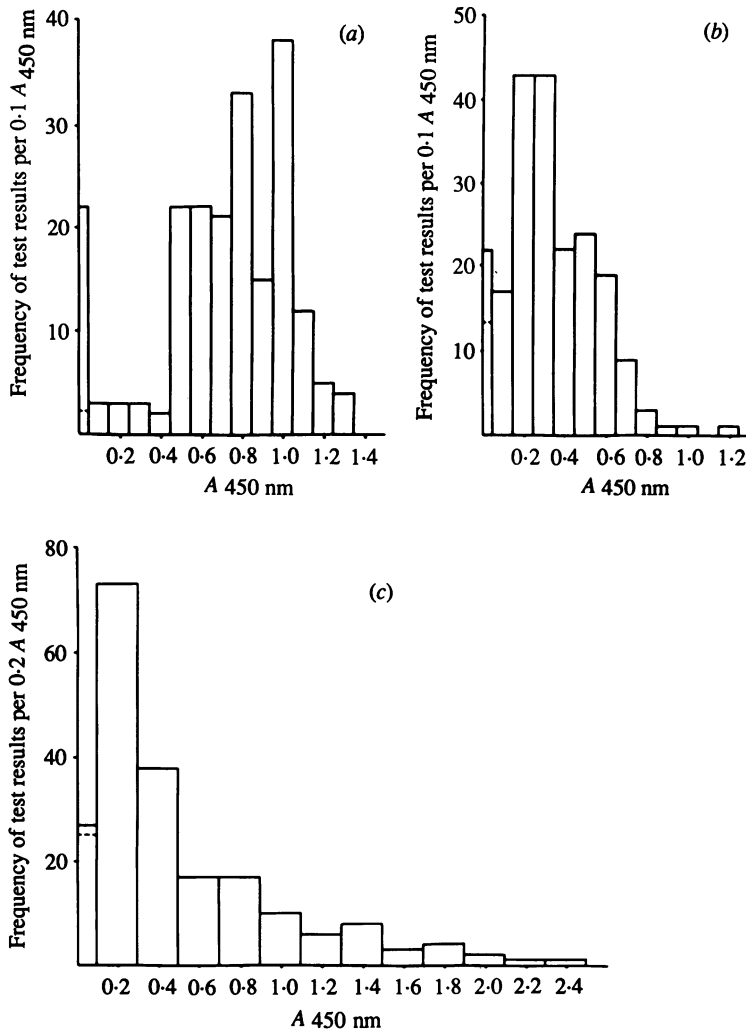
Fig. 1. Frequency distribution of results of ELISA tests for *T. evansi* in camels with patent infection, November 1985 to December 1986. (In the first column, animals with ELISA values greater than zero are plotted below the dotted line.) Number of camels, 193. (*a*) Conventional ELISA test for antibody to *T. evansi*. (*b*) Protein A ELISA test for antibody to *T. evansi*. (*c*) ELISA test for *T. evansi* antigens.

of zero (to two decimal places) is indicated by a dotted line in the left-hand bar of each figure, values of zero being plotted above the dotted line.

Fig. 1 *a–c* depicts the distribution of the results of the three ELISAs from the 193 camels which had patent infection. In Fig. 1 *a,b* for those camels with ELISA values greater than zero (to two decimal places), the shape of the histograms is suggestive of the Normal. The shape of the histogram in Fig. 1 *c*, again for camels with ELISA values greater than zero, is suggestive of a lognormal distribution.

The remaining Figs. (2*a*–4*c*) refer to camels without patent infection (i.e. camels which are either non-patently infected or non-infected). No patent infections were recorded in the first 10 months of the survey in a relatively large sample of 173 animals; this was therefore probably a time during which no current infections
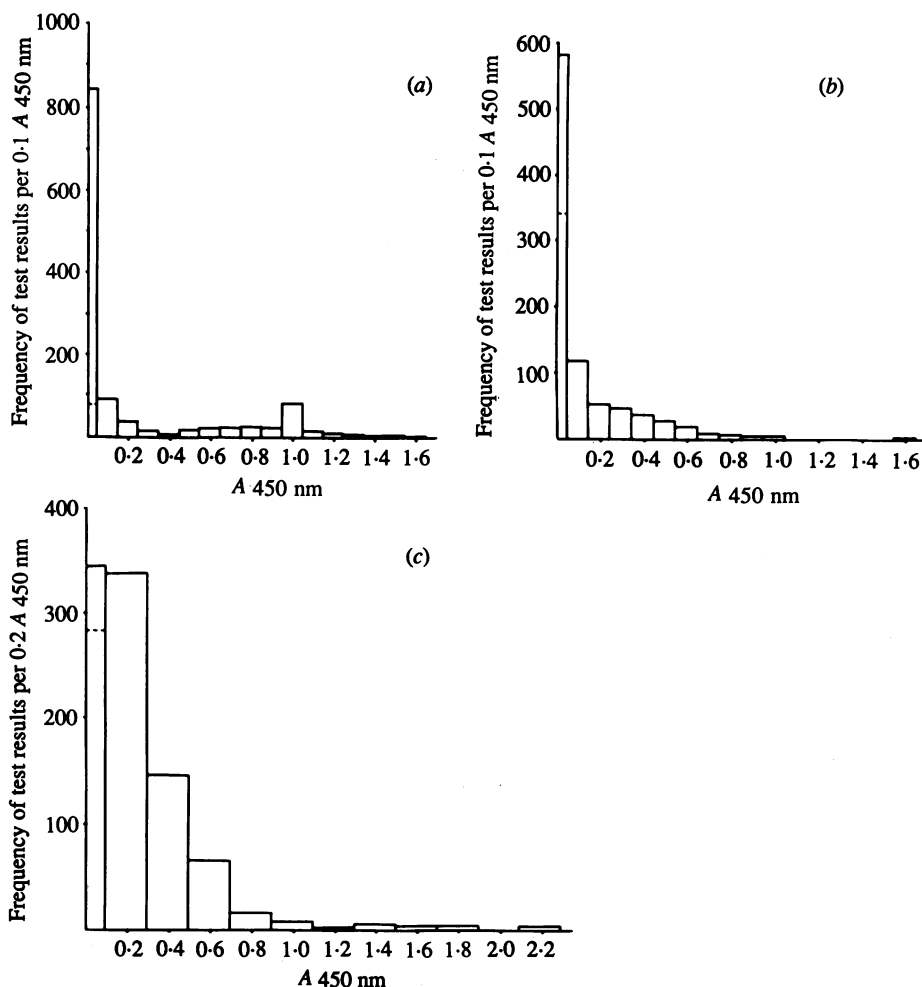
Fig. 2. Frequency distribution of results of ELISA tests for *T. evansi* in camels without patent infection, January 1985 to December 1986. (In the first column, animals with ELISA values greater than zero are plotted below the dotted line.) Number of camels, 751. (*a*) Conventional ELISA test for antibody to *T. evansi*. (*b*) Protein A ELISA test for antibody to *T. evansi*. (*c*) ELISA test for *T. evansi* antigens.

were present. This serological survey therefore was divided into two distinct periods:

(1) January to October 1985: during which no patent infections were identified;

(2) November 1985 to December 1986: during which 193 infections were detected in 771 camels sampled.

The relationships among Figs. 2*a*–4*c* are shown in Table 1. Fig. 2*a*–*c* relates to camels during the total study period (January 1985 to December 1986). The form of the histogram in Fig. 2*a* suggests that the results of ELISA Test 1 for camels with ELISA values greater than zero may be a mixture of two distributions: exponential and Normal. The form of the histograms in Fig. 2*b,c* (representing Tests 2 and 3, respectively, for camels with ELISA values greater than zero) is indicative of an exponential distribution.

Table 1. *Figures representing histograms of test results for camels without patent infection. The histograms of the figures listed in the fourth column are formed by adding together the two histograms represented by the figures in parentheses*

	Study period*		
Test	1	2	3
Conventional ELISA	3a	4a	2a (3a, 4a)
Protein A ELISA	3b	4b	2b (3b, 4b)
ELISA test for *T. evansi* antigens	3c	4c	2c (3c, 4c)

* 1, January 1985 to October 1985; 2, November 1985 to December 1986; 3, January 1985 to December 1986.
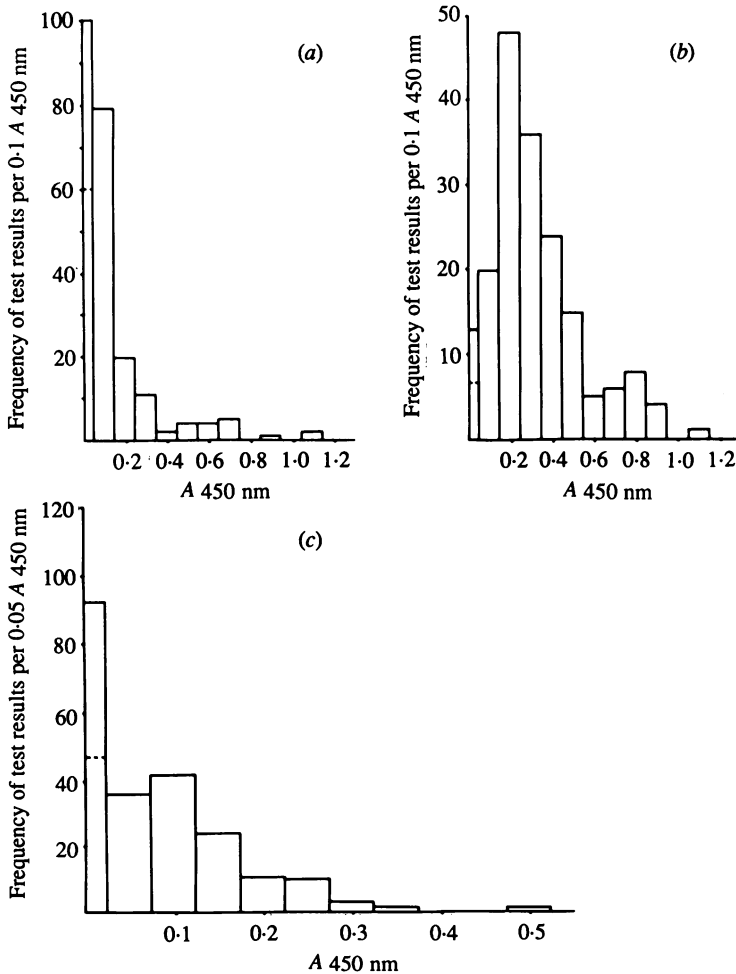

Fig. 3. Frequency distribution of results of ELISA tests for *T. evansi* in camels without patent infection, January 1985 to October 1985. (In the first column, animals with ELISA values greater than zero are plotted below the dotted line.) Number of camels, 173. (*a*) Conventional ELISA test for antibody to *T. evansi*. (*b*) Protein A ELISA test for antibody to *T. evansi*. (*c*) ELISA test for *T. evansi* antigens.
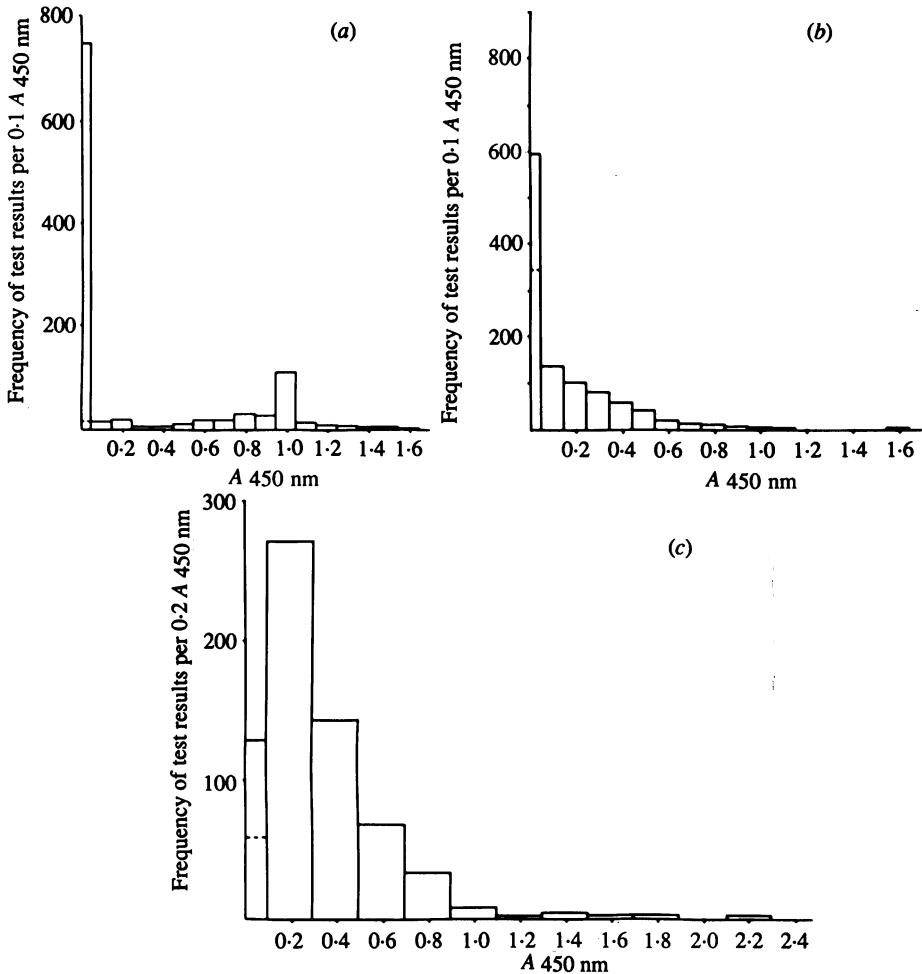
Fig. 4. Frequency distribution of results of ELISA tests for *T. evansi* in camels without patent infection, November 1985 to December 1986. (In the first column, animals with ELISA values greater than zero are plotted below the dotted line.) Number of camels, 578. (*a*) Conventional ELISA test for antibody to *T. evansi*. (*b*) Protein A ELISA test for antibody to *T. evansi*. (*c*) ELISA test for *T. evansi* antigens.

Fig. 3*a–c* illustrates distributions of ELISA values during the initial 10-month period of study (January to October 1985) when no patent infections were identified. Fig. 3*a,c*, again for camels with ELISA values greater than zero, suggests an exponential distribution. In contrast, Fig. 3*b* is similar to a Normal distribution.

Fig. 4*a–c* shows frequency distributions of ELISA values for camels not exhibiting patent infection during the second half of the study period (November 1985 to December 1986). It was during this period that all the patent infections were identified. A mixture of an exponential and a Normal distribution (heavily weighted towards the Normal) is suggested for animals with ELISA values greater than zero in Fig. 4*a*. Fig. 4*b* indicates an exponential distribution, whereas Fig. 4*c* suggests a lognormal one (again, for animals with ELISA values greater than zero).

DISCUSSION

Diagnosis of trypanosomiasis in individual camels at risk from infection with *T. evansi* is dependent on a number of factors: those relating to host–parasite interaction including levels of parasitaemia and presence or absence of trypanosomal antibodies, and those relating to the type of diagnostic test used, particularly its detection limits. Hence, in chronic infections parasites may be few in number and difficult to detect. In camels with active infections, examination of blood microscopically fails to identify less than half of those animals shown to be infected by rodent inoculation (Godfrey & Killick-Kendrick, 1962). Serological assays may provide a convenient alternative to examination of blood films for identification of infection with *T. evansi*. Previous surveys for trypanosomiasis in camels have shown that in areas where the disease occurs large numbers of camels show serological evidence of infection (Luckins *et al.* 1979). However, problems in the interpretation of the results of serological assays such as ELISA tests means that it is difficult to determine the infection status of individual animals. Experimental evidence indicates the extent of these problems. Thus, in camels and rabbits infected with *T. evansi*, antibodies appear within 14 days of infection and remain throughout the duration of the infection. Following treatment and elimination of infection, although there is decline, antibodies persist in the circulation for up to 100 days after treatment (Luckins, Gray & Rae, 1978; Luckins *et al.* 1979). Antigens released by the parasites can be detected in serum as early as 5 days after infection. After trypanocidal drug treatment, antigen is no longer detectable 7 days after treatment (Rae & Luckins, 1984). Hence, whilst both tests are capable of indicating the presence of trypanosomes, the test for antigen detection seems capable of identifying animals with current infections, which may be either patent or non-patent. Most work involving the use of ELISA in animal trypanosomiasis has relied on the interpretation of results based on an arbitrary absorbance value to distinguish between positive and negative results. The data collected from this survey are being used to provide statistically valid methods of interpretation and these preliminary investigations present the frequency distributions of the three immunoassays.

Two probability distributions (an exponential, and a Normal and related distribution – the lognormal) are suggested by the Figures. An exponential distribution would be considered consistent with the decay of antibodies stimulated by a previous infection, in the absence of current infection. Recently stimulated antibodies, along with many other biological variables, are Normally distributed, or their distribution can be transformed to Normality. Antibodies accompanying current infection frequently follow such a pattern (e.g. Herbert, 1970). Identification of these two different patterns (exponential or Normal) may therefore allow definition of an animal as being either currently infected or uninfected (either with or without waning antibody levels resulting from previous infection).

The distribution of ELISA values for Tests 1–3 in patently infected animals (Fig 1*a–c*) is consistent with their current infection. Fig. 3*a,c* shows exponential distributions, consistent with both antibody and antigen decay in the absence of current infection. Tests 1 and 3 thus appear to reflect accurately an animal's infection status; either infected or non-infected. However, the histogram

representing the results of Test 2 (Fig. 3*b*) is similar to that depicted in Fig. 1*b* relating to camels with patent infection. Thus, Test 2 is not good at discriminating between camels with and without current infection.

The second period (November 1985 to December 1986) was characterized by both patently and not-patently infected animals and therefore may have included animals with ELISA values resulting from both current and previous infections. Furthermore, camels that are not patently infected may have non-patent infections: Godfrey & Killick-Kendrick (1962) showed that approximately 60% of camels without patent infections are non-patently infected. Fig. 4*a*–*c* thus relates to camels which may, or may not, be non-patently infected, in the approximate ratio of 6:4 respectively. Test 1 (Fig. 4*a*) implies current infection (a Normal distribution) and previous infection with waning antibody levels (an exponential distribution), with the two distributions clearly separated, and could thus be used to identify current infection (patent or non-patent). Fig. 2*a*, depicting the results of this test for the full study period, substantiates this inference. Test 2 (Fig. 4*b*) does not display a Normal component and therefore is unsuitable for detecting current infections. In contrast, Test 3 (Fig. 4*c*) has a lognormal distribution. This can be transformed to a Normal one and therefore may be suitable for identifying current infections, either patent or non-patent.

This investigation of three ELISAs suggests that the test for circulating antibody (Test 1) is likely to be the best test for differentiating between non-infected and infected (either patently or non-patently) camels. Test 3 is also capable of identifying infection.

Further work is required to determine the best fit of a statistical distribution to the continuous data that result from the tests. It appears from the histograms that this fit will be achieved by consideration of exponential, Normal and lognormal distributions.

REFERENCES

BOID, R., LUCKINS, A. G., RAE, P. F., GRAY, A. R., MAHMOUD, M. M. & EL MALIK, K. H. (1980). Serum immunoglobulin levels and electrophoretic patterns of serum proteins in camels infected with *Trypanosoma evansi*. *Veterinary Parasitology* 6, 333–345.

BOID, R., JONES, T. W. & LUCKINS, A. G. (1986). Protozoal diseases of camels. In *The Camel in Health and Disease* (ed. A. Higgins), p. 44. London: Bailliere Tindall.

GODFREY, D. G. & KILLICK-KENDRICK, R. (1962). *Trypanosoma evansi* of camels in Nigeria: a high incidence demonstrated by the inoculation of blood into rats. *Annals of Tropical Medicine and Parasitology* 56, 14–19.

HERBERT, W. J. (1970). *Veterinary Immunology*, p. 190. Oxford: Blackwell Scientific Publications Limited.

LANHAM, S. M. & GODFREY, D. G. (1970). Isolation of salivarian trypanosomes from man and other animals using DEAE cellulose. *Experimental Parasitology* 28, 521–534.

LUCKINS, A. G. (1977). Detection of antibodies in trypanosome infected cattle by means of a microplate enzyme linked immunosorbent assay. *Tropical Animal Health and Production* 9, 53–62.

Luckins, A. G. (1986). Antigens and antibodies in fungal and parasitic diseases. *Trypanosoma brucei.* In *Methods in Enzymic Analysis*, (ed. R. F. Masseyeff), pp. 351–367. Federal Republic of Germany: Vch Verlagsgesellschaft.

Luckins, A. G., Boid, R., Rae, P., Mahmoud, M. M., El Malik, K. H. & Gray, A. R. (1979). Serodiagnosis of infection with *Trypanosoma evansi* in camels in the Sudan. *Tropical Animal Health and Production* **11**, 1–12.

Luckins, A. G., Gray, A. R. & Rae, P. (1978). Comparison of the diagnostic value of serum immunoglobulin levels, an enzyme-linked immunosorbent assay and a fluorescent antibody test in experimental infections with *Trypanosoma evansi* in rabbits. *Annals of Tropical Medicine and Parasitology* **72**, 429–441.

Luckins, A. G. & Mehlitz, D. (1978). Evaluation of an indirect fluorescent antibody test, enzyme linked immunosorbent assay and quantification of immunoglobulin in the diagnosis of bovine trypansomiasis. *Tropical Animal Health and Production* **10**, 149–159.

Rae, P. F. & Luckins, A. G. (1984). Detection of circulating trypanosomal antigens by enzyme immunoassay. *Annals of Tropical Medicine and Parasitology* **78**, 587–596.

Ruitenberg, E. J., Van Amstel, J. A., Brosi, B. J. M. & Steerenberg, P. A. (1977). Mechanization of the enzyme linked immunosorbent assay (ELISA) for large scale screening of sera. *Journal of Immunological Methods* **16**, 351–359.

Ryan, B. F., Joiner, B. L. & Ryan, T. A (1985). Minitab Handbook, 2nd edition. Boston: Prindle, Weber and Schmidt.

Schoening, H. W. (1924). Trypanosomiasis in camels. Its detection by complement fixation. *Journal of Infectious Diseases* **34**, 608–613.

Thalhamer, J. & Freund, J. (1984). Cascade immunization: a method of obtaining polyspecific antisera against crude fractions of antigens. *Journal of Immunological Methods* **66**, 245–251.

Wilson, A. J., Schwartz, H. J., Dolan, Rosemary & Olahu, W. M. (1983). A simple classification of different types of trypanosomiasis occurring in four camel herds in selected areas of Kenya. *Tropenmedizin und Parasitologie* **34**, 220–224.

Wilson, M. B. & Nakane, P. K, (1978). Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies. In *Immunofluorescence and Related Staining Techniques*. New York: Elsevier/North Holland Biochemical Press.

Woo, P. T. K. (1970). The haematocrit centrifuge technique for the diagnosis of African trypanosomiasis. *Acta Tropica* **27**, 384–386.

Zweygarth, E., Sabwa, C. & Röttcher, D. (1985). An enzyme linked immunosorbent assay for the detection of antibodies to *Trypanosoma (T.) brucei evansi* in camels (*Camelus dromedarius*) using peroxidase-conjugated protein A. *Tropenmedizin und Parasitologie* **37**, 105–106.