Antigen ELISAs for Trypanosomes

Evaluation of the Performance

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The International Livestock Research Institute (ILRI) began operations on 1 January 1995. ILRI is one of 16 centres in a worldwide agricultural research network sponsored by the Consultative Group on International Agricultural Research (CGIAR). ILRI conducts strategic research in the biological, animal and social sciences to improve livestock productivity in sustainable agricultural systems throughout the developing world.

The objectives of the institute's research programmes are to improve animal health, nutrition and productivity (milk, meat, traction) in ways that are sustainable over the long term, to characterise and conserve the genetic diversity of indigenous tropical forage species and livestock breeds, to promote sound and equitable national policies for animal agriculture and natural resource management, and to strengthen the animal husbandry research programmes of developing countries.

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Foreword

Tsetse-transmitted trypanosomosis continues to be a major constraint to livestock productivity in sub-Saharan Africa. The four important livestock trypanosomes, *Trypanosoma congolense, T. vivax, T. brucei* and *T. evansi,* cause high mortality in the acute phase and severe production losses in the chronic phase of the infection. The control of the disease is still principally based on vector management and the use of a limited number of trypanocidal drugs available in the market. Alternative control measures involve the use of trypanotolerant breeds of cattle, particularly in West Africa where trypanosomosis is a constant threat. No new drugs, vaccines or alternative control measures have been developed and are unlikely to be forthcoming in the near future.

Accurate diagnosis of trypanosomosis is an important component of the disease control and still remains a challenge due to very low parasitaemias in the majority of infections. In an attempt to improve detection of trypanosomes in subclinical and chronic infections, antigen detection ELISAs (Ag-ELISAs), based on species-specific monoclonal antibodies were developed in 1989 by the International Laboratory for Research on Animal Diseases (ILRAD). After the successful validation exercise in the laboratories in Kenya, the assays were distributed to 13 laboratories in sub-Saharan Africa through the joint FAO/IAEA Division, Vienna, for field validation and wider evaluation. Following this exercise, it was found necessary to modify the procedure for the Ag-ELISA. This was done by joint division of FAO/IAEA and subsequently distributed through the FAO/IAEA Co-ordinated Research Programme to a number of African countries for routine use in the control of trypanosomosis.

A number of workshops has been held to discuss the performance of the Ag-ELISA; the most recent of these was organised by FAO/IAEA in 1995 and held at ILRI's campus in Addis Ababa, Ethiopia. The most important conclusions and recommendations made at this workshop were that the Ag-ELISAs have a high specificity of between 95 and 99%, and were valuable for detection of chronic and mixed infections. However the assays exhibited poor sensitivity necessitating concurrent use of a parasitological test such as the buffy coat technique (BCT) in field surveys.

Since this workshop, and through continuing collaboration with FAO/IAEA and the partners in the national laboratories in Africa, it has become clear that the modified version of Ag-ELISAs shows poor sensitivity in the field and their performance appears to vary significantly between laboratories. As part of continuing development and evaluation of these tests, ILRI organised a workshop to assess the current status of Ag-ELISAs and identify ways of improving them. The workshop entitled 'Evaluation of the Performance of Antigen-ELISAs for the Detection of Trypanosomes' was held at ILRI between 9 and 11 December, 1996 with the following main objectives:

- to define the circumstances of use and requirements of trypanosome antigen detection assays;
- to develop quantitative standards on which their performance can be assessed;
- to examine the problems of trypanosome antigen-ELISAs and devise ways and means of circumventing them; and
- to recommend protocols for the development, standardisation and validation of future antigen detection assays for trypanosomes.

In order to seek advice from a wide range of disciplines in the workshop, a number of scientists from eight countries representing epidemiologists, field workers, laboratory technologists, serologists and test developers were invited. The workshop was held over a period of three days. The first day was devoted to presentations of key papers covering the epidemiology of trypanosomosis and the requirements for diagnostics, the performance of Ag-ELISAs in various laboratories, technical aspects of Ag-ELISAs and the use and value of alternative tests. The remaining two days were devoted to addressing two specific issues: a) shortcomings of Ag-ELISAs and possible solutions; and b) diagnostic needs for trypanosomosis and quantitative standards.

This publication contains all the formal presentations, summaries of discussions and a set of recommendations made at the workshop. These will be extremely valuable for both ILRI and the joint division of FAO/IAEA in defining research agenda for improvement of the Ag-ELISAs and development of new diagnostics for detection of trypanosomes.

The organising committee would like to thank a number of ILRI scientists who assisted in formulating the programme for the workshop, the rapporteurs for their timely submissions of the summaries of the discussion after formal presentations, and the invited participants for keeping the discussion open, honest and interactive. We thank Dr John McDermott for assisting in summarising the group discussions. We are also grateful to Dr Rob Eley for his help and assistance in organising the conference and Mr Peter Werehire for proofreading the text and typesetting the book.

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Diagnostic tests requirements, development, standardisation and validation

Requirements for diagnostic tests to improve field investigations into the epidemiology and control of trypanosomosis

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Summary

There are multiple requirements for diagnostic tests in the study of epidemiology and control of trypanosomosis. These tests need not be perfect; what is crucial is that they provide results that are sufficient for the purposes demanded. In this paper different circumstances are outlined for which tests are needed for epidemiological investigations of trypanosomosis; the requirements for diagnostic tests are described for each of these circumstances. The focus, as in this workshop, is on diagnostic tests for livestock rather than for tsetse populations. The requirements for the performance of trypanosomosis diagnostic tests in terms of their sensitivity and specificity to estimate the seroprevalence, disease incidence and other epidemiological parameters can vary widely when these tests are required to answer different objectives. Increased sensitivity and specificity will be needed for the estimation of parameters for smaller, specifically defined population groups.

Epidemiology of trypanosomosis, basic patterns influencing control programmes

A key feature of the epidemiology of trypanosomosis in Africa is the variability of both its distribution and importance between different ecological areas and farming systems. As with other vector-borne diseases, tsetse distribution and density are most influenced by spatial factors such as climate, vegetation and land utilisation. Given these spatial differences, it is important to be able to characterise areas so that trypanosomosis control programmes can be targeted to areas in which maximum benefits will be achieved. Three important criteria for characterising areas as to their relative suitability for control programmes are: 1) epidemiologic disease status (endemic stability; prevalence or incidence of infection and disease), 2) economic costs (morbidity, mortality and other production effects) and 3) cost and efficacy of measures to control trypanosomosis. Given the variability of incidence of trypanosomosis from area to area, usually a large number of areas, with relatively few farms and animals, need to be sampled to obtain confidence intervals of prevalence or incidence estimates which are sufficiently narrow to detect area-to-area differences.

Superimposed on this basic spatial pattern are temporal changes (seasonal or longer-term trends) and differences among animals (e.g. species, breed, age), so that the occurrence and impact of trypanosomosis depends on tsetse challenge and on a number of other factors such as host distribution, livestock breeds, farming practices and trypanosomosis control practices. Defining the size and boundaries of target areas of interest for both epidemiological studies and control programmes can be difficult. Trypanosomosis risk can vary both by larger agro-

ecological areas which would correspond in many cases to administrative units for control planning purposes or by much smaller areas which exhibit local variations in vegetation within a grazing area (Wacher et al 1994). In this paper, the areas referred to are administrative areas for disease control purposes. Since control programmes are usually targeted at areas (or if targeted at herds and animals it is usually within high risk areas), it is sensible to try and classify areas epidemiologically, taking into account major risk factors. For tick-borne diseases, this has been done by classifying areas as to their endemic stability (Norval et al 1992; Perry and Young 1995).

Endemic stability has not been a concept commonly applied to studies of the epidemiology of trypanosomosis but it could be a useful paradigm for helping to consider the important interrelationships of factors influencing trypanosomosis occurrence and impact. Endemic stability is a relative term, describing the stability of the relationships between host(s), agent, vector, farming system, chemotherapy regime and environment. Where endemic stability exists there is a balance between host factors, pathogen factors and disease challenge. Endemic diseases occur at predictable rates in an area, whereas epidemic diseases occur much in excess of predictions. Combining the concepts of endemicity and stability is useful in planning control programmes, particularly in circumstances where sustained eradication is not feasible. Thus, for the given environmental circumstances, different combinations of control measures (e.g. introduction of trypanotolerant breeds, changes in grazing practice, strategic tsetse control and chemotherapy) can be tried in order to move towards an endemically stable state with low disease incidence. The principal challenge of this strategy is to minimise disease losses in a cost-beneficial fashion while the desired endemically stable trypanosomosis disease control state is being established. Figure 1 depicts common relationships between seroprevalence, disease prevalence, endemic stability and risk factors for many vector-borne diseases. As can be seen on the right hand Y-axis of Figure 1, for simple classifications into either 1) very low challenge, 2) endemically unstable and 3) moderate to high challenge endemic stable categories, only modest diagnostic test performance is required.

Generally, in endemically stable areas, trypanosomosis risk for most animals will be relatively constant. However, in endemically unstable areas, trypanosomosis risk will be more heterogeneous and can vary by animal (e.g. breed, age), farm (e.g. grazing system, control measures) and micro-area (e.g. forests, riverbanks). In these areas, diagnoses by animal and farm are often demanded.

Studies to investigate the epidemiology and improve the control of trypanosomosis

To comment on the needs and requirements for diagnostic tests, it is first necessary to summarise the types of studies needed to investigate the epidemiology and improve the control of trypanosomosis. These are somewhat arbitrarily categorised into four main groups based on objectives: 1) studies to characterise the status of areas, 2) studies to assess changes in status over time, 3) controlled trials for testing different treatment or clinical decisions, and 4) studies to estimate parameters for modelling. These categories are not mutually exclusive. Within each category, the information that might be obtained and the relative test performance needed to achieve it with the required precision can then be considered. These are summarised in Table 1.

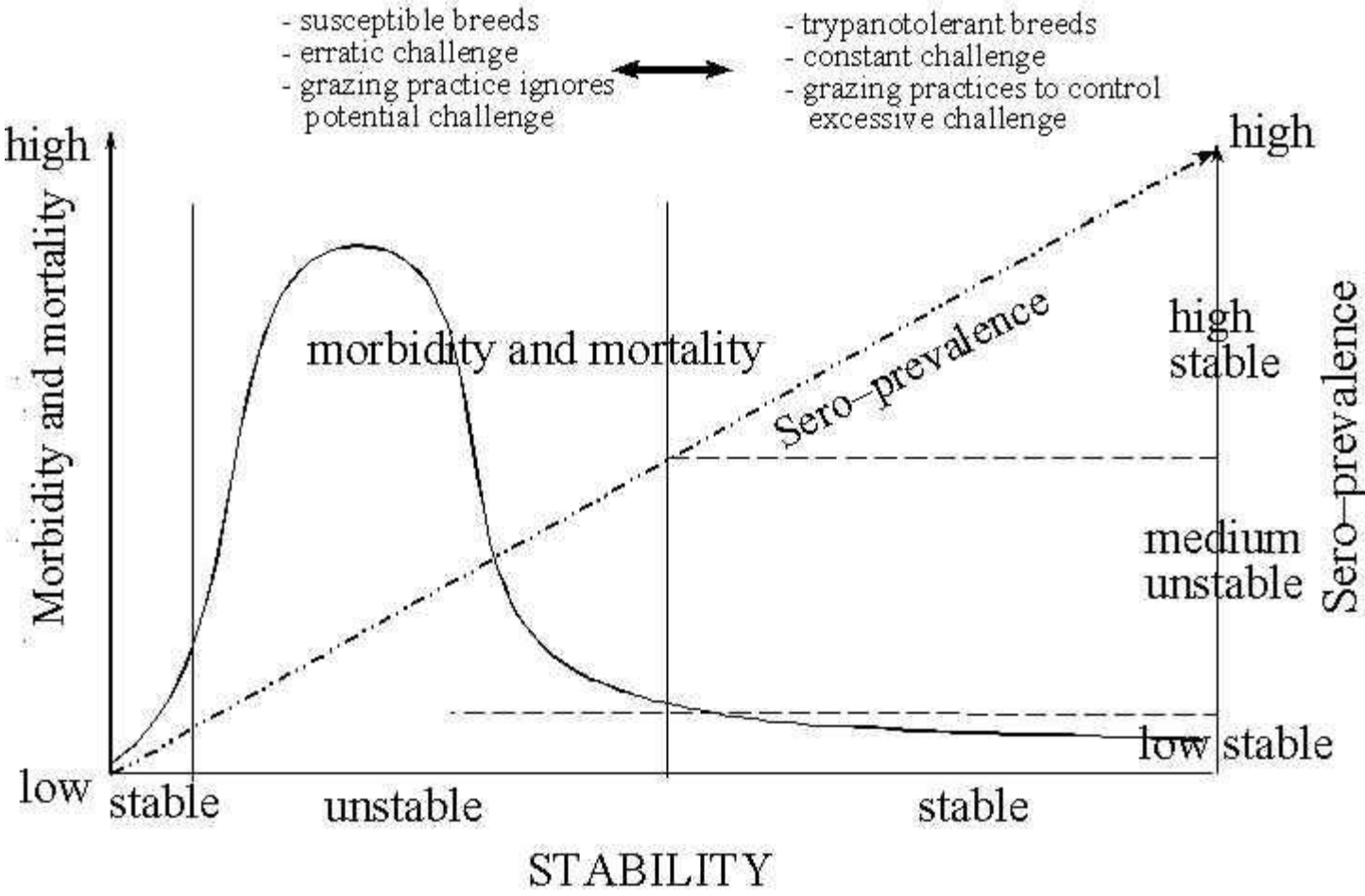

Figure 1. *Hypothesised relationships between seroprevalence, morbidity and mortality rates and factors associated with different epidemiological states used for a number of vector-borne diseases (modified from Perry and Young 1995)*.

Studies to characterise target areas for control

For cross-sectional or longitudinal studies to characterise endemic stability and to estimate the relative incidence or prevalence of trypanosomal infection and morbidity in different areas, relatively low sensitivity (Se = 0.2–0.5) is acceptable (Table 1; Figure 1) as long as: 1) the combination of test sensitivity and specificity are noticeably better than occurrence by chance (i.e. the test predicts better than tossing a coin), 2) test sensitivity and specificity are consistent across areas, farms and animals and 3) trypanosomosis is not rare. If test errors are random, deficiencies in test performance can be compensated for by increasing sample size. Unfortunately, an assumption that the sensitivity and specificity of some tests (antigen- and perhaps antibody-detection ELISA tests) are homogenous across areas (beyond within-laboratory repeatability and between-laboratory differences) may not always be correct and needs to be evaluated.

Table 1. *Studies to investigate the epidemiology and improve the control of trypanosomosis: objectives, study design, information that might be obtained and relative test performance.*

Study objectives	Study design	Information that might be obtained	Relative test[†] performance
Characterise target areas for control	Observational: cross-section or longitudinal	1. Presence of infection	Low–moderate[‡]
		2. Identification of parasite	
		3. Morbidity/ mortality rate	
		4. Effects on production	
		5. Potential control benefits	
Estimate changes in occurrence or impact over time to assess effects of control programme Observational: longitudinal	Observational: longitudinal	1. Presence of infection	Moderate–high[§]
		2. Identification of parasite	
		3. Morbidity/mortality rate	
		4. Effects on production	
Test different treatment or clinical decisions for use in control programmes Experimental: clinical field trials	Experimental: clinical field trials	1. Presence of infection	Moderate–high[§]
		2. Identification of parasite	
		3. Morbidity/mortality rate	
		4. Effects on production	
		5. Other specialised measures[¶]	
Estimate parameters for disease models	Observational: longitudinal Experimental: field and laboratory	1. Presence of infection	Moderate–high[§]
		2. Identification of parasite	
		3. Morbidity/mortality rate	
		4. Immune responses	
		5. Other specialised measures[¶]	

[†]For tests with a dichotomous outcome; approximate sensitivity (Se) and specificity (Sp) values for relative performances presented are: low (Se: 0.2–0.4; Sp: 0.4–0.7), moderate (Se: 0.4–0.8; Sp: 0.7–0.9) and high (Se: >0.8; Sp>0.9); see Martin et al (1987) for additional discussion on sensitivity and specificity.
[‡]Low–moderate for sensitivity, parasite identification by skilled personnel should have a specificity of 1.
[§]Interpretation of infections in individual animals requires higher performance overall than in herds or areas and is a function of the relative costs of false positives and false negatives.
[¶]e.g. host genetic markers, level of infection, measures of host response for trypanotolerance; in some areas and cases species or other identification may be important to assess transmission.

The performance requirements for serological or other tests to link infection to morbidity and mortality events and (subclinical) production losses will require at least a moderate (Se: 0.4–0.8; Sp: 0.7–0.9) level of performance since the number of animals used to estimate these parameters will usually be limited (Table 1). The performance of microhaematocrit (Woo 1971) or buffy coat techniques (Murray et al 1977), by comparison, has lower sensitivity (<0.4 in many circumstances) and high specificity (essentially 1 with experienced personnel). When sample sizes are small, using either of these as the diagnostic test may restrict the ability to find significant associations with production losses, although, the low sensitivity can be improved with repeated sampling. In addition, there has been interest in assessing whether there are any production effects of chronic infections in livestock having levels of parasites below the threshold detected by buffy coat examination. This would require that tests designed to assess infection status, such as the antigen-detection ELISA (Nantulya and Lindqvist 1989), be used semi-quantitatively rather than just qualitatively to estimate what levels of infection might

interfere with production. Again, moderate levels of performance may suffice for mean effect estimates in an area or over a number of areas. However, if the test is to be used for individual animal diagnosis and treatment decisions, high (Se: >0.8; Sp >0.9) performance might be required depending on the incidence of trypanosomosis and the costs of correct and incorrect treatment decisions (Table 1).

Observational studies of changes in occurrence or impact over time to assess effects of control programmes

Relative test performance requirements for tests used to estimate changes in trypanosomosis occurrence or impact over time will also depend on the size of the differences in infection, morbidity, mortality rates and other production losses to be compared. Requirements will be higher than for characterising areas and will increase in proportion to how small a difference needs to be detected (Table 1). Increasingly, estimates of the impact of control programmes are required more quickly, so that small differences over short time periods will be used to predict likely trends and revise programmes in progress. However, such assessments of changing incidence or prevalence over time are subject to problems of confounding with other factors that change over time (Rowlands 1994; Rowlands et al 1996), thus, requiring similar trends to be observed over multiple time periods and/or sites to give us sufficient confidence that any effects observed are important.

Clinical trials to assess control and treatment programmes

Clinical field trials are, by design, more powerful in estimating differences between groups to be contrasted because other factors which can confound the contrast of interest are on average balanced by random allocation. However, depending on the study costs per experimental unit, there may be severe restrictions on the potential sample size. Test errors will, of course, increase the required sample size.

For both observational and experimental studies, the unit of observation or randomisation may be area, farm, animal or combinations of these. As described above, specific decisions at the animal level relying on diagnostic tests will be completely susceptible to individual test errors while for herd- or area-level decision-making individual test errors are averaged together with correct test results. If mistakes in individual animal treatment decisions are costly, high test performance will be required (Table 1). In the past, field trials for comparing control options between trypanosomosis control areas have rarely been conducted. The major difficulty is that a number of control areas need to be included. One possible strategy would be to subdivide the target area for a national or regional trypanosomosis control programme into a number of smaller control areas and begin control options to be compared in a randomly selected subset of these. This would allow for both the assessment and delivery of the control programme to be integrated.

Studies to estimate parameters for disease models

For longitudinal studies or clinical trials to estimate parameters for modelling the transmission dynamics of trypanosomosis, the data are divided more and more finely to classify animals into different epidemiologic states. Basic states include: susceptible, latent, infectious and immune (Anderson and May 1991). However, these may need to be subdivided by age or breed to account for potentially important variations in transmission. The more that data from studies to

estimate these parameters need to be subdivided, the higher are the levels of test performance required (Table 1). Relative to some other livestock and human diseases, transmission models for trypanosomosis are undeveloped. This is likely a function of both the complexity of modelling trypanosomal infections (indirectly transmitted microparasites) and the only recent interest in integrated methods to control trypanosomosis.

For transmission models, the population and infection dynamics of both the tsetse and animal populations are important. In the animal population, not only the presence of infection, but also relative levels of infectiousness and immunity may need to be assessed. Thus, no one test will be sufficient. Because of the crucial importance of temporal as well as spatial patterns of animal infections in assessing transmission dynamics, antigen-detection methods such as the ELISA have a potentially important role to play. However, because of the detail of information required, the standards of test performance demanded to meet these objectives are high (Table 1).

Diagnostic tests—needs and priorities

For all categories of studies listed in Table 1, direct microscopic identification of parasites and/or other methods to detect the presence of infection in animals are important in estimating both the effects of infection on production (e.g. morbidity, mortality, weight loss) and transmission dynamics. Less commonly required is the detection of immune response to previous infection. In practice, available antibody detection assays (e.g. Luckins et al 1978) are used primarily as a surrogate measure of previous infection status rather than a measure of the effectiveness of the immune response (its use for this latter objective needs to be evaluated).

In the field, direct microscopic examination of the buffy coat of spun microhaematocrit tubes (Woo 1970, 1971) or its refinements (Murray et al 1977) are standard and robust techniques which can and have been used effectively to characterise areas (Nankodaba et al 1988), assess control success (Leak et al 1995) and diagnose trypanosomal infections in individual animals for subsequent animal or herd treatment. Their high specificity (1 by experienced persons) is a major advantage relative to antibody and antigen detection systems. However, if detection of low levels of parasitaemia is either important or required, then the sensitivity of the buffy coat technique is relatively low compared to what might be achieved by other techniques. As with other tests with a specificity of 1, it is possible to increase the sensitivity by examining multiple tubes (Woo (1970) recommends three) or multiple samples over time. The threshold level of parasitaemia which results on average in significant production effects needs further elaboration. It has been hypothesised that parasitaemia levels below what can be detected by buffy coat examination may cause production losses (Trail et al 1992a, 1992b) but this has yet to be fully demonstrated. The other advantage of buffy coat examination is that it simultaneously provides an estimate of reductions in PCV, which have also been shown to be associated with production loss (Rowlands et al 1995; Trail et al 1991).

As described above, characterising or comparing areas is a very important component of the epidemiological assessment of trypanosomosis control. In comparing areas, it is crucial that test performance is consistent from area to area. This may be more difficult to achieve for indirect methods such as antigen- or antibody-detection ELISAs, for which variations in trypanosomal populations might cause variations in test performance. Thus, the assessment of these methods prior to their widespread distribution in the field needs to consider test consistency in a wide variety of circumstances.

Some of the tests in current use were developed to distinguish infections of different species of trypanosomes. Differentiating between infections of trypanosomes which are potentially 'pathogenic'*(Trypanosoma brucei, T. congolense* and *T. vivax)* has been considered useful in some situations. Trail et al (1992b) in data from a ranch in Gabon, found production differences for animals detected by Ag-ELISA to be infected by *T. congolense,* but no differences were detected between animals positive and negative for any trypanosome, ignoring species. It is difficult to know how much emphasis should be placed on species differentiation for routine screening tests. Species differentiation will be important in the research domain and perhaps its importance should be well justified there first, before greater efforts are made to develop species-specific screening tests for widespread use.

There are a number of other diagnostic testing needs with potential widespread application which also require further refinement in the research domain. Two of particular importance are diagnostic methods for assessing trypanotolerance of animal populations (Trail et al 1992a, 1994), e.g. level of trypanosomal infection (for associations with production effects), measures of host immune response and host genetic markers, and methods for determining drug sensitivity of trypanosomal populations (e.g. assays to detect circulating drug levels). In the research domain, a broad range of potential diagnostic methods should be considered and critically compared (including immunological and nucleic acid methods) by skilled technical staff using samples from both controlled laboratory and field situations. The assessment and interpretation of the results would be best debated among a wide range of researchers (molecular biologists, immunologists, epidemiologists) and field users before widespread distribution of these tests is considered.

What are the priority needs for improving the use and interpretation of diagnostic tests for field investigations of the epidemiology and control of trypanosomosis? A key requirement is to link the use and interpretation of tests to specific diagnostic objectives. There are three main priority areas for improving the routine diagnostic tests for trypanosomosis discussed in the paper. The first is that the epidemiological states to be diagnosed must be clearly defined (e.g. levels of infection and immunity). Thereafter, tests should be evaluated based on their ability to classify areas correctly into these states. The second is that any tests for widespread distribution, particularly indirect tests, should be developed so that their results are consistent from laboratory to laboratory and area to area. Thirdly, increased sensitivity is required for some but not all applications.

There is still much to be learned about the epidemiology and improved control of trypanosomosis. Improvements in our understanding will depend on better study design, more intelligent and consistent application of existing diagnostic methods, and, in some circumstances, development, standardisation and evaluation of newer methods.

References

Anderson R.M. and May R.M. 1991. *Infectious Diseases of Humans: Dynamics and Control.* Oxford University Press, Oxford, UK. 757 pp.

Leak S.G.A., Mulatu W., Rowlands G.J. and d'Ieteren G.D.M. 1995. A trial of a cypermethrin 'pour-on' insecticide to control *Glossina pallidipes, G. fuscipes fuscipes* and *G. morsitans submorsitans*(Diptera: Glossinidae) in south-west Ethiopia. *Bulletin of Entomological Research* 85:241–251.

Luckins A.G., Gray A.R. and 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 *T. evansi* in rabbits*. Annals of Tropical Medicine and Parasitology* 72:429–441.

Martin S.W., Meek A.H. and Willeberg P. 1987. *Veterinary Epidemiology, Principles and Methods.* Iowa State University Press, Ames, Iowa, USA. 343 pp.

Murray M., Murray P.K. and McIntyre W.I.M. 1977. An improved parasitological technique for the diagnosis of African trypanosomosis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 71:325–326.

Nankodoba G., Coulibaly L., Hecker P., Leak S.G.A. and Schuetterle A. 1988. Trypanosome prevalence in cattle herds exposed to a range of tsetse challenge levels in northern Côte d'Ivoire. In:*Livestock Production in Tsetse-affected Areas of Africa. Proceedings of a Meeting Held in Nairobi, Kenya, 1987 at ILCA/ILRAD.* pp. 55–60.

Nantulya V.M. and Lindqvist K.J. 1989. Antigen-detection immunoassays for diagnosis of *Trypanosoma vivax, T. congolense* and *T. brucei* infections in cattle. *Tropical Medicine and Parasitology*40:267–272.

Norval R.A.I., Perry B.D. and Young A.S. 1992. *Epidemiology of Theileriosis in Africa.* Academic Press, London. 481 pp.

Perry B.D. and Young A.S. 1995. The past and future roles of epidemiology and economics in the control of tick-borne diseases of livestock in Africa. *Preventive Veterinary Medicine* 25:107–120.

Rowlands G.J., d'Ieteren G.D.M., Coulibaly L., Hecker P.A., Leak S.G.A. and Nagda S.M. 1996. Assessment of the effect of tsetse control on livestock productivity—a case study in northern Côte d'Ivoire. *Preventive Veterinary Medicine* 28:17–32.

Rowlands G.J., Mulatu W., Nagda S.M. and d'Ieteren G.D.M. 1995. Variations in packed red cell volume and trypanosome prevalence and relationships with reproductive traits in East African zebu cows exposed to drug-resistant trypanosomes. *Acta Tropica* 59:105–116.

Rowlands G.J. 1994. Assessing impacts of animal health interventions on productivity. In: Rowlands G.J., Kyule M.N. and Perry B.D. (eds), *Proceedings of the 7th International Symposium on Veterinary Epidemiology and Economics, Nairobi, Kenya. Kenyan Veterinarian* 18:353–355.

Trail J.C.M., Wissocq N., d'Ieteren G.D.M., Kakiese O. and Murray M. 1994. Quantitative phenotyping of N'Dama cattle for aspects of trypanotolerance under field tsetse challenge. *Veterinary Parasitology* 55:185–195.

Trail J.C.M., d'Ieteren G.D.M., Maille J.C., Yangari G. and Nantulya V.M. 1992a. Use of antigen-detection enzyme immunoassays in assessment of trypanotolerant N'Dama cattle. *Acta Tropica*50:11–18.

Trail J.C.M., d'Ieteren G.D.M., Viviani P., Yangari G. and Nantulya V.M. 1992b. Relationships between trypanosome infection measured by antigen-detection enzyme immunoassays, anaemia and growth in trypanotolerant N'Dama cattle. *Veterinary Parasitology* 42:213–223.

Trail J.C.M., d'Ieteren G.D.M., Feron A., Kakiese O., Mulungo M. and Pelo M. 1991. Effect of trypanosome infection, control of parasitaemia and control of anaemia development on productivity of N'Dama cattle. *Acta Tropica* 48:37–45.

Wacher T.J., Milligan P.M., Rawlings P. and Snow W.F. 1994. Spatial factors in the assessment of trypanosomosis challenge. In: Perry B.D. and Hansen J.W. (eds), *Modelling Vector-Borne and Other Parasitic Diseases. Proceedings of a Workshop Organised by ILRAD in collaboration with FAO held at ILRAD, Nairobi.* ILRAD (International Laboratory for Research on Animal Diseases), Nairobi, Kenya. pp. 119–129.

Woo P.T.K. 1970. The haematocrit centrifuge technique for the diagnosis of African trypanosomosis. *Acta Tropica* 27:384–386.

Woo P.T.K. 1971. Evaluation of the haematocrit centrifuge and other techniques for the field diagnosis of human trypanosomosis and filariasis. *Acta Tropica* 28:298–303.

Development, standardisation and validation of enzyme immunoassays: an integrated process

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Summary

The development, standardisation and validation of an enzyme immunoassay should be viewed as an integrated and ongoing process which requires comprehensive planning from the onset and critical evaluation at various stages along the way. Specific requirements in terms of analytical criteria, diagnostic performance, standardisation, quality assurance, data expression, production, technology transfer and validation need to be considered. This necessitates input and cooperation from appropriate research, production, diagnostic, field and programme groups. Without this type of integrated approach, the likelihood that some factor will be overlooked is virtually guaranteed. A small factor, which may seem insignificant in the early stages of the process, may prove to be a significant barrier to the final acceptance of an assay. Coordination of this process is not an easy task and it is best handled by a methods development team. This is the group that defines the above requirements and ensures that they are met. Based on a number of years' experience in methods development, the following discussion describes some of the less obvious factors which can make or break an enzyme immunoassay with respect to it becoming a valuable diagnostic asset.

Introduction

The virtues of the enzyme immunoassay (EIA) have been extolled for over a quarter of a century and need not be reiterated time and again. Advances in biotechnology, specifically monoclonal antibodies and recombinant antigens, have even further enhanced the potential of this technique. However, very few EIAs have made the successful transition from the research laboratory into the diagnostic laboratory. The reasons for this are numerous and, for the most part, are preventable. The development, standardisation and validation of an enzyme immunoassay should be viewed as an integrated and ongoing process. A process which requires comprehensive planning from the onset and critical evaluation at various stages along the way. Specific requirements in terms of analytical criteria, diagnostic performance, standardisation, quality assurance, data expression, production, technology transfer and validation need to be considered. This necessitates input and cooperation from appropriate research, production, diagnostic, field and programme groups. Perhaps the single greatest challenge is getting the appropriate people from the appropriate groups to work together in the planning and evaluation stages. The best approach to this is to form a methods development team with representation from all of the concerned groups.

Considerations

The following highlight a number of considerations which, if not properly addressed, may pose significant barriers to the usefulness and acceptance of an assay. Some may seem intuitively obvious but all are nonetheless important.

Analytical criteria

- Host species: application to single or multiple species may have an affect on assay design strategies and the need for species specific reagents (especially for antibody detection).
- Immunoglobulins: detection of antibodies of certain immunoglobulin isotypes may be more diagnostically relevant than others (e.g. IgG1 vs. IgM).
- Antigens: detection of some antigenic epitopes or antibodies against them may be more appropriate than others (e.g. group specific vs. serotype-specific epitopes).
- Cross reactions: detection of cross reacting antigenic epitopes on other organisms or antibodies against them may lead to false positive reactions.
- Vaccination: detection of persistent or residual antibody following vaccination may lead to false positive reactions (especially where vaccination programmes are not regulated).
- Test Samples: nature and stability of the test sample and processing requirements may affect assay repeatability and reproducibility (especially for antigen detection).
- Immunological reagents: choice of polyclonal vs. monoclonal antibodies may impact on analytical and diagnostic performance (i.e. analytical specificity and binding avidity).
- Antigenic reagents: choice of crude, purified or recombinant antigens may impact on both analytical and diagnostic performance and on assay design (particularly for antibody detection).
- Enzyme/substrate systems: some systems are more sensitive than others in terms of signal generation; this does not necessarily translate to enhanced diagnostic performance but rather may have a negative impact.
- Assay design: all of the foregoing considerations may impact on the choice of assay design with respect to detection strategies (i.e. indirect, competitive, inhibition, blocking, capture, etc.).

Diagnostic performance

- Application: programme applications (for both antibody and antigen detection assays) should dictate diagnostic performance requirements (e.g. control, vaccination, elimination, surveillance, import/export, survey, treatment, etc.).
- Interpretation: level of interpretation should be considered (e.g. herd or individual animal levels); interpretation with or without other test results may affect diagnostic performance (e.g. serial or parallel interpretation).
- Diagnostic sensitivity: a minimum, acceptable diagnostic sensitivity target should be established based on application and interpretation.
- Diagnostic specificity: a minimum, acceptable diagnostic sensitivity target should be established based on application and interpretation.
- Prevalence: prevalence of infection or disease and the impact of false positive or negative results may impact on minimum targets for sensitivity and specificity.

Standardisation

- Biological reagents: international reference standards should be used; if unavailable, standards should be created and characterised for future reference and assay calibration; standards should be the basis for the development of internal controls.
- Buffer systems: standard buffer systems (composition, pH, ionic strength) and detergents should be adopted for wash, diluent and substrate buffers; addition of biological blocking agents should be avoided unless absolutely necessary.

- Physical parameters: storage conditions and volumes, dilution factors, incubation times and temperatures, wash cycles, plate shaking, batch processing, etc. should suit the assay application with respect to volume of tests to be performed and expected turn around time for results.
- Microplates: microplates chosen should be of consistent batch-to-batch quality and preferably from the same manufacturing facility; for some applications, good quality untreated plates may be better than treated (i.e. high protein-binding capacity) plates.
- Water quality: minimum standard should be established and performance of reference reagents and internal controls should be characterised under these conditions; changes in water quality may first be evident in the activity of internal controls.
- Equipment: equipment used for development should be the same or equivalent to equipment used in the diagnostic laboratory (e.g. of the same quality, accuracy and precision).
- Suppliers: for all of the above, sufficient information should be documented to allow diagnostic laboratory to obtain identical or equivalent materials and equipment (e.g. chemical grade, pipette specifications, etc.).
- Protocols: a standard format for assay protocols should be adopted; all of the above information should be included in the protocols; protocols should meet International Standards Organization (ISO) guidelines.

Quality assurance

- Biological reagents: should be stable upon shipping and storage; method of preservation, shelf-life and special handling instructions should be documented.
- Buffer systems: acceptable pH ranges, shelf-lives and storage conditions should be documented.
- Physical parameters: acceptable ranges for times, temperatures, etc. should be documented.
- Internal controls: should cover the range of expected reactions, including strong, weak and negative reactions; results should always be unequivocal with respect to positive/negative status; upper and lower control limits should be established in diagnostic laboratory setting.
- Acceptance criteria: acceptance of a given plate or run should be established based on the performance of the internal controls; clear criteria should be documented; final decision should be a `human' decision.
- Test samples: acceptance of test sample results should also be defined; clear criteria are required for reactivities near the positive/negative cut-off; in some assays, a suspicious zone may be appropriate.
- Control charts: daily control charts of internal controls should be kept and used to monitor process control and developing trends.
- Proficiency testing: where possible, external check sample programmes should be implemented and used to certify or recognise diagnostic laboratory proficiency.
- Protocols: all of the above information should be included in the assay protocols; protocols should meet International Standards Organization (ISO) guidelines.

Data expression

- Choices: for some applications, qualitative data (e.g. positive or negative) may be appropriate; for others, quantitative or semi-quantitative data may be more suitable (e.g.

percent inhibition or percent positivity); raw data (e.g. absorbency values) are not comparable between laboratories.
- Reference standards: internal controls should be calibrated against reference standards; data should be expressed relative to calibrated, internal control (e.g. strong positive antibody standard in the indirect ELISA or monoclonal antibody control in competitive type assays).
- Cut-off: should be expressed in the same manner as test data (i.e. percent inhibition or percent positivity); cut-off values should be established as part of the field validation process.
- Laboratory interpretation: reactivity status should be based on analytical and quality assurance criteria; additional interpretation may be required if serial or parallel testing is performed.
- Field interpretation: reported data must be in a format which is acceptable to and easily interpreted by field staff; no internationally adopted formats exist for ELISA's, although some have been proposed for antibody assays.

Production

- Protocols: should be documented for the production of all biological materials, including antigens, antibodies, conjugates, controls and panels; protocols should include production details, standardisation processes and quality assurance checks.
- Schedules: should be established with respect to production frequency and batch requirements, including quantity, titer, aliquot size, safety tests, preservation methods and expiry.

Technology transfer

- Scope: should suit target groups and general level of laboratory proficiency; both technical and scientific staff may require training; more than one session may be required.
- Troubleshooting: target laboratories should be surveyed with respect to deficiencies in utilities, equipment, supplies, personnel, etc. and corrective actions should be recommended.
- Proficiency: as part of the technology transfer process, an external check sample programme should be established to certify diagnostic laboratories as proficient or 'ready to perform'.
- Backstopping: contingencies should be established to assist laboratories which experience unforeseen problems with assay performance.

Validation

- Design: should suit the intended application of the assay with respect to target population(s); sample sizes should give appropriate confidence for estimates of diagnostic sensitivity and specificity.
- Definition: test populations should be realistic and clearly defined; `text book' groupings may not be achievable.
- Testing site(s): ideally, validation should be conducted under routine diagnostic laboratory conditions and in more than one laboratory.
- Cut-off: should be established after the testing of defined populations; method of analysis should be compatible with method of data expression; one cut-off may not be

suitable for all laboratories depending on background activity associated with local test populations.

- Prevalence: cut-off values may require readjustment as the prevalence of disease changes; in general cut-off values and prevalence are inversely related.
- Other factors: validation should not only include estimates of diagnostic performance but assay accuracy, precision, repeatability and reproducibility as well.
- Monitoring: validation should be a continuous process which requires constant monitoring, assessment and updating at the diagnostic laboratory level.

Conclusion

As may be seen in the foregoing 50 or so points, the development, standardisation and validation of an enzyme immunoassay is a complex and integrated process. Undoubtedly other points may be added to the list, but the preceding serves to illustrate that this process requires comprehensive planning from the outset and critical evaluation at various stages along the way. Confidence in the assay by diagnostic laboratory, programme and field personnel is critical to its acceptance and use. One weak link in the process and confidence may be jeopardised. In order to ensure a smooth transition, a methods development team must ensure that all of the potentially critical points, as minor as some may seem, have been addressed.

Ag-ELISAs for trypanosomes

Antigen-ELISA for detection of trypanosomes

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Summary

Diagnosis is essential in the management of disease, both at the level of individual animal and at the level of disease control in populations. The routinely employed methods for detection of trypanosomes are capable of revealing infection during the acute phase but as the disease becomes chronic, the power to detect parasites greatly reduces. This led to development and application of a new and more appropriate technique for diagnosis of African trypanosomes. Sensitive and easy to perform antigen-detection ELISAs, based on monoclonal antibodies (MAbs), raised against trypanosome species have been developed. The performance of these assays has been evaluated both in the laboratory and field and found to be more sensitive than the routinely applied parasitological assays. The sensitivity of these assays is not optimal and requires further refinement to enable them to detect, if possible, all the parasitaemic cases. Steps have been taken to achieve this but the changes likely to improve the performance of the assays have not yet been effected. The specificity of the monoclonal antibodies in capturing trypanosome antigens was evaluated using bovine sera obtained from trypanosomosis-free areas and shown to be over 92 per cent specific.

Introduction

Trypanosomosis is a major constraint to livestock production in sub-Saharan Africa and to a lesser extent in Latin America and Asia. The distribution of the disease is influenced by the existence of tsetse and biting flies. Tsetse-transmitted trypanosomosis is encountered in 39 sub-Saharan African countries. The non-tsetse transmitted trypanosomosis occurs in Latin America, Middle East, Asia, Eastern Europe and to a lesser extent Africa.

Trypanosomosis, a disease of man and livestock, is endemic in much of tropical Africa. The trypanosomes responsible for the disease in domestic animals belong to several species which include*Trypanosoma congolense, T. simiae, T. vivax, T. brucei, T. evansi* and the sexually transmitted *T. equiperdum.* Among these, *T. vivax* and *T. evansi* are the most widely spread, since they occur not only in tropical Africa, but also in South America (Applewhaite 1990; Shaw and Lainson 1972; Wells 1984) and southeastern Asia (Boid and Mleche 1985). *Trypanosoma evansi* causes disease in horses, camels, pigs, buffaloes and cattle, while *T. equiperdum* affects horses and donkeys. Typically, trypanosomosis in livestock is characterised by anaemia, weight loss, compromised immunological and endocrinological functions and waves of parasitaemia (Losos and Ikede 1972; Masake 1980). The disease syndromes associated with *T. vivax* infections vary from acute and fatal disease seen in West Africa, to relatively milder forms of infection of cattle in East Africa. However, in the latter region, some stocks of *T. vivax* cause a severe haemorrhagic disseminated intravascular coagulation (DIC)-like syndrome (Assoku and Gardiner 1989).

Thus, there appears to be no consistent set of clinical signs to facilitate diagnosis of trypanosome infections. In view of this, microscopy has been employed traditionally in the positive identification of the causative organism (Woo 1971; Murray et al 1977). Standard diagnostic techniques applied in the detection of trypanosome infection are heavily dependant on the identification of the parasite in accessible body fluids of infected animals. The easiest of the parasitological techniques used is the direct microscopic examination of wet blood film or stained blood smears for the presence of trypanosomes. Detection of parasites in wet blood films allows speciation on the basis of motility while examination of Giemsa-stained blood smears makes classification on the basis of morphology possible. Although these techniques are easy to perform, their sensitivity is poor. In an effort to improve the sensitivity of the direct microscopy, Woo (1971) introduced the microhaematocrit technique (Woo technique) in which trypanosomes are concentrated at the vicinity of the buffy coat. Further improvements of the technique was achieved by cutting the micro-capillary tube and exuding the buffy coat and adjacent red blood cells onto a microscope slide. The contents are subsequently examined under phase contrast/dark ground microscopy to identify trypanosomes (Murray et al 1977). This modification, often referred to as buffy coat technique, is useful in detection of *T. congolense* which tends to stick to red cells unlike the Woo technique which is better in the detection of *T. brucei.*Other concentration methods in use involve removal of blood cells either by lysing them or by anion exchange chromatography. Despite all the modifications introduced, the paucity of trypanosomes in the blood of chronically infected animals limits the use of the parasitological techniques alone in diagnosis. This has proved to be notoriously difficult in field studies designed to examine the prevalence of trypanosomosis (Nantulya 1990). These shortcomings necessitated the development of alternative diagnostic techniques, one of which is an antibody detection ELISA (Nantulya 1990; Luckins 1977). These assays provide information about exposure of animals to the parasite, but cannot differentiate current from past infections.

Antigen-detection enzyme-linked immunosorbent assay (Ag-ELISA)

Tremendous efforts have gone into the development of highly sensitive and specific diagnostic techniques, which detect current infections. To do this, species-specific monoclonal antibodies (MAbs) were generated based on the hybridoma technology. Monoclonal antibodies have been raised against non-variable trypanosomal antigens which distinguish between *T. brucei, T. congolense* and *T. vivax* (Nantulya et al 1987). Monoclonal antibodies reacting with *T. congolense* and *T. vivax* are species-specific while those capturing *T. brucei* antigens have been found to react with *T. b. gambiense, T. b. rhodesiense, T. evansi* and *T. equiperdum.* The MAb raised against the Trypanozoon group of parasites recognises an antigen on the plasma membrane whose epitope is resistant to periodate oxidation (Nantulya and Lindquist 1989). These genus- and species-specific MAbs have been incorporated into an antigen-trapping ELISA for detecting trypanosome antigens in the blood of infected animals and human patients.

In cattle the MAbs capture trypanosome antigens in blood before or coinciding with the onset of parasitaemia. The trypanosome antigens have been shown to increase and persist in the blood even when parasites were not demonstrable by the haemoconcentration techniques (Figure 1; Nantulya and Lindquist 1989; Masake et al 1991, 1995). The ability of the assay to detect infections associated with very low numbers of parasites in circulation has facilitated its use in revealing presence of the trypanosomes in chronically infected animals. To assess the specificity of the Ag-ELISA, serum samples were obtained from aparasitaemic goats residing in areas known to be either endemic with or free of trypanosomosis. These were examined for the presence of trypanosome antigens. Out of 131 samples collected from goats in endemic areas,

106 were positive for trypanosome antigens, while all the 24 sera from trypanosome-free regions were negative on Ag-ELISA (Figure 2). The sensitivity of a monoclonal antibody-based Ag-ELISA for the diagnosis of *T. congolense* was evaluated using sera from experimentally infected goats and cattle. Ten goats (Galla × East African Maasai) and seven steers (Bos indicus) were infected with different clones of *T. congolense* and left to run a chronic course for 46 and 24 months, respectively. During this period, monthly blood samples were collected and analysed for the presence of trypanosomes and their antigens in peripheral blood. Of 383 caprine blood samples examined, 361 were positive for circulating antigens whereas only 43 had demonstrable trypanosomes as revealed by the microhaematocrit centrifugation technique. In cattle, 570 (82%) out of 691 blood samples were Ag-ELISA positive compared to 136 (20%) samples with detectable trypanosomes (Table 1; Masake and Nantulya 1991).

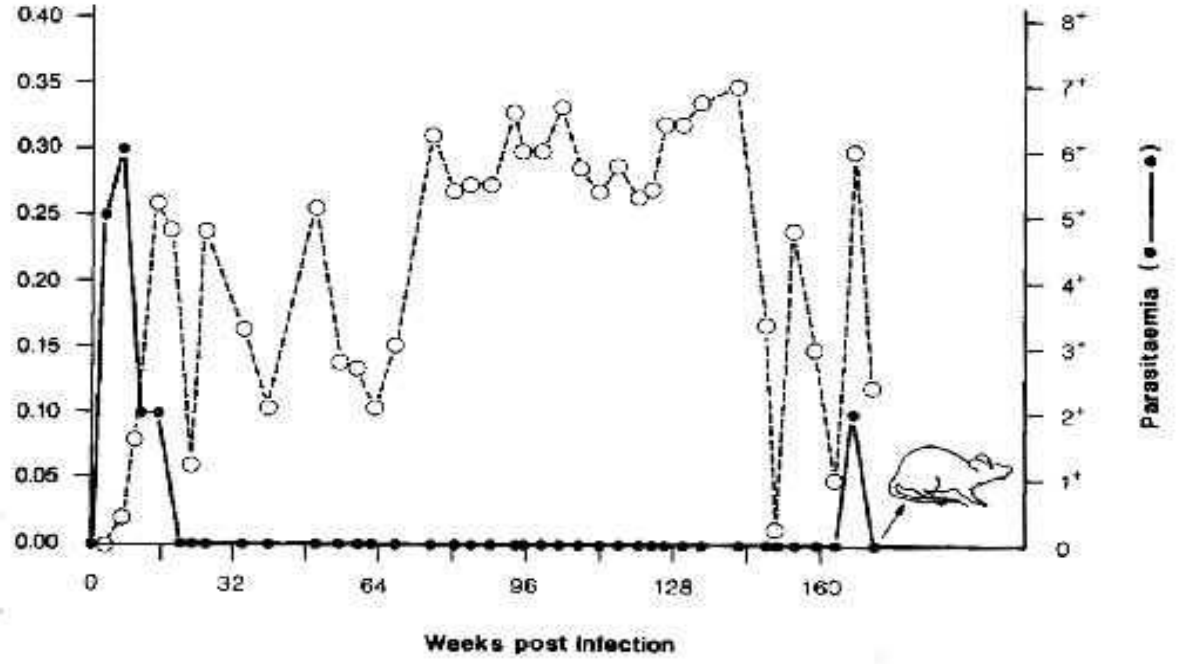

Figure 1. *Comparison of the sensitivity of the antigen-ELISA and the buffy coat technique (BCT) in the diagnosis of* Trypanosoma congolense *infection in goat number 0–474. A mouse inoculated with blood from the goat 176weeks post-infection became infected with* T.congolense *parasites. Note the persistence of trypanosome antigens in the absence of dectable parasitaemia. Optical density (OD) values of 0.05 and above were considered positive.*

Similarly, sensitivity of Ag-ELISA for *T. brucei* was evaluated by infecting four Boran cattle with *T. brucei* using *Glossina morsitans centralis* and which were left untreated throughout the experimental period of 18 months. During this period, sequential blood samples were collected and examined for the presence of anti-trypanosome antibodies and trypanosome antigens. Using the microhaematocrit centrifugation technique, trypanosomes were detected in 38 of the 233 (16%) blood samples. Unlike the microhaematocrit centrifugation technique, Ag-ELISA diagnosed infections in 189 (81%) of the blood samples (Table 2; Masake et al 1995).

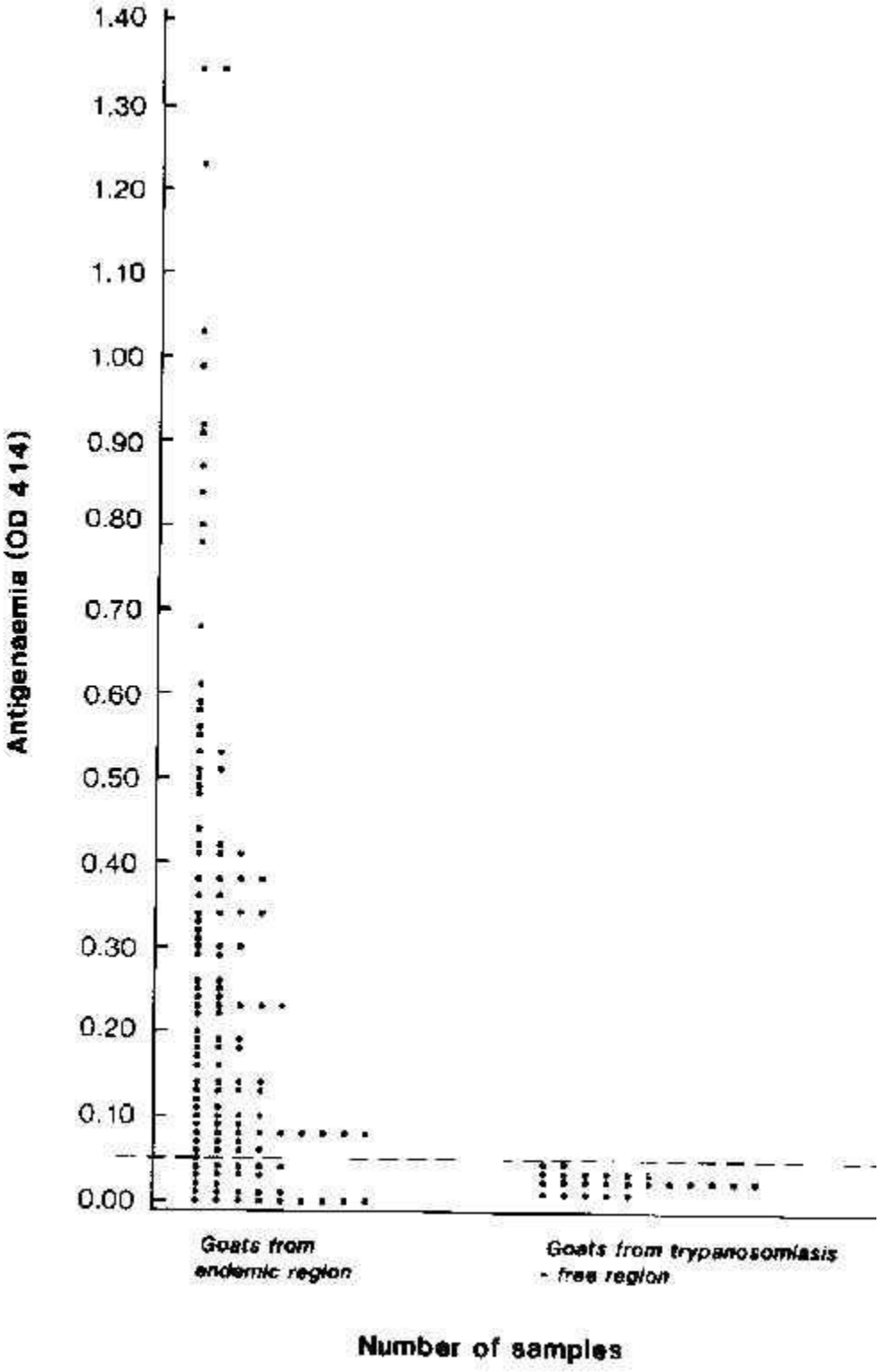

Figure 2. *The ability of Tc39-based Ag-ELISA to capture trypanosome antigens in (a) field sera obtained from aparasitaemic goats residing in a trypanosomiasis endemic area and (b) a*

trypanosomosis-free area. Note the high proportion of Ag-ELISA positive sera from the endemic area as opposed to those negative in trypanosomosis-free area. Optical density (OD) values of 0.05 (interrupted horizontal line) and above were regarded as positive.

The ability of Tc39-based Ag-ELISA to capture trypanosome antigens in field sera obtained from aparasitaemic goats residing in (a) a trypanosomosis-endemic area and (b) a trypanosomosis-free area. Note the high proportion of Ag-ELISA positive sera from the trypanosomosis-endemic area as opposed to those negative in trypanosomosis-free area. Optical density (OD) values of 0.05 (interrupted horizontal line) and above were regarded as positive.

Table 1. C*omparison of the sensitivity of antigen-ELISA and the buffy coat technique (BCT) in the detection of infections in cattle experimentally infected with* T. congolense.

Animal No.	Number of samples tested	Number positive by BCT (%)	Ag-ELISA (%)
D532	99	26 (26)	76 (77)
D533	99	18 (18)	89 (90)
D534	99	8 (8)	61 (62)
D538	98	29 (30)	94 (96)
D541	97	22 (23)	76 (78)
D542	99	11 (11)	85 (87)
D638	100	22 (22)	89 (89)
Total	691	136 (20)	570 (83)

Table 2. *Comparison of the sensitivity of antigen-detection ELISA and the buffy coat (BCT) in diagnosing* T. brucei *infection in experimentally infected cattle.*

Animal No.	Number of samples tested	Number positive by BCT (%)	Ag-ELISA (%)
BH329	57	14 (25)	46 (81)
BH346	59	8 (14)	34 (58)
BH347	59	8 (14)	53 (90)
BH349	58	8 (14)	56 (97)
Total	233	38 (16)	(189) (81)

Further, an Ag-ELISA based on monoclonal antibody specific for Trypanozoon group was tested for its reactivity with *T. b. gambiense* (Nantulya et al 1992) and *T. b. rhodesiense* (Nantulya 1989; Komba et al 1992; Gichuki et al 1994). Cerebral spinal fluid (CSF) and sera were collected from human clinical cases of *T. b. gambiense* sleeping sickness in Daloa and Kinshasa clinics. The sera and CSF from parasitologically confirmed cases were examined by Ag-ELISA for the presence of trypanosomes. Out of 265 cases, 241 were positive (91%) on Ag-ELISA. In four of these Ag-ELISA positive cases, trypanosome antigens were found only in the CSF (Nantulya et al 1992). Clearance of trypanosome antigens in the peripheral blood and CSF occurred following chemotherapeutic intervention. Control sera for the evaluation of the assay were obtained from 165 blood donors and 40 patients with malaria, 2 with hydatidosis and 12

with leishmaniasis. The optical density (OD) reading of these sera on Ag-ELISA all fell below the cut off OD 0.05.

The performance of the Ag-ELISA in revealing *T. b. rhodesiense* infections was evaluated in humans and monkeys (Komba et al 1992; Gichuki et al 1994). In the case of human trypanosomosis, 363 patients seen in four different clinics in Uganda, Tanzania and Zambia with detectable parasites either by thick blood film, haematocrit centrifugation technique, miniature anion-exchange chromatography or on single and double centrifugation of CSF were examined by Ag-ELISA for the presence of circulating trypanosome antigens. Three hundred and thirty-two out of 363 were positive on Ag-ELISA. This represented a detection rate of approximately 92%. More importantly, no cross reactivity with bacterial, viral and parasitic diseases prevailing in these areas was observed in 111 patients. However, out of 20 blood donors residing in an area known to be endemic for trypanosomosis one was positive on Ag-ELISA (Komba et al 1992). Prior to the above study, 375 blood donor sera from Uganda were examined and shown to be negative on Ag-ELISA using the recommended dilution factor and no cross-reactivity occurred with sera from either 10 leishmaniasis, 17 schistosomiasis or 23 malaria patients (Nantulya 1989). However, when 211 serum samples from clinically suspected cases of trypanosomosis were subjected to Ag-ELISA, 136 were positive (64%). All of these patients were negative by all the parasitological tests employed. Hence Ag-ELISA can serve as a complementary test to parasitological diagnosis as well as assist in obtaining correct diagnosis at certain stages of the disease. Furthermore, the assay can be employed in the assessment of efficacy of trypanocidal drugs (Gichuki et al 1994).

Trypanosome antigens captured by the monoclonal antibodies applied in Ag-ELISA

Trypanosoma vivax-specific antigen recognised by monoclonal antibody Tv27 is a 10 kDa protein. The protein is diffusely distributed throughout the cytosol and nucleus of metacyclics, bloodstream form parasites and procyclic-like elongated trypomastigotes, but was not detectable in the epimastigotes of *T. vivax.* The gene encoding the 10 kDa protein has been isolated and sequenced. The gene is repeated in tandem with a monomeric unit length of 900 bp. Amino acid analysis did not reveal any significant identity with any known proteins. Thus the function of this protein is unknown.

The monoclonal antibody (Tc38.51 and Tc39) for capturing *T. congolense* antigens recognises a protein doublet of 38/40 kDa. The antigen is expressed by all stages of *T. congolense.* The gene encoding this antigen was identified, isolated and sequenced. The DNA isolated was 1.8 kb. The size of hybridising genes varied in size from one isolate to another giving sizes such as 2.8, 3.6, 5.4, and 6.6 kb. These sizes were suggestive of a tandemly repeated cDNA. This was subsequently proven using a time course digestion of genomic DNA derived from different types of *T. congolense*and shown to exhibit 20–30 repeats. The DNA insert obtained hybridised to the *T. congolense* genome but not to that of *T. vivax.* Cross reactivity was observed with the *T. brucei* genome at low stringency washings (Jaye 1993).

Amino acid analysis of the gene revealed extensive homology (96%) with another *T. congolense* cysteine protease sequenced by Fish et al (1995). It is apparent that there is a family of cysteine proteases within the Nannomonas group. Furthermore, there was a 69% homology with a cysteine protease isolated from *T. b. brucei* and 70% identity to that of *T. b. rhodesiense* (Pamer et al 1990).

Trypanosoma brucei antigen captured by the Tr7 monoclonal antibody has not been isolated. Nevertheless, the antigen captured is located on the plasma membrane and the epitope recognised is a protein since it is resistant to periodate oxidation.

New generation of monoclonal antibody-based Ag-ELISA

Despite the high sensitivity displayed by Ag-ELISA, false negatives were sometimes observed in the presence of detectable parasitaemia. This was particularly evident in the field validation report obtained from Mali (Bamako) whilst the field studies carried out in Uganda recorded less than 9.3% false negatives. In order to improve further the sensitivity of Ag-ELISA IgG1 isotype monoclonal antibodies have been made. Studies conducted using IgG1 monoclonal antibodies derived against Trypanozoon groups T43 and *T. congolense* (Tc38.51)-specific in the laboratory yielded similar results to those obtained using IgM (Tr7 and Tc39) isotypes. The sensitivity of MAb T43 ranged from 32% to 90% when tested on serial serum samples recovered from individual animals but gave an average sensitivity of 70% as opposed to 37% obtained on parasitological examination.

Bovine serum samples were collected from a trypanosomosis endemic area and examined for the presence of *T. brucei* antigens. Of 80 samples, 26 had detectable trypanosome antigens; when a similar exercise was conducted on 80 bovine sera obtained from a trypanosome-free region, all results were negative. Studies on the *T. congolense* Ag-ELISA based on Tc38.51 yielded data similar to that of Tc39 (IgM isotype currently in use). Reactivity of Ag-ELISA based on Tc39 (IgM) and Tc38.51 (IgG) were compared on 80 bovine sera from each of the areas endemic for, or free of, trypanosomosis; 18 out of the 80 sera from the endemic area were positive for the two MAbs and none of the sera from trypanosomosis-free area was positive.

These studies were expanded and the new and original monoclonal antibodies tested on 200 serum samples obtained from Kapiti plains. The farm, which is located next to the main highway connecting the Kenya coast and Nairobi, is believed to be free from trypanosomosis. The four monoclonal antibodies were examined for their specificity in capturing trypanosome antigens on the 200 bovine sera as shown in Table 3. The results obtained indicate a slightly higher specificity (98.5 and 99.5%) for the IgG1 isotype when compared to the IgM (92.5 and 95%).

Table 3. *Specificity of T43, Tr7, Tc38.51 and Tc39 in capturing trypanosome antigens in bovine sera obtained from Kapiti plains.*

Mabs	Isotype	No. of bovine sera	No. of positive sera	No. of negative sera	Specificity of the MAbs (%)
T43	IgG_1	200	1	199	99.5
Tr7	IgM	200	10	190	95.0
Tc39	IgM	200	15	185	92.5
Tc38.51	IgG_1	200	3	197	98.5

Table 4. *The sensitivity of Ag-ELISA based on T43, Tr7, Tc38.51 and Tc39 when used for trapping trypanosome antigens in bovine sera obtained from 4 trypanosome endemic areas*

Origin	No. of samples	No. and percentage of sera positive by Ag-ELISA based on

Apatit	72	57 (79%)	15 (21%)	12 (17%)	59 (82%)
Katelenyang'	96	71 (74%)	27 (28%)	30 (31%)	85 (89%)
Ng'elechom	163	95 (58%)	26 (16%)	35 (21%)	138 (85%)
Rukada	181	22 (12%)	70 (39%)	70 (39%)	129 (71%)

Subsequently more bovine sera were obtained from endemic areas, namely Apatit, Katelenyang', Ng'elechom and Rukada. These were assayed for the presence of trypanosome antigens using ag-detection ELISA based on T43, Tr7, Tc38.51 and Tc39. This study showed that T43 is much more sensitive than Tr7 in the field while Tc39 trapped antigen in more sera than Tc38.51. It was even more interesting to note that Ag-ELISA based on T43 captured antigens in a high proportion of bovine sera obtained from cattle residing at Apatit, Katelenyang' and Ng'elechom, which are endemic for human sleeping sickness (Table 4). Furthermore the cattle found to be positive for *T. brucei* on parasitological test were also positive using T43-based Ag-ELISA. The same correlation was observed between Tc39-based Ag-ELISA and the parasitological technique.

The number of animals found to be harbouring trypanosome antigens using T43 were much less in Rukanda, a place not associated with human sleeping sickness. The field data would therefore suggest that T43 trapped Trypanozoon antigens since the specificity of the Ag-ELISA based on this Mab is approximately 99%. On the contrary Mab Tc38.51 performed poorly when compared to the IgM isotype (Tc39). Tc38.51 failed to trap antigens in most of the parasitologically positive cases of *T. congolense.*

Steps taken to optimise the sensitivity of Ag-ELISA

Attempts were made to improve the performance of Ag-ELISA by altering various parameters such as temperature, chromogen system and volume of test material in the reaction mixture. The assay is usually run at room temperature at ILRI. Change of temperature from prevailing room temperature to 37°C resulted in minimal increase in optical density reading. But when changes in temperature were combined with use of 3',3', 5',5'-tetramethylbenzidine (TMB) instead of Azino bis (3-ethyl)-benzthiazoline-6-sulphonic (ABTS), the increase in optical density readings were significant. Further increases in optical density readings were obtained when the volume of sera introduced into the microtiter plate was increased from 10 al to 40 al in a reaction volume of 100 al. This increase in volume of the test sample did not cause a rise in background reading as seen in the Figure 3.

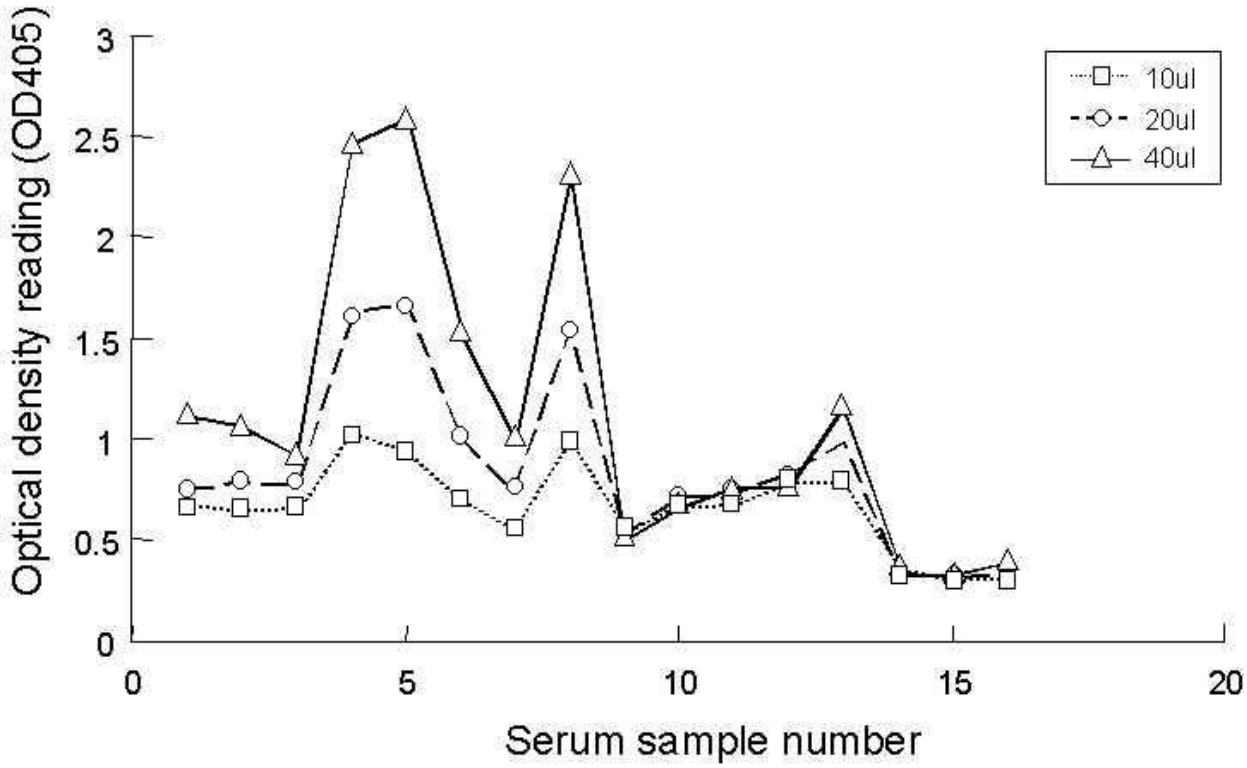

Figure 3. *Effect of volume on optical density (OD) readings.*

References

Applewhaite L.M. 1990. Small ruminant trypanosomiasis in Guyana: a preliminary report. *British Veterinary Journal* 146:93–94.

Assoku R.K.G. and Gardiner P.R. 1989. Detection of antibodies to platelets and erythrocytes during infection with haemorrhage-causing *Trypanosoma vivax* in Ayrshire cattle. *Veterinary Parasitology*31:209–216.

Boid R. and Mleche W.C.H. 1985. Isoenzyme analysis of stocks of trypanosomes isolated from cattle in Indonesia. *Research in Veterinary Science* 39:388–389.

Fish W.R., Nkhungulu Z.M., Muriuki C.W., Ndegwa D.M., Lonsdale-Eccles J.D. and Steyaert J. 1995. Primary structure and partial characterization of a lifecycle-regulated cysteine protease from*Trypanosoma (Nannomonas) congolense. Gene* 161:125–128.

Gichuki C.W., Nantulya V.M. and Sayer P.D. 1994. *Trypanosoma brucei rhodesiense:* use of an antigen-detection enzyme immunoassay for evaluation of response to chemotherapy in infected vervet monkeys *(Cercopithecus aethiops). Tropical Medicine and Parasitology* 45:237–242.

Jaye A. 1993. Characterisation of a *Trypanosoma (Nannomonas) congolense*-specific antigen: identification as a thiol protease precursor. PhD Thesis, Brunel University, Uxbridge, England.

Komba E., Odiit M., Mbulamberi D.B., Chimfwembe E.C. and Nantulya V.M. 1992. Multicentre evaluation of an antigen-detection ELISA for the diagnosis of *Trypanosoma brucei rhodensiense*sleeping sickness. *Bulletin of the World Health Organisation* 70:57–61.

Losos G.J. and Ikede B.O. 1972. Review of pathology of diseases in domestic and laboratory animals caused by *Trypanosoma congolense, T. vivax, T. brucei, T. rhodesiense* and *T. gambiense. Veterinary Pathology* 9 (supplement):1–72.

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

Masake R.A. 1980. The pathogenesis of infection with *Trypanosoma vivax. Veterinary Record* 107:551–557.

Masake R.A. and Nantulya V.M. 1991. Sensitivity of an antigen-detection enzyme immunoassay for diagnosis of *Trypanosoma congolense* infections in goats and cattle. Journal of Parasitology 77: 231–236.

Masake R.A., Moloo S.K., Nantulya V.M., Minja S.H., Makau J.M. and Njuguna J.T. 1995. Comparative sensitivity of antigen-detection enzyme immunosorbent assay and microhaematocrit centrifugation technique in the diagnosis of *Trypanosoma brucei* infections in cattle. *Veterinary Parasitology* 56:37–46.

Murray M., Murray P.K. and McIntyre W.I.M. 1977. An improved parasitological technique for the diagnosis of African trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 71:325–326.

Nantulya V.M. 1989. An antigen-detection enzyme-linked immunoassay for the diagnosis of rhodesiense sleeping sickness. *Parasite Immunology* 11:69–75.

Nantulya V.M. 1990. Trypanosomiasis in domestic animals: the problem of diagnosis. *Revue Scientifique et Technique de I'Office International des Epizooties* 9:357–367.

Nantulya V.M. and Lindqvist K.J. 1989. Antigen-detection enzyme immunoassays for diagnosis of *Trypanosoma vivax, T. congolense* and *T. brucei* infections in cattle. *Tropical Medicine and Parasitology* 40: 267–272.

Nantulya V.M., Doua F. and Molisho S. 1992. Diagnosis of *Trypanosoma brucei gambiense* sleeping sickness using an antigen-detection enzyme-linked immunosorbent assay. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 86:42–45.

Nantulya V.M., Musoke A.J., Rurangirwa F.R., Saigar M. and Minja S.H. 1987. Monoclonal antibodies that distinguish *Trypanosoma congolense, T. vivax* and *T. brucei. Parasite Immunology* 9: 421–431.

Pamer E.G., Davis C.E. Eakin A. and So M. 1990. Cloning and sequencing of the cysteine protease cDNA from *Trypanosoma brucei rhodesiense. Nucleic Acids Research* 18: 6141.

Shaw J.J. and Lainson R. 1972. *Trypanosoma vivax* in Brazil. *Annals of Tropical Medicine and Parasitology* 66:25–32.

Wells E.A. 1984. Animal trypanosomiasis in South America. *Preventive Veterinary Medicine* 2:31–41.

Woo P.T.K. 1971. Evaluation of the haematocrit centrifuge and other techniques for the field diagnosis of human trypanosomiasis and filariasis. *Acta Tropica* 28:298–303.

Performance of a direct sandwich enzyme-linked immunosorbent assay for detection of trypanosomal antigens

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Summary

Following the initial development of an enzyme-linked immunosorbent assay (ELISA) at the International Livestock Research Institute (ILRI, formerly at ILRAD), the methodology and the monoclonal antibody producing hybridoma cell lines were transferred from ILRI to the Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA) Agriculture and Biotechnology Laboratory in Seibersdorf. Subsequently, it was necessary to perform various assay adjustments in order to standardise and transform the assay to a kit form for distribution to research and diagnostic laboratories in Africa. Initial results on the sensitivity of the test were obtained by experimental infections in goats, while results from negative populations in the field were used for the assessment of the test specificity. Training of scientists in ELISA techniques and the transfer of the methodology was successfully achieved during the six years of a Co-ordinated Research Programme funded by the Government of the Netherlands and executed by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture (1987–1992). During an additional two years the test was used in combination with standard parasitological techniques for monitoring tsetse and trypanosomosis control programmes with funding from the IAEA Technical Co-operation Department and the British Government (1993–1995). A second Co-ordinated Research Programme funded by the Netherlands Government has been initiated to develop and use improved antibody- and antigen-detection ELISA for the diagnosis of trypanosomosis and the monitoring of control and eradication programmes in Africa.

Introduction

The Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, Austria, operates the sub-programme of Animal Production and Health, which is one of five currently existing sub-programmes. Support for the operation of these sub-programmes is provided through five sections within the Joint FAO/IAEA Division and five units of the FAO/IAEA Agriculture and Biotechnology Laboratory in Seibersdorf.

The mandate of the Animal Production and Health Sub-programme is to improve livestock production in developing countries through the support of problem-oriented research that identifies the constraints of production and develops cost-effective and sustainable solutions (using nuclear-based technologies). Improvements in animal health are approached by supporting the introduction and use of nuclear and related techniques in developing countries through Co-ordinated Research and Technical Co-operation Programmes. A Co-ordinated

Research Programme (CRP) entitled 'Improving the Diagnosis and Control of Trypanosomosis and Other Vector-borne Diseases of African Livestock Using Immunoassay Methods' was executed from 1987 to 1992 with funding from the Directorate General of Development Co-operation (DGIS) of the Government of the Netherlands. The International Livestock Research Institute (ILRI) in Nairobi, Kenya, succeeded in the development of enzyme-linked immunosorbent assay (ELISA) systems for the detection of cytoplasmic antigens of *Trypanosoma congolense, T.vivax* and *T. brucei* in cattle (Nantulya et al 1987). For the purpose of the CRP, a ready-to-use ELISA kit form was assembled, consumables and training were provided and small research projects for field validation of the ELISA were initiated. A network which originally consisted of 13, but was subsequently reduced to 11, national agricultural research systems (NARS) was set up with the active collaboration of ILRI and the Centre of Tropical Veterinary Medicine (CTVM) in the UK. The reports by the various research contract holders were compiled and published as a technical document (IAEA 1993) together with conclusions, recommendations and reports by experts in the field. The continuation of the programme will be briefly described with emphasis on the performance of the antigen-detection ELISA following the transfer of biological reagents from ILRI to the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria in December 1992.

Laboratory activities (1993–1996)

Training and transfer of biological reagents and methodology

ILRI developed monoclonal antibodies (MAbs) against the conserved, invariant, non-secreted internal antigens of tsetse fly transmitted *T. brucei, T. congolense* and *T. vivax.* The antigens are released upon parasite destruction. During the period from October to December 1992 a FAO/IAEA staff member undertook a scientific visit to ILRI to become familiar in the handling of hybridoma cell lines and in production and quality assurance aspects of specific monoclonal antibodies and conjugates used in the direct sandwich ELISA. Following the training period, the MAbs-producing hybridoma cell lines, trypanosome stabilates and the relevant methods were transferred to the FAO/IAEA Agriculture and Biotechnology Laboratory.

Establishment of a cell culture laboratory and laboratory animal facilities

At the laboratory's Animal Production Unit (APU), a cell culture laboratory and facilities for accommodation of mice, rats and rabbits for experimental purpose were established to provide technical capabilities that are crucial for quality assured routine bulk production of biological reagents. The laboratory has the capacity for

- thawing, cryopreserving, and propagation of hybridoma cell lines including quality control of both cell lines and media components for, in particular, mycoplasma, IBR-, BVD-, BRS- and PI-3 virus contamination
- production of MAbs in Balb/c mice ascitic fluid by injection of monoclonal antibody producing hybridoma cells (T.b.7/47.34.25 IgM anti-*T. brucei*; T.c. 39/30.38.11 IgM anti-*T. congolense*; T.v. 27/9.45 IgG1 anti-*T. vivax).* Since this method is no longer accepted by the scientific community for ethical reasons, production methods have been changed to replace the in vivo system by an in vitro one recommended as the preferred method for routine bulk production of monoclonal antibodies
- purification of MAbs using fast performance liquid chromatography (FPLC) system

- production of direct-reporting monoclonal antibodies by conjugation of purified MAbs with horseradish peroxidase enzyme
- sodium dodoecyl sulphate, polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot, as well as ELISA technique used for quality assurance of MAb production
- propagation of trypanosome bloodstream forms in mice *(T. vivax* IL2160) and rats *(T. brucei* IL2616, *T. congolense* IL3266)
- routine diagnosis of trypanosomosis by standard parasitological methods such as wet blood smears, Giemsa-stained thin and thick blood smears, micro haematocrit centrifuge technique and phase contrast/dark ground technique for microscopic examination of the buffy coat, separation of trypanosomes from blood by anion exchange chromatography column
- preparation of trypanosome antigen lysates
- experimental infection studies on goats/cattle to produce monospecific trypanosomal antigen reference serum samples and to assess diagnostic sensitivity and specificity of the direct sandwich ELISA.

At the end of 1993, the MAbs and their conjugates produced at the APU were tested and cross-referenced to those produced at ILRI to ensure that no aberrations occurred during the transfer of hybridoma cell lines to Seibersdorf. A comparative titration of these reagents performed according to the ILRI protocol of the antigen-detection ELISA indicated that the Seibersdorf-produced reagents did not differ from the ILRI reagents.

Table 1. *Minor assay adjustments incorporated in the protocol of the FAO/IAEA direct sandwich ELISA for trypanosomal antigen detection.*

1	High binding plate (Nunc Immuno Plate MaxiSorp™).
2	Constant shaking for 15 minutes at 37°C.
3	Control and test serum samples diluted 1:20.
4	Addition to the serum diluent buffer of 0.5 % normal mouse serum as a liquid phase blocking agent.
5	Addition to the conjugate diluent buffer of 1.0 % bovine serum albumin as a liquid phase blocking agent.
6	Use of 3', 3' ,5', 5' tetramethylbenzidine chromogen and hydrogen peroxide
7	Use of non corrosive 1M phosphoric acid as stopping solution.

Assay adjustments

In 1994, the APU's cell culture laboratory went into full-scale production of MAbs in ascitic fluid for use in the trypanosomosis ELISA kit. Minor adjustments as listed in Table 1 were incorporated in the protocol of the FAO/IAEA direct sandwich ELISA to increase the diagnostic specificity of the assay and improve the robustness of the test for use under laboratory conditions in Africa. Moreover, standardised internal quality control samples were incorporated including strong positive, moderate positive and negative antigen control, as well as conjugate control samples. All test samples were expressed as percent positivity relative to the strong positive antigen control, calculated by a computer software programme developed for the ELISA kit.

In addition, the counterpart laboratories using ELISA technology were assessed for proficiency as part of the FAO/IAEA external quality assurance programme for diagnostic laboratories.

ELISA sensitivity

Two goats were experimentally infected with 1.5 × 108 *T. vivax* bloodstream forms. Blood samples were taken daily. The course of infection was assessed by determining the appearance of parasites and antigen in the systemic circulation, packed red cell volume (PCV) values, and rectal body temperature. When severe clinical symptoms were observed, the goats were treated with half the recommended dosage of Berenil® to induce chronic infection.

Trypanosomes first appeared in both goats on day 5 post infection (pi), followed by antigen on day 5 pi (goat #20) and day 7 pi (goat #191), respectively. The presence of antigen detected by ELISA did not always coincide with the appearance of parasites detected by the buffy coat technique (BCT). After 83 days pi (goat #20) and 103 days pi (goat #191), neither parasites in goats #20 and #191 nor antigen in goat #20 were observed. Interestingly, goat #191 remained positive by ELISA until the end of experimental period (416 days pi). Optical density (OD) values were similar high to those previously observed at parasite-confirmed stages; false positive results might have been due to circulating anti-idiotypic antibodies produced during chronic infections. At certain stages of infection, the ELISA failed to give positive results although circulating trypanosomes were demonstrated microscopically. A full dosage of Berenil® for final treatment was administered on day 103 pi (goat #20) and day 125 pi (goat #191). On day 396 pi, an induced relapse of parasitaemia and antigenaemia was unsuccessfully attempted by non-steroidal drug treatment (Flurbiprofen®) over a period of seven days. The experiment was terminated at day 416 pi. For goat #20, the BCT sensitivity was 41.3% and the ELISA sensitivity 30.7%. For goat #191, the diagnostic sensitivity of the BCT and the ELISA was 40.2% and 45.1%, respectively. Although the results demonstrated that the ELISA used in combination with BCT improved the diagnosis of trypanosomosis in goats, the sensitivity of the test needed improvement. The low sensitivity of the test can be attributed to i) low concentration of detecting antibody used, ii) masking of trypanosomal antigens by host-produced antibodies forming immune complexes during the course of infection, and iii) competition of capture and detecting antibodies for the same *T. vivax*-specific epitope.

In comparisons with other diagnostic techniques the results from positive cattle populations in tsetse-infested areas in Africa demonstrated that the ELISA was more sensitive than parasitological techniques in the detection of *T. brucei* but less sensitive in the detection of *T. congolense* or *T. vivax.*

ELISA specificity

The ELISA had a diagnostic specificity in the range of 94–100% using a cut-off value of 10 percent positivity *(T. brucei* 96% ± 2%, *T. congolense* 99.5% ± 1%, *T. vivax* 99% ± 1%) when applied to negative cattle populations in tsetse-free areas in Africa.

Training activities

The FAO/IAEA direct sandwich ELISA was demonstrated at the FAO/IAEA/ILRI training course on 'Diagnostic Methods for Animal Trypanosomiasis', held from 16 May to 10 June 1994 at the International Livestock Research Institute (ILRI), Nairobi, Kenya and the FAO/IAEA inter-

regional training course on 'The Use of Immunoassay and Molecular Methods for Animal Disease Diagnosis and Control', held at Seibersdorf from 24 October to 25 November 1994. Furthermore, ELISA training was provided at the APU to researchers from Zanzibar on individual basis.

Technical co-operation programme (TCP) RAF/5/028, 1993–1995

Following the completion of the CRP which terminated at the end of 1992, the IAEA Department of Technical Co-operation and the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture provided support for a regional TCP (RAF/5/028) for an additional two years from mid-1993 to mid-1995. Financial assistance for this TCP was obtained from a voluntary contribution by the Overseas Development Agency of the United Kingdom (ODA-UK) and from funds allocated by the IAEA Department of Technical Co-operation for this purpose. The TCP assisted in strengthening disease diagnosis in nine national agricultural research systems (NARS) and supported the introduction of improved diagnostic techniques in five additional institutes. In addition, the TCP provided equipment to install serum banks, fellowships for additional training in data analysis and expert missions to resolve specific problems. The majority of the institutes that participated in the original FAO/IAEA CRP were assisted for an additional two years. At the initiation of the TCP, five institutes in Burkina Faso, Cameroon, Côte d'Ivoire, Ethiopia and Nigeria were added, while two institutes in Morocco and the Gambia were removed from the list of those being supported (Table 2). The shipment of FAO/IAEA direct sandwich ELISA kits to 14 research contract holders, and to Zanzibar (IAEA technical co-operation project on tsetse fly eradication from the island using the sterile insect technique) was completed at the beginning of 1995. The scientific results obtained by the participants at the FAO/IAEA/ILRI epidemiology workshop on the monitoring of trypanosomosis and tsetse control programmes, held at the International Livestock Research Institute (ILRI) in Addis Ababa, Ethiopia, from 17–28 April 1995 have been published as an IAEA technical document (IAEA 1997).

Table 2. *African institutes assisted by the IAEA department of technical co-operation through TCP RAF/5/028.*

Country	Research institute	Principal investigator
Burkina Faso	CIRDES	Z. Bengaly
Cameroon	LANAVET	C. Ndamkou
Côte d'Ivoire	LANADA	A. N'Depo
Ethiopia	NTTICC	N. Tewelde
Ghana	Central Veterinary Lab.	C. Doku
Kenya	KETRI	W. Olaho-Mukani
Mali	Lab. Central Veterinaire	O. Diall
Nigeria	NVRI	S. Ajayi
Senegal	ISRA/LNERV	M. Seye
Sudan	University of Khartoum	E. Elamin
Uganda	LIRI	N. Okuna
United Republic of Tanzania	ADRI	H. Mbwambo
Zambia	CVRL	L. Sinyangwe

Zimbabwe	CVL	R. Ries

Co-ordinated Research Programme (CRP), 1995–1999

In 1995, the Directorate General of Development Co-operation (DGIS) of the Government of the Netherlands started funding the FAO/IAEA Co-ordinated Research Programme entitled 'The Use of an Immunoassay Method to Improve the Diagnosis of African Trypanosomosis and its Application to Monitor Disease Control Programmes' in which 15 research contracts (Figure 1) and four research agreements were awarded. The research agreement holders are scientists based at the University of Glasgow, Centre for Tropical Veterinary Medicine (CTVM) in Edinburgh, ILRI, Nairobi, and the Centre de coopération internationale en recherche agronomique pour le développement—départment d'élevage et de médicine vétérinaire (CIRAD-EMVT) in Montpellier. The shipment of FAO/IAEA direct sandwich ELISA kits to 15 research contract holders, and to Zanzibar (IAEA technical co-operation project) was completed at the beginning of 1996. The results were presented at the first research co-ordinated meeting which was held from 9–13 September 1996 in Dakar, Senegal.

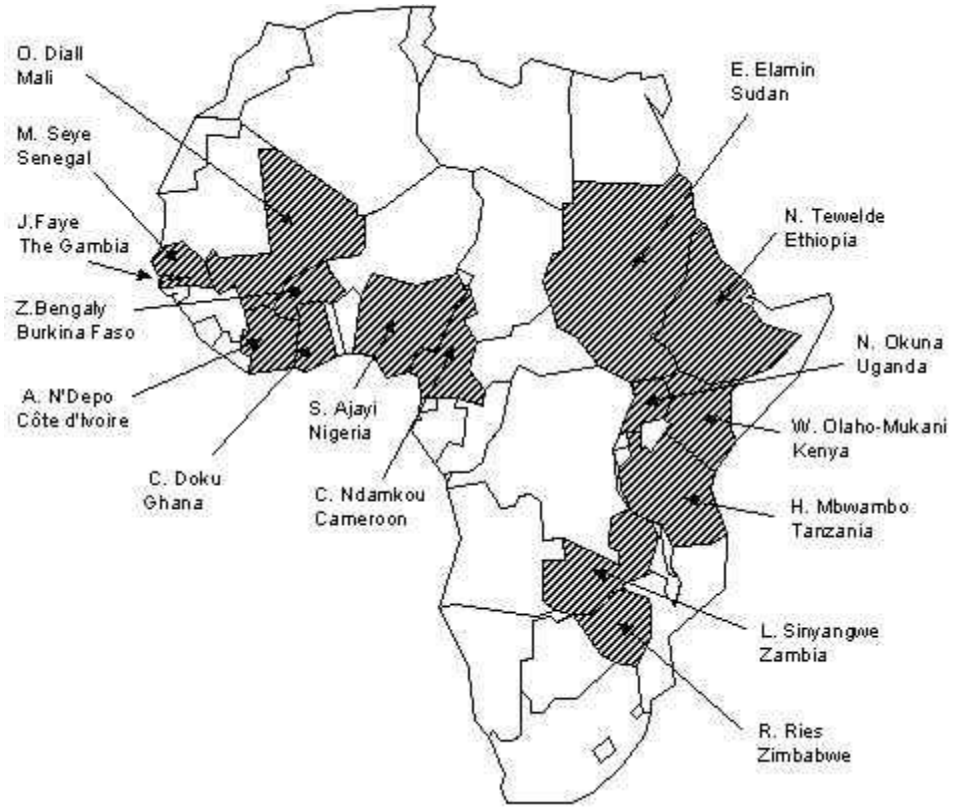

Figure 1. *African institutes participating in the joint FAO/IAEA Co-ordinated Research Programme (CRP), 1995–1999.*

The results confirmed the diagnostic specificity of the ELISA in the range of 90–100% using a cut-off value of 10 percent positivity. However, the sensitivity had not been improved. Therefore, a reassessment of the antigen-ELISA performance was recommended. The research contract holders received ELISA reagents to initiate the FAO/IAEA external quality assurance programme to assess individual laboratory proficiency.

Conclusions

ELISA technology was successfully established in 15 African laboratories belonging to the NARS. Consequently, an extensive network has become available for the application of an antigen-detection ELISA used in combination with parasitological techniques for improved diagnosis of trypanosomosis and monitoring trypanosomosis control programmes. Through the project initiated by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, Austria, various international research and diagnostic institutes in Africa have been assisted in the collection and analysis of ELISA and parasitological data from field and experimental studies during the past eight years. The results indicated that the diagnostic sensitivity of the ELISA was unsatisfactory compared with standard parasitological techniques. Therefore, the improvement and reassessment of the antigen-detection ELISA was initiated under supervision of a wider group of scientists. The group will be requested to prioritise and execute research activities for development of a new generation of immunoassays required for detection of anti-trypanosomal antibodies and trypanosomal antigens.

References

Nantulya V.M., Musoke A.J., Rurangwira F.R., Saigar M. and Minja S.H. 1987. Monoclonal antibodies that distinguish *Trypanosoma congolense, T. vivax* and *T. brucei. Parasite Immunology* 9:421–431.

IAEA (International Atomic Energy Agency). 1993. Improving the diagnosis and control of trypanosomiasis and other vector-borne diseases of African livestock using immunoassay methods. *IAEA-TECDOC–707.* IAEA, Vienna, Austria.

IAEA (International Atomic Energy Agency). 1997. Application of an immunoassay method to improve the diagnosis and control of African trypanosomosis. *IAEA-TECDOC–925.* IAEA, Vienna, Austria.

Evaluation of Ag-ELISA in the field

Sensitivity and specificity of antigen-ELISAs for diagnosis of *Trypanosoma congolense* and *Trypanosoma vivax* infections in cattle

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Summary

Sensitivity and specificity of the Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA) antigen-ELISA kits for diagnosis of bovine trypanosomosis were investigated using sera from cattle experimentally infected by tsetse challenge with cloned populations of *Trypanosoma congolense* (three populations) or *T. vivax* (one population). The kits are based on monoclonal antibodies that recognise internal antigens of tsetse-transmitted trypanosomes. In general, while overall specificities were high, sensitivities of the antigen-ELISAs were poor. For example, at a cut-off of 5% positivity, the sensitivities of the antigen-ELISAs were 11% for samples (n=1162) from *T. congolense*-infected cattle (n=30), and 24% for samples (n=283) from *T. vivax*-infected cattle (n=10). The corresponding specificity values were 95 and 79%, respectively. At a cut-off of 2.5% positivity, sensitivity for *T. congolense* was 25%, and for *T. vivax* 35%; corresponding specificity values were 85 and 63%, respectively. There were no values of the positive-negative threshold at which both sensitivity and specificity were satisfactory. Restricting the analyses to samples taken more than two weeks after tsetse challenge did not improve sensitivity estimates. Trypanosome species-specificities of the antigen-ELISAs were also poor. In contrast to the antigen-ELISA, the sensitivity of the buffy coat technique when applied to the same experimental animals was fairly high at 67% for *T. congolense* infections and 60% for *T. vivax* infections. For samples taken more than two weeks after tsetse challenge, high sensitivity estimates of 96% for *T. congolense* and 76% for *T. vivax* infections were obtained.

Introduction

The FAO/IAEA trypanosomosis direct antigen-ELISA kit has been distributed to scientists holding research contracts under an IAEA co-ordinated research programme in 14 sub-Saharan African countries. It is intended for improved diagnosis of bovine tsetse-transmitted trypanosomosis as an aid to epidemiological studies and to national trypanosomosis control programmes. The method was developed at the International Livestock Research Institute's Nairobi laboratories by Nantulya and Lindqvist (1989), and subsequently modified at the Animal Production and Health Unit at the IAEA Agriculture Laboratory, Seibersdorf, Austria. The most significant of these modifications are:

- use of a blocking agent (0.5% normal mouse serum) in the sample diluent buffer
- use of the chromogen 3,3', 5,5' tetramethylbenzidine (TMB) in place of ABTS

- use of the percentage positivity data expression system
- incorporation of internal quality assurance target values

In spite of these modifications, the basic assay configuration remains the same as that of Nantulya and Lindqvist (1989). The anti-trypanosomal monoclonal antibodies are from the original hybridoma cell lines (derived at ILRI), and are used in the original assay configuration.

In the work described here, the sensitivity and specificity of the FAO/IAEA trypanosomosis direct antigen-ELISA kit were assessed in relation to trypanosome infections in cattle. ELISA responses were measured in serum of previously naive cattle following primary experimental infection with cloned populations of *Trypanosoma congolense* and *T. vivax* originating from geographically distinct locations in East and West Africa.

Materials and methods

Experimental cattle

Fifty-six Boran cattle *(Bos indicus)* aged 12 to 18 months, were obtained from ILRI's breeding herd at Kapiti Ranch on the Athi Plains, a trypanosomosis non-endemic area of Kenya. Cattle were loose-housed with free access to drinking water, and fed on hay and concentrates. Cattle infected with *T. vivax* and their uninfected cohorts were housed similarly, but in fly-proof accommodation.

Trypanosomes

Three cloned populations of *T. congolense,* representing parasites isolated in different locations, and one cloned population of *T. vivax* were used for infection of cattle. These were *T. congolense* IL 2642 originating from Uganda (Morrison et al 1978, 1982); *T. congolense* IL 1180 (Geigy and Kauffmann 1973; Nantulya et al 1984) from Tanzania; *T. congolense* IL 2281 from Nigeria (Moloo and Kutuza 1988); and *T. vivax* IL Dat 1.2 derived from an isolate made in Nigeria (Gardiner et al 1986).

Infection of cattle

Cattle were infected by the application of five infective *Glossina morsitans centralis* to the skin of the flank. Six weeks before infection of cattle, goats were inoculated intramuscularly with one of the four trypanosome populations. Twelve to fourteen days later, trypanosomes were detected in peripheral blood. After another seven days, teneral *G. m. centralis* were fed daily on the infected goats for 25 days. Flies were then starved for two days prior to examination for infection by microscopic examination of salivary probes. Tsetse flies with metacyclic trypanosomes in their saliva were used to challenge cattle.

Parasitological monitoring

Cattle were examined for the presence of trypanosomes in the circulation by the phase contrast buffy coat technique (Murray et al 1977), using blood collected into EDTA vacutainers (Becton-Dickinson), and an SM Lux microscope (Leitz, Wetzlar, Germany) with a 25 × magnification phase contrast objective. Care was taken to ensure that every microscopic field of the buffy coats was examined thoroughly.

Collection and separation of serum

Whole blood samples were collected from the cattle by jugular venipuncture into 10 ml siliconised plain vacutainers (Becton Dickinson) and incubated for four hours at 37°C to maximise clotting. Samples were then incubated at 4°C overnight, after which they were centrifuged at 1200 g for 15 min at 4°C, and serum aspirated. This procedure resulted in the maximum yield of serum, and no visible haemolysis.

Experimental design

Experiments were conducted using a double-blind system: neither those collecting the samples nor those conducting subsequent analyses knew which animals were infected and with which parasites until completion of the experiment.

In each of four groups of 14 cattle, ten cattle were infected with trypanosomes, while the remaining four cattle were uninfected controls. Within each group, all ten infected cattle were infected with a single trypanosome population; group 1 with *T. congolense* IL 2642, group 2 with *T. congolense* IL 1180, group 3 with *T. congolense* IL 2281 and group 4 with *T. vivax* IL Dat 1.2.

Beginning the day after application of tsetse flies, parasitological examination was conducted daily for three weeks, and bi-weekly thereafter until the following number of days after initiation of infections: groups 1 and 2, 108 days; group 3, 60 days; group 4, 67 days. Blood samples for separation of serum were collected on the same days as parasitological examination.

Individual cattle were removed from the experiment if either the PCV fell below 14% or their clinical condition warranted removal on welfare grounds.

Double-blind sample encryption and storage of serum

Two aliquots (each 2 ml) of serum were stored in racks at -70°C in cryovials (Greiner). A random number coding system was used for the identification of the serum samples; the cryovials were labelled only with their unique code numbers. The identity of infected and control cattle were not revealed to individuals responsible for collection or subsequent testing of experimental samples.

Antigen-ELISA

Trypanosome Ag-ELISA was conducted using the FAO/IAEA Trypanosome Antigen-ELISA kits (serial numbers TRP95/GLA/01, TRP95/GLA/01, TRP95/06/18) provided by the FAO/IAEA Joint Division for Nuclear Techniques, Vienna, in accordance with the kit manual. The monoclonal antibodies contained in these kits were:

T. brucei	T.b.r.7 (IgM)
T. congolense	T.c.39 (IgM)
T. vivax	T.v.27 (IgG1)

Briefly, the FAO/IAEA kit protocol was as follows:

Maxisorp (Nunc) 96-well polystyrene microtitre plates were coated overnight with 100 µl per well of monoclonal antibody (T.b.r.7, T.c.39 or T.v.27) diluted to 3.3 µg/ml in 50 mM carbonate bicarbonate buffer, pH 9.6.

Test and control sera were diluted 1/20 in PBS (10 mM phosphate buffered saline, pH 7.4, containing 0.5% Tween 20 [Sigma]) and 0.5% (v/v) normal mouse serum, in polypropylene tubes arranged in 96-well format racks (Micronics systems, Nunc), by adding 40 µl serum to 760 µl diluent. Each dilution of each test sample or control serum was subsequently included in three microtitre plates, i.e. one coated with each of the three monoclonal antibodies. Four replicates of each control and two replicates of each test serum were included in adjacent wells of each microtitre plate.

Coated plates were emptied by flicking the contents into a sink, and blotted on paper towel. The wells were then filled with washing buffer (PBST) consisting of PBS containing 0.5% Tween 20 (Sigma), and immediately emptied by flicking the contents into a sink, and blotted on paper towel.

Diluted test and control sera were immediately added (100 µl per well) to the coated microtitre plates, and incubated with orbital shaking at 37°C for 15 min, after which plates were emptied and washed once as described above. Horseradish peroxidase (HRP)-conjugated homologous monoclonal antibody, appropriately diluted in PBST containing 1% (w/v) bovine serum albumin, was then added to all wells (100 µl per well). Microtitre plates which were then incubated with orbital shaking at 37°C for 15 min.

Following incubation with HRP-conjugated monoclonal antibody, microtitre plates were emptied and washed once as described above, and immediately refilled with washing buffer in which wells were left to soak for 10 min. Plates were again emptied, refilled and soaked for 10 min for two further cycles, after which they were emptied and blotted on paper towel. A two component proprietary hydrogen-peroxide/3,3', 5,5' tetramethylbenzidine substrate-chromogen system (TMB, Kirkgard-Perry Laboratories) at 4°C was then added (100 µl per well). Substrate-chromogen was incubated in the microtitre plates for 20 min with orbital shaking at 37°C, after which the reaction was quenched by the addition of 100 µl per well of 1 M orthophosphoric acid. Absorbances were read using a multichannel photometer (Immunoskan Plus, Labsystems) connected to the serial port of an IBM-compatible personal computer via an RS232C cable.

Quality assurance and data analysis

The quality assurance (QA) aspects of the FAO/IAEA trypanosome antigen-ELISA kit protocol were strictly adhered to. This protocol broadly follows the recommendations of Wright et al (1993). Hence, strong positive (C++), weak positive (C+), negative (C-) and conjugate controls (CC; serum diluent buffer without serum) were included on every plate. Results for individual ELISA plates were accepted or rejected based on strict QA acceptance criteria (FAO/IAEA kit protocol; Rebeski, personal communication). Results for test samples were expressed as percentage positivity (PP) values based on the median absorbance of the four replicates of the strong positive control serum. Acceptance of QA data was based on two levels of criteria. Firstly, the median absorbance of the four replicates of the strong positive control had to fall within a prescribed range. Secondly, the percentage positivity of three of the four absorbances of each of the four controls (C++, C+, C- and CC) had to fall within prescribed ranges. Proprietary software (Hopquik, M.C. Eisler, unpublished) was used to perform the QA

calculations and to output the percentage positivity data for test samples as a database file for subsequent analysis (see below).

Sensitivity, specificity and diagnostic efficiency calculations

Sensitivity of the phase contrast buffy coat technique and the Ag-ELISA for each trypanosome species was calculated separately for each *T. congolense*- and *T. vivax*-infected animal. Sensitivity was calculated either including observations from day 1 following tsetse challenge, or including observations from day 14 following tsetse challenge. Similarly, specificity of the Ag-ELISA for each trypanosome species was calculated separately for each non-infected control animal. Sensitivity was calculated as

$$\frac{n_p}{n}.100\% \qquad 1$$

where np is the number of samples, from a total of n samples from an infected animal, with percentage positivity values greater or equal to the cut-off. Specificity was calculated as

$$\frac{n_n}{n}.100\% \qquad 2$$

where nn is the number of samples, from a total of n samples from an uninfected animal, with percentage positivity values less than the cut-off.

Combined estimates (CS) of sensitivity (or specificity) were obtained as weighted means for groups of infected or uninfected animals respectively, using the following formula:

$$CS = \frac{\sum_{i=0}^{N}(S_i n_i)}{\sum_{i=0}^{N} ni} \qquad 3$$

where Si is the estimate of sensitivity (or specificity) for the ith of N cattle, and ni is the number of samples on which the sensitivity (or specificity) estimate for the ith animal is based. Finally 95% confidence intervals for combined sensitivity (or specificity) estimates were calculated using the combined mean ± t × SEw where t is the value of student's t distribution at p=0.05, and N – 1 degrees of freedom, and SEw is the weighted standard error, calculated by:

$$SE = \sqrt{\frac{\sum_{i=0}^{N}[(S_i - CS)^2 \cdot ni]}{\sum_{i=0}^{N}[n_i] \cdot (N-1)}} \qquad 4$$

The diagnostic efficiencies of the Ag-ELISAs at any given percentage positivity cut-off value were calculated as the product of the sensitivity and specificity at that percentage positivity cut-off value.

The cross-reactivity of each of the three Ag-ELISAs for heterologous salivarian trypanosome infections was considered equivalent to the sensitivity for such infections.

For these analyses, the Hopquik output database file (see above) was imported into Microsoft Access Version 2.00 (Microsoft Corporation) databases and Lotus 1-2-3 Release 5 for Windows (Lotus Development Corporation) spreadsheets specifically designed for this purpose.

Results

Phase contrast buffy coat technique

All 40 cattle infected with *T. congolense* (IL 2642 [n=10], IL 1180 [n=10] or IL 2281 [n=10]) or *T. vivax* (IL Dat 1.2 [n=10]) had detectable parasitaemias during the experiments. No parasitaemia was detected in any of the uninfected control cattle [n=16] at any stage. After an initial pre-patent period of infection, parasites were detected in *T. congolense*-infected cattle on the great majority of occasions of sampling. Sensitivity estimates for the phase contrast buffy coat technique are shown in Table 1.

Table 1. *Sensitivity estimates for the phase contrast buffy coat technique trypanosome used on trypanosome-infected cattle.*

	Trypanosome Species	No. of cattle	No. of samples	Sensitivity (%)	LCL (%)	UCL (%)
Start D.[†]	*T. congolense*	30	1224	67.0	63.4	70.6
	T. vivax	10	358	59.5	56.5	62.5
Start D. 14[‡]	*T. congolense*	30	834	96.0	94.5	97.6
	T. vivax	10	228	76.3	68.7	83.9

[†]Sensitivity calculated using observations from day 1 following tsetse challenge.
[‡]Sensitivity calculated using observations from day 14 following tsetse challenge.
LCL: lower 95% confidence limit.
UCL: upper 95% confidence limit.

The sensitivity of the phase contrast buffy coat technique, calculated using all observations from the first day following tsetse challenge, was at least 60% for all three of the *T. congolense* populations tested (data not shown). Individual lower 95% confidence limits (LCL 95%) ranged from 51.5 to 66.4% and individual upper 95% confidence limits (UCL 95%) ranged from 71.8 to 73.6%. The overall value for sensitivity for *T. congolense* infections was 67.0% (95% confidence intervals [C.I.] 63.4 to 70.6%). The corresponding sensitivity for the one *T. vivax* population tested was 59.5% (95% C.I. 56.5 to 62.5%).

The corresponding sensitivity calculated using only observations made from the 14th day following tsetse challenge was at least 95% for all three of the *T. congolense* populations tested (data not shown). Individual lower 95% confidence limits (LCL 95%) ranged from 92.7 to 93.5%

and individual upper 95% confidence limits (UCL 95%) ranged from 98.2 to 99.7%. The overall value for sensitivity for *T. congolense* infections was 96.0% (95% C.I. 94.5 to 97.6%). The corresponding sensitivity for the one *T. vivax* population tested was 76.3% (95% C.I. 68.7 to 83.9%).

Ag-ELISA

Sensitivity estimates and their 95% confidence intervals (C.I.) for the three antigen-ELISAs are presented in Tables 2 to 4.

Sensitivity of the *T. congolense* Ag-ELISA

Sensitivity estimates for the *T. congolense* Ag-ELISA are presented in Table 2. Its overall sensitivity (based on a 5% positivity cut-off, and including results for all samples collected from day 1 following tsetse challenge) for all *T. congolense*-infected cattle (n=30) was 10.5% (95% C.I. 4.3 to 16.7%). Restricting the analysis (based on a 5% positivity cut-off) to those samples collected from day 14 following tsetse challenge did not appreciably affect the overall sensitivity estimate, which became 12.4% (95% C.I. 5.4 to 19.5%).

On lowering the cut-off to 2.5% positivity, the sensitivity (including results for all samples collected from day 1 following tsetse challenge) increased to 25.1% (95% C.I. 18 to 32.2%). On raising the cut-off to 10% positivity, the sensitivity (including results for all samples collected from day 1 following tsetse challenge) decreased to 4.0% (95% C.I. 1.1 to 6.8%).

Table 2. *Sensitivity estimates for the* T. congolense *ELISA.*

Analysis from day[†]	Cut off (PP) (%)	Trypanosome Species	No. of cattle	No. of samples	Sensitivity (%)	LCL (%)	UCL (%)
1	2.5	*T. congolense*	30	1162	25.1	18.0	32.2
		T. vivax	10	283	39.2	28.6	49.8
	5	*T. congolense*	30	1162	10.5	4.3	16.7
		T. vivax	10	283	22.3	10.9	33.6
	10	*T. congolense*	30	1162	4.0	1.1	6.8
		T. vivax	10	283	14.8	5.4	24.3
14	2.5	*T. congolense*	30	773	26.8	18.6	34.9
		T. vivax	10	153	51.6	39.0	64.3
	5	*T. congolense*	30	773	12.4	5.4	19.5
		T. vivax	10	153	37.9	22.2	53.7
	10	*T. congolense*	30	773	5.7	1.5	9.9
		T. vivax	10	153	26.1	11.4	40.8

[†]Analysis including samples from day 1 or from day 14 following tsetse challenge.
PP: Percentage positivity.

LCL: lower 95% confidence limit.
UCL: upper 95% confidence limit

Cross-reactivity of *T. congolense*-infected cattle in the *T. brucei* Ag-ELISA

The cross-reactivity (i.e. sensitivity) of the *T. brucei* Ag-ELISA (based on a 5% positivity cut-off, and including results for all samples collected from day 1 following tsetse challenge) for *T. congolense*-infected cattle (n=30) was 9.0% (95% C.I. 5.3% to 12.6%), (see Table 3). Restricting the analysis (based on a 5% positivity cut-off) to those samples collected from day 14 following tsetse challenge slightly increased the overall sensitivity estimate to 14.2% (95% C.I. 8.6 to 19.8%) (see Table 3).

Table 3. *Sensitivity estimates for the* T. brucei *ELISA.*

Analysis from day[†]	Cut off (PP) (%)	Trypanosome Species	No. of cattle	No. of samples	Sensitivity (%)	LCL (%)	UCL (%)
1	2.5	*T. congolense*	30	1159	28.5	24.0	33.0
		T. vivax	10	283	23.0	16.9	29.0
	5	*T. congolense*	30	1159	9.0	5.3	12.6
		T. vivax	10	283	5.3	0.6	10.0
	10	*T. congolense*	30	1159	2.9	0.7	5.1
		T. vivax	10	283	1.4	0.0	3.1
14	2.5	*T. congolense*	30	669	44.5	38.2	50.9
		T. vivax	10	153	32.0	22.2	41.9
	5	*T. congolense*	30	669	14.2	8.6	19.8
		T. vivax	10	153	9.2	1.4	16.9
	10	*T. congolense*	30	669	4.6	1.3	8.0
		T. vivax	10	153	2.6	0.0	0.0

[†]Analysis including samples from day 1 or from day 14 following tsetse challenge.
PP: Percentage positivity.
LCL: lower 95% confidence limit.
UCL: upper 95% confidence limit.

Cross-reactivity of *T. congolense*-infected cattle in the *T. vivax* Ag-ELISA

The cross-reactivity (i.e. sensitivity) of the *T. vivax* Ag-ELISA (based on a 5% positivity cut-off and including results for all samples collected from day 1 following tsetse challenge) for all *T. congolense*-infected cattle (n=30) was 15.0% (95% C.I. 11.1 to 19.0%), (see Table 4). Restricting the sensitivity data to those samples collected from day 14 following tsetse challenge had little effect on the sensitivity estimate, which became 13.3% (95% C.I. 9.3 to 17.3%) (Table 4).

Table 4. *Sensitivity estimates for the* T. vivax *ELISA.*

Analysis from day†	Cut off (PP) (%)	Trypanosome Species	No. of cattle	No. of samples	Sensitivity (%)	LCL (%)	UCL (%)
1	2.5	*T. congolense*	30	1163	35.1	30.1	40.0
		T. vivax	10	283	35.3	23.5	47.2
	5	*T. congolense*	30	1163	15.0	11.1	19.0
		T. vivax	10	283	24.4	10.5	38.3
	10	*T. congolense*	30	1163	5.3	2.3	8.4
		T. vivax	10	283	14.5	4.0	25.0
14	2.5	*T. congolense*	30	773	32.2	26.3	38.1
		T. vivax	10	153	41.8	26.5	57.2
	5	*T. congolense*	30	773	13.3	9.3	17.3
		T. vivax	10	153	32.7	15.0	50.4
	10	*T. congolense*	30	773	4.3	1.5	7.0
		T. vivax	10	153	19.6	4.4	34.8

†Analysis including samples from day 1 or from day 14 following tsetse challenge.
PP: Percentage positivity.
LCL: lower 95% confidence limit.
UCL: upper 95% confidence limit.

Sensitivity of the *T. vivax* Ag-ELISA

Sensitivity estimates for the *T. vivax* Ag-ELISA are presented in Table 4. The overall sensitivity (based on a 5% positivity cut-off, and including results for all samples collected from day 1 following tsetse challenge) for *T. vivax*-infected cattle (n=10) was 24.4% (95% C.I. 10.5 to 38.3%). Restricting the analysis (based on a 5% positivity cut-off) for *T. vivax*-infected cattle to those samples collected from day 14 following tsetse challenge moderately increased the overall sensitivity estimate to 32.7% (95% C.I. 15.0 to 50.4%).

On lowering the cut-off to 2.5% positivity, the overall sensitivity for *T. vivax*-infected cattle, including results for all samples collected from day 1 following tsetse challenge, was 35.3% (95% C.I. 23.5 to 47.2%). On raising the cut-off to 10% positivity, the overall sensitivity for *T. vivax*-infected cattle, including results for all samples collected from day 1 following tsetse challenge, was 14.5% (95% C.I. 4.0 to 25.0%).

Cross-reactivity of *T. vivax*-infected cattle in the *T. brucei* Ag-ELISA

The cross-reactivity (i.e. sensitivity) of the *T. brucei* Ag-ELISA (based on a 5% positivity cut-off, and including results for all samples collected from day 1 following tsetse challenge) for *T. vivax*-infected cattle (n=10) was 5.3% (95% C.I. 0.6 to 10.0%) (Table 3). Restricting the analysis (based on a 5% positivity cut-off) for *T. vivax* IL Dat 1.2-infected cattle to those samples

collected from day 14 following tsetse challenge moderately increased the overall sensitivity estimate to 9.2% (95% C.I. 1.4 to 16.9%) (Table 3).

Cross-reactivity of *T. vivax*-infected cattle in the *T. congolense* Ag-ELISA

The cross-reactivity (i.e. sensitivity) of the *T. congolense* Ag-ELISA (based on a 5% positivity cut-off, and including results for all samples collected from day 1 following tsetse challenge) for *T. vivax* IL Dat 1.2-infected cattle (n=10) was 22.3% (95% C.I. 10.9 to 33.6%) (Table 2). Restricting the analysis (based on a 5% positivity cut-off) for *T. vivax* IL Dat 1.2-infected cattle to those samples collected from day 14 following tsetse challenge moderately increased the overall sensitivity estimate to 37.9% (95% C.I. 22.2 to 53.7%, Table 2).

Specificity of the trypanosome Ag-ELISAs

The specificity of the trypanosome antigen-ELISAs based on the results for uninfected control cattle (n=16) are presented in Table 5.

Specificity of the *T. brucei* Ag-ELISA

The overall specificity (based on a 5% positivity cut-off) of the *T. brucei* Ag-ELISA for uninfected cattle (n=16) was 94.2% (95% C.I. 90.5 to 97.8%). On lowering the cut-off to 2.5% positivity, the overall specificity of the *T. brucei* Ag-ELISA for uninfected cattle, was 75.8% (95% C.I. 71.4 to 80.3%). On raising the cut-off to 10% positivity, the overall specificity of the *T. brucei* Ag-ELISA for uninfected cattle was 98.3% (95% C.I. 96.0 to 100.0%).

Specificity of the *T. congolense* Ag-ELISA

The overall specificity (based on a 5% positivity cut-off) of the *T. congolense* Ag-ELISA for uninfected cattle (n=16) was 94.9% (95% C.I. 90.8 to 99.1%). On lowering the cut-off to 2.5% positivity, the overall specificity of the *T. congolense* Ag-ELISA for uninfected cattle, was 85.4% (95 C.I. 78.9% to 91.0%). On raising the cut-off to 10% positivity, the overall specificity of the *T. congolense* Ag-ELISA for uninfected cattle was 98.3% (95% C.I. 95.6 to 100.0%).

Table 5. *Specificity estimates for the trypanosome Ag-ELISAs.*

Cut off (PP) (%)	ELISA nominal specificity	No. of cattle	No. of samples	Specificity (%)	LCL (%)	UCL (%)
2.5	*T. brucei*	16	530	75.8	71.4	80.3
5	*T. brucei*	16	631	94.2	90.5	97.8
10	*T. brucei*	16	636	98.3	96.0	100.0
2.5	*T. congolense*	16	530	85.4	79.8	91.0
5	*T. congolense*	16	631	94.9	90.8	99.1
10	*T. congolense*	16	636	98.3	95.6	100.0
2.5	*T. vivax*	16	530	62.6	50.4	74.7
5	*T. vivax*	16	631	78.9	66.6	91.3
10	*T. vivax*	16	636	92.3	83.7	100.0

P: Percentage positivity.
LCL: lower 95% confidence limit.
UCL: upper 95% confidence limit.

Specificity of the *T. vivax* Ag-ELISA

The overall specificity (based on a 5% positivity cut-off) of the *T. vivax* Ag-ELISA for uninfected cattle (n=16) was 78.9% (95% C.I. 66.6 to 91.3%). On lowering the cut-off to 2.5% positivity, the overall specificity of the *T. vivax* Ag-ELISA for uninfected cattle, was 62.6% (95% C.I. 50.4 to 74.7%). On raising the cut-off to 10% positivity, the overall specificity of the *T. vivax* Ag-ELISA for uninfected cattle was 92.3% (95% C.I. 83.7 to 100.0%).

Relationship between cut-off value, sensitivity, specificity and diagnostic efficiencies of the *T. congolense* and *T. vivax* Ag-ELISAs

The relationships between cut-off value, sensitivity, specificity and diagnostic efficiency of the *T. congolense* and *T. vivax* Ag-ELISAs are shown in Figures 1 and 2.

Discussion

The sensitivity and specificity of the FAO/IAEA trypanosome antigen detection ELISAs have been formally investigated using sera from defined laboratory infections of cattle in a double-blind trial. Three unrelated cloned populations of *T. congolense* originating from different areas of sub-Saharan Africa and a single cloned population of *T. vivax* were used for these studies. The phase contrast buffy coat technique (Murray et al 1977) was used as a reference method for parasitological diagnosis. These investigations showed that although the Ag-ELISAs had good diagnostic specificity for trypanosome infections, trypanosome species specificity was poor, and diagnostic sensitivity and hence overall diagnostic efficiency were unsatisfactory.

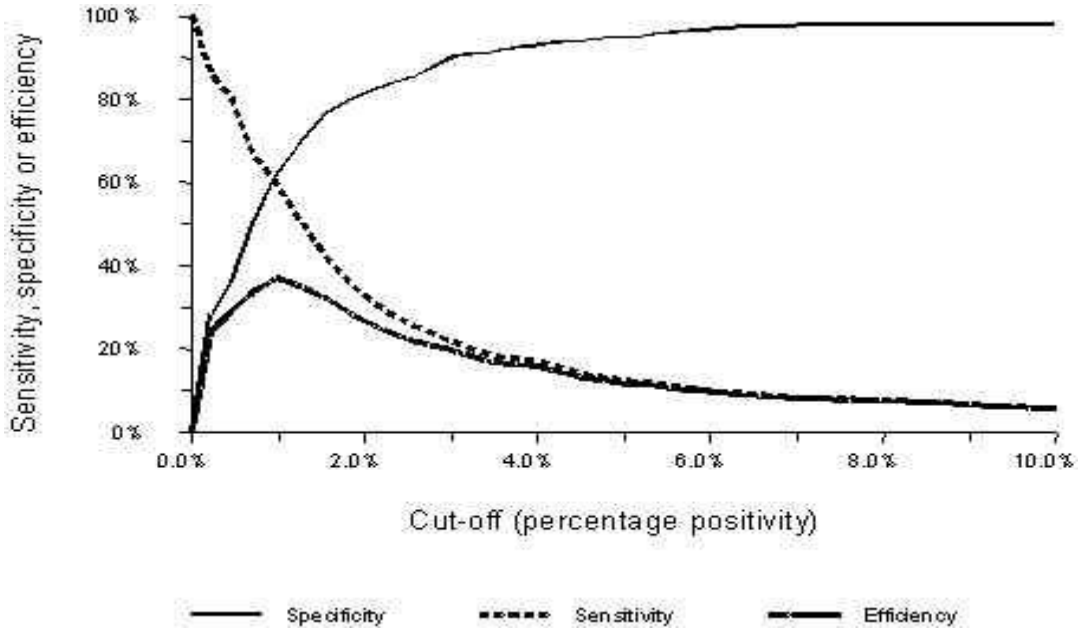

Figure 1. *Sensitivity, specificity and efficiency of the* T.congolense *Ag-ELISA in relation to cut-off. Analyses include samples from day 14 following tsetse challenge.*

The sensitivity of the phase contrast buffy coat technique for *T. congolense* infections was reasonably high (67.0%), when results for all samples collected from day 1 following tsetse challenge were included in the analysis. When only those samples collected 14 days or more after tsetse challenge were included, the diagnostic sensitivity for this trypanosome species was very high (96.0%). These studies were conducted on experimentally infected cattle in excellent animal handling facilities in close proximity to a well-equipped laboratory. The sensitivity estimates obtained here for the phase contrast buffy coat technique therefore may not reflect the situation under field conditions. The sensitivity of this technique for *T. vivax* infections was lower, but nevertheless reasonably high (76.3%) when only those samples collected 14 days or more after tsetse challenge were included.

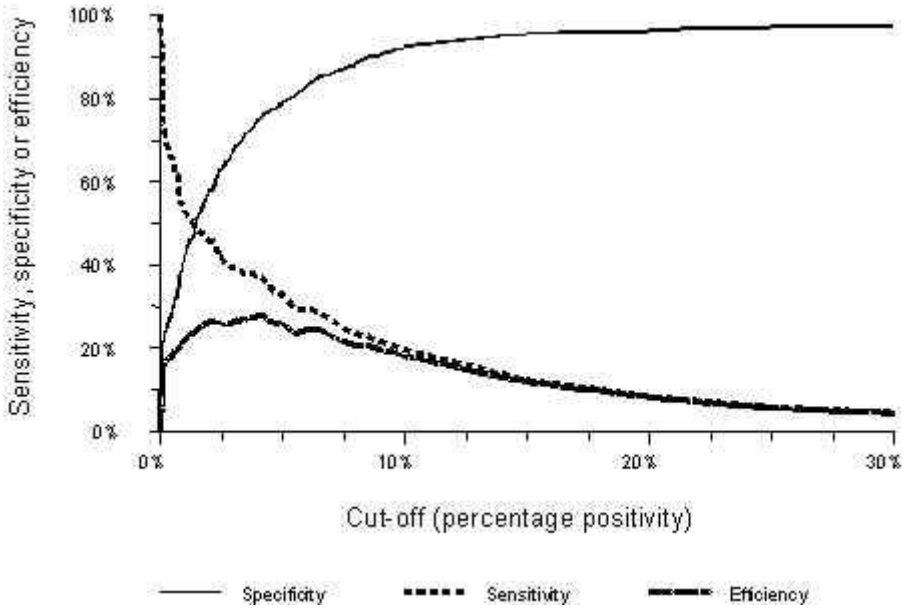

Figure 2. *Sensitivity, specificity and efficiency of the* T.vivax *Ag-ELISA in relation to cut-off. Analyses include samples from day 14 following tsetse challenges.*

In contrast to the phase contrast buffy coat technique, the sensitivities of the *T. congolense* and *T. vivax* Ag-ELISAs for infections with homologous species parasites were very low, at the recommended cut-off value of 10% positivity. This was regardless of whether or not the results for samples taken during the first 14 days following tsetse challenge were included (see Tables 2–4). Lowering the cut-off percentage positivity improved the sensitivity estimates, as shown in Figures 1 and 2, but only at the expense of specificity. The optimal diagnostic efficiency for the *T. congolense*ELISA occurred at a cut-off of approximately 1% positivity, at which both sensitivity and specificity were approximately 60%. For the *T. vivax* ELISA there was no value for the cut-off at which both sensitivity and specificity were above 50%. In fact, the best possible diagnostic efficiency for this trypanosome species, which occurred when the cut-off was in the range of approximately 2–5% positivity was scarcely better than 25% (the value that would be expected from tossing an unbiased coin). Since no *T. brucei*-infected cattle were investigated, it was not possible to assess the sensitivity or diagnostic efficiency of the *T. brucei* ELISA in these studies.

In addition to poor sensitivity, the results obtained here suggest that the species specificity of these ELISAs for salivarian trypanosome infections is also poor, at least for *T. congolense* and *T. vivax*infections.

In summary, the diagnostic sensitivities of the current FAO/IAEA Ag-ELISAs for *T. congolense* and *T. vivax* are inadequate for these tests to be useful in sero-epidemiological investigations of bovine trypanosomosis. In contrast, it was shown that at least under optimised conditions, good diagnostic sensitivity is possible using the phase contrast buffy coat technique.

Despite the poor sensitivity results for the Ag-ELISAs, and the excellent sensitivity results for the phase contrast buffy coat technique obtained here, the development of a serological diagnostic method for bovine trypanosomosis remains a worthwhile objective. Improvement in sensitivity is not the only reason why a serological diagnostic for trypanosomosis would be a desirable addition to parasitological techniques. Indirect diagnostic methods, such as serological methods, offer many potential advantages over direct methods. Examples are testing under controlled laboratory conditions, non-subjectivity of testing, provision of quality assurance, and the possibility of repeat testing of samples stored under optimised conditions. Enzyme-linked immunosorbent assay (ELISA)-based serological methods offer further advantages of high sample throughput suitable for large-scale epidemiological investigations, and automated processing and analysis of the large body of resultant data using widely available microcomputer technology.

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References

Gardiner P.R., Thatthi R., Gathuo H., Nelson R. and Moloo S.K. 1986. Further studies of cyclical transmission and antigenic variation of the ILDar 1 serodeme of *Trypanosoma vivax.* Parasitology 92:581–593.

Geigy R. and Kauffmann M. 1973. Sleeping sickness in Serengeti area (Tanzania), 1971. I. Examination of large mammals for trypanosomes. *Acta Tropica* 30:12–23.

Moloo S.K. and Kutuza S.B. 1988. Comparative study on the infection rates of different laboratory strains of *Glossina* species by *Trypanosoma congolense. Medical and Veterinary Entomology* 2:253–257.

Morrison W.I., Roelants G.E., Mayor-Withey K.S. and Murray M. 1978. Susceptibility of inbred strains of mice to *T. congolense:* correlation with changes in spleen lymphocyte populations. *Clinical and Experimental Immunology* 32:25–40.

Morrison W.I., Black S.J., Paris J., Hinson C.A. and Wells P.W. 1982. Protective immunity and specificity of antibody responses elicited in cattle by irradiated *T. brucei. Parasite Immunology* 4:395–407.

Murray M., Murray P.K. and McIntyre W.I.M. 1977. An improved parasitological technique for the diagnosis of African trypanosomosis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 71:325–326.

Nantulya V.M. and Lindqvist K.J. 1989. Antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma vivax, T. congolense* and *T. brucei* infections in cattle. *Tropical Medicine and Parasitology* 40:251–390.

Nantulya V.M., Musoke A.J., Rurangirwa F.R. and Moloo S.K. 1984. Resistance of cattle to tsetse-transmitted challenge with *Trypanosoma brucei* or *Trypanosoma congolense* after spontaneous recovery from syringe-passaged infections. *Infection and Immunity* 43:735–738.

Wright P.F., Nilsson E., Van Rooij E.M.A., Lelenta M. and Jeggo M.H. 1993. Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. *Revue Scientifique et Technique O.I.E.* 12:435–450.

Evaluation of antigen-detection ELISAs in Mali

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Summary

The efficacy of a tsetse-control programme can be measured through tsetse-density monitoring on the one hand and through infection rate and packed cell volume (PCV) monitoring in cattle on the other hand. The objective of this study was to determine the suitability of the antigen-ELISA (Ag-ELISA) as an additional method for assessing the efficacy of a tsetse-control programme based on the use of traps and screens impregnated with deltamethrine. The buffy coat test was used as a reference to evaluate the available Ag-ELISA (using two kits: one from the International Livestock Research Institute (ILRI) and another from the International Atomic Energy Agency (IAEA)). Herd monitoring was carried out in 8 localities: 4 in the test area and 4 in an untreated area, and the results were compared over 12 months. The apparent tsetse density in the treated area was reduced by nearly 95% after one month of control, and few flies were caught thereafter. A similar trend was observed for rates of infection in cattle which were reduced from 6 to 2% in the treated area but increased from 3 to 10% in the untreated control area. At the beginning of the experiment, mean PCV was the same (29.4%) for cattle in both treated and untreated areas. After 12 months of operation, the mean PCV in the treated area was higher than that in the untreated area. Positive antigen prevalence did not correlate with trypanosomal infection rate in cattle, nor with apparent tsetse density. The two Ag-ELISAs suffered from low sensitivity as measured on a parasite positive population for both *Trypanosoma congolense* and *T. vivax* and the Ag-ELISA should not be recommended for trypanosomosis monitoring on its own.

Introduction

There is a move in Mali towards tsetse and trypanosomosis control using insecticide-impregnated traps and screens. There is a need for an improved trypanosomosis diagnosis for monitoring such control operations, as the currently available tests have limited sensitivity. The antigen-ELISA is a candidate test. In 1988 ILRAD proposed an Ag-ELISA for trypanosomosis diagnosis based on monoclonal antibodies specific for the three main species of cattle trypanosomosis *(T. vivax, T. congolense* and *T. brucei)* (Nantulya 1990; Nantulya and Lindqvist 1989; Masake and Nantulya 1991). The test was validated in ten African laboratories. The different results (Diall et al 1993) called for refinements of the test by IAEA.

The aim of the present study was twofold:

1. Evaluation of the Ag-ELISA as a tool for assessing the impact of tsetse control programmes on the incidence of trypanosomal infections in livestock.
2. Evaluation of the sensitivity and the specificity of the Ag-ELISA, and comparison of ILRI's and IAEA's kits.

Materials and methods

The sector of Niena in Mali was chosen for the pilot tsetse-control programme. It covers 500 km2 with 56,000 cattle. Two species of flies are prevalent: *(Glossina palpalis gambiensis, G. tachinoides)*alongside the River Bagoé and its affluents. The annual rain fall is 800–1000 mm.

Tsetse monitoring

Impregnated traps were deployed alongside the River Bagoé and its tributaries within the limits of the control area. Entomological surveys were carried out every 1–2 months using biconical traps to monitor the apparent density of tsetse flies at selected points on the river banks. For comparison purposes the same studies were conducted at other sites selected in a neighbouring untreated area.

Cattle infection monitoring and packed cell volume

Fifty animals were selected in each of eight villages. Four of the villages were located in the test area, while the four others were located in the non-treated (control) area. These animals were ear-tagged and bled every two months for parasite and antigen monitoring. The parasite monitoring was done using the buffy coat technique (BCT). Sera were collected and stored at -20°C for antigen-detection.

Mean packed red cell volume was compared at the beginning of the experiment and 12 months later. One hundred sera from trypanosome-negative (using BCT) animals with low packed cell volume (PCV) (PCV ≥ 25%) and 100 sera from trypanosome-negative (using BCT) animals with normal PCV (PCV ≥ 25%) were tested by Ag-ELISA in order to determine if there were significantly more antigen-positive cases in the low PCV group than in the normal PCV group for *T. congolense* and *T. vivax.*

Data analysis

To determine the effect of the tsetse control on trypanosomal prevalence in cattle, the results obtained in the treated area were first compared over different sampling periods and then with those in the control area over the same periods. Correlations were also made between tsetse fly density and trypanosomosis prevalence in both treated and control areas on one hand and with antigen positivity on the other hand.

Results

Entomolosgy

Before the beginning of tsetse control, the apparent tsetse densities were relatively high for both tsetse species and in both treated and untreated areas. One month after the beginning of the control, the reduction in apparent density was 93% for *G. p. gambiensis* and 98% for *G. tachinoides* (Figure 1). One year after the deployment of the targets hardly any flies were captured in the treated area. Apparent tsetse densities of *G. p. gambiensis* were 11.6 and 16.7 in untreated and treated areas, respectively, in March 1993, one month before the start of tsetse control, and 3.25 and 0, respectively, 13 months later in April 1994. Corresponding apparent densities for *G. tachinoides* were 2.4 and 3.7, before tsetse control, and 1.90 and 0, respectively, 12 months after tsetse control.

Cattle infections and PCV

At the beginning of the project the prevalence of trypanosomal infections in cattle in the selected herds was 6.2% in the treated area and 3.4% in the untreated area. It should be noted, however, that before the study animals were being regularly treated by the owners using either Berenil® or Trypamidium®, and this may account for the low trypanosomal infection rates in the cattle at the beginning of the experiment. Once the study started, however, animals were not treated independently by the owners and the detection of the parasite was the only indication for treatment. During the study, fluctuations were observed in infection rates in both areas.

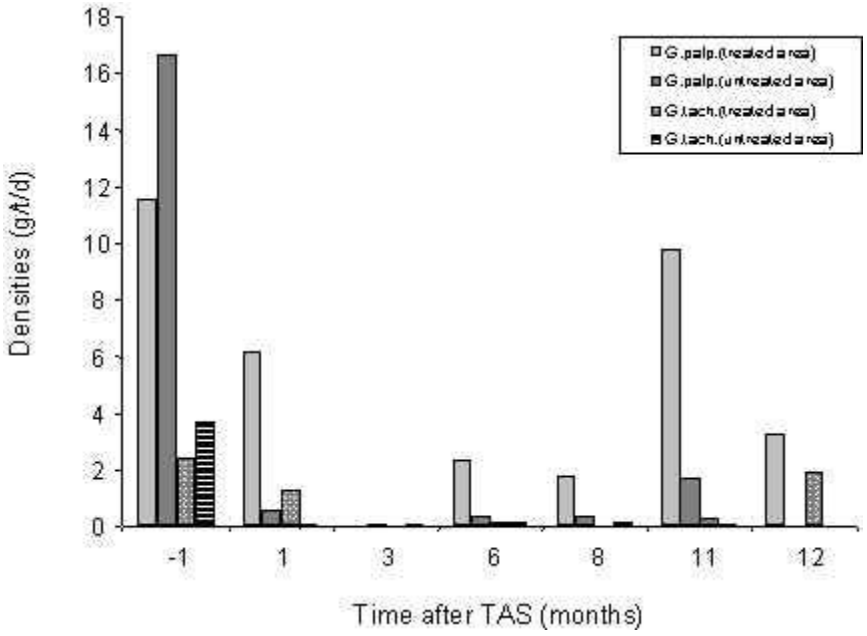

Figure 1. *Variations of the densities in the treated and untreated areas.*

One year after the beginning of the study, the prevalence of trypanosomal infections in the treated area was lower (2%) than in the untreated area (9%) (Table 1).

With regard to the PCV, the results of the antigen-detection ELISA tests using both kits are shown in Table 2 for samples from cattle with normal PCVs (≥ 25%) and low PCVs (<25%).

Table 1. *Summary of parasitological and haematological results in eight villages.*

Area	Village	Start March 93 Prev†	PCV	+1 month May 93 Prev	Pcv	+3 months July 93 Prev	Pcv	+8 months Nov. 93 Prev	Pcv	+11 months Feb. 94 Prev	Pcv	+12 months April 94 Prev	Pcv
Test	1	3.6	31.7	0.0	29.5	0.0	45.7	0.0	45.7	6.8	35.7	0.0	32.4
	2	8.0	29.8	14.0	28.8	2.0	32.0	13.0	32.1	6.7	31.0	4.8	28.4
	3	6.0	28.6	10.0	31.5	4.0	34.0	2.1	34.0	2.4	33.8	0.0	33.2
	4	7.9	27.2	2.0	29.7	0.0	30.7	0.0	35.4	0.0	31.9	3.0	31.8
	Mean	6.2	29.4	6.5	29.9	1.5	33.1	3.7	36.8	4.2	33.2	2.0	31.3

Control	5	1.0	30.6	16.0	29.8	0.0	31.9	26.3	30.6	21.6	30.1	14.3	27.9
	6	2.0	26.9	4.0	32.1	0.0	31.9	0.0	33.0	2.8	32.3	6.2	25.7
	7	4.0	32.0	14.0	31.2	12.5	32.4	4.3	33.5	0.0	33.8	2.8	26.7
	8	5.9	27.9	18.0	24.9	13.5	29.7	16.7	29.2	2.6	28.9	13.2	26.2
	Mean	3.5	29.4	13.0	29.5	6.2	31.6	10.8	31.6	6.7	31.3	9.0	26.6

[†]Prev: trypanosomal prevalence

Table 2. *Results of the antigen-detection ELISA test using IAEA and ILRI kits.*

PCV	No. of sera	Species	No. positive (IAEA)	No. positive (ILRI)
Normal (≥25%)	100	*T. brucei*	7	12
		T. congolense	5	15
		T. vivax	10	9
Low (<25%)	100	*T. brucei*	24	42
		T. congolense	10	8
		T. vivax	10	8

χ^2 analysis was performed to determine if the observed differences in numbers detected positive between normal and low PCV samples were statistically significant (Table 3).

Table 3. *χ^2 values for comparing numbers detected positive between normal and low PCV samples.*

	ILRI		IAEA	
	χ^2	P value	χ^2	P value
T. congolense	1.77	0.18	1.15	0.28
T. vivax	0	0	0.06	0.81
T. brucei	21.33	<0.001	9.77	<0.01

Table 4. *Results of the antigen-ELISA test for BCT positive sera using AIEA and ILRI kits.*

Positive by BCT	Monoclonal	IAEA		ILRI	
		No. of sera	No. positive by Ag-ELISA	No. of sera	No. positive by Ag-ELISA
T. congolense	*T. brucei*	80	7 (9) [†]	80	0 (0)
	T. congolense		37 (46)		65 (81)
	T. vivax		11 (14)		2 (2.5)
T. vivax	*T. brucei*	60	1 (2)	50	1 (2)

	T. congolense		13 (22)		20 (40)
	T. vivax		15 (25)		17 (34)

[†]Percentage in parentheses.

The results indicated that only for T. brucei was the difference statistically significant for both kits.

Correlation between parasitological and haematological results

Monthly parasitological and haematological parameters were recorded. The data (Figures 2A and 2B) demonstrate no correlation between antigen and parasitaemia results in either area. PCVs in animals treated and untreated areas were similar until month 11 but were lower in animals in untreated areas in month 12.

Evaluation of sensitivity and specificity

Of 1201 samples collected and tested using the BCT, 80 were positive to *T. congolense* and 50 to *T. vivax.* Using ILRI's and IAEA's reagents, these sera were further examined and the sensitivities and specificities, and the positive and negative predictive values (using the three monoclonals for each kit) were calculated and compared to BCT results. The c2 test was used to compare the performance of the ILRI and IAEA tests. The specificity of the Ag-ELISA was also determined on 596 sera collected from a trypanosome-free zone in Nara in Mali, but using only IAEA reagents.

The results of the evaluations are shown in Table 4 for IAEA and ILRI reagents, respectively.

Ag-ELISA test statistics on samples positive to *T. congolense* by BCT are shown in Table 5. The performance of both sets of reagents was poor when using monoclonal *T. brucei* and monoclonal *T. vivax*. The results given in Table 5 when monoclonal *T. congolense* was used show that ILRI's reagents detected more positive cases than did IAEA's reagents.

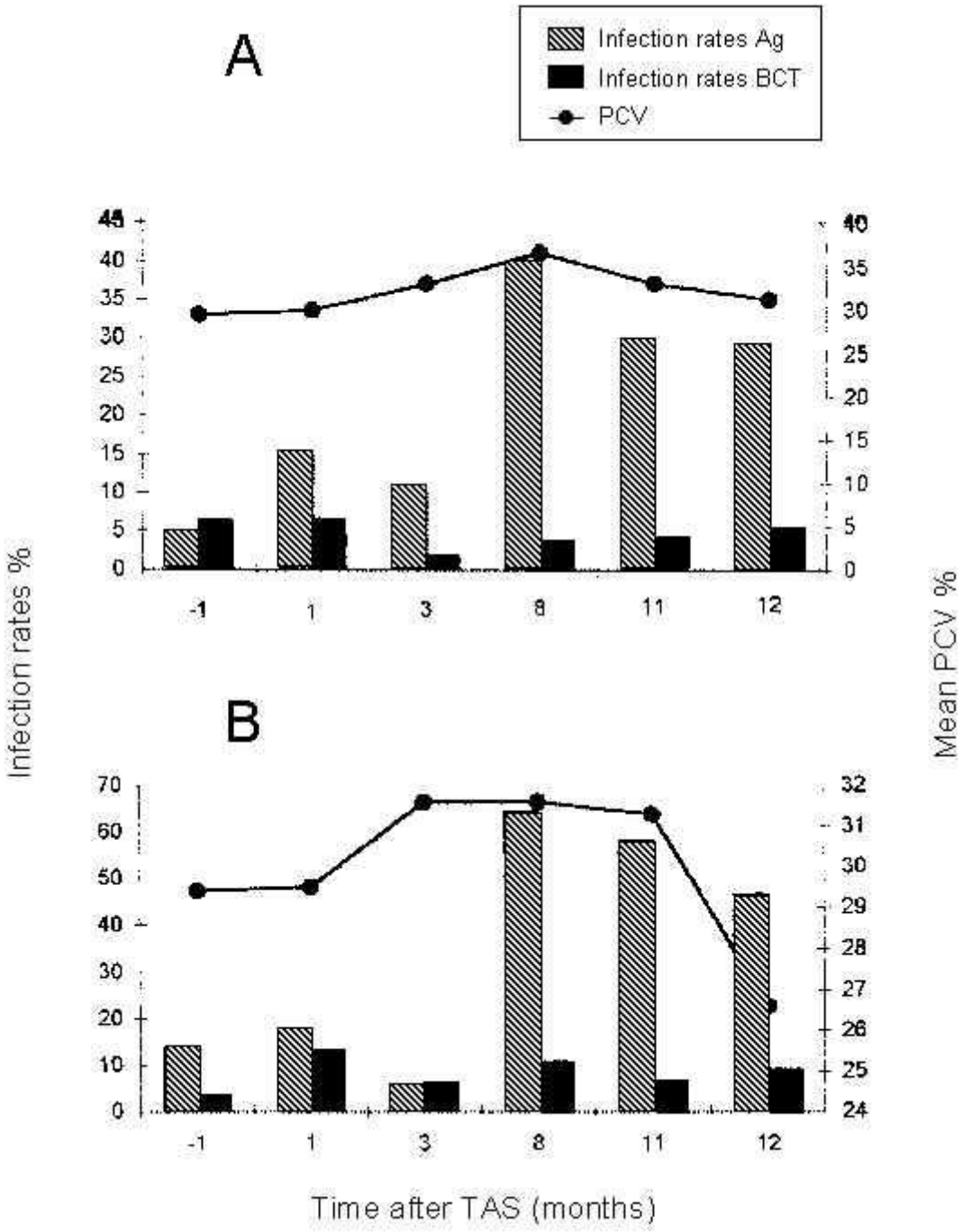

Figure 2A. *Validations of the mean PCV, infection rates using the Ag-ELISA and BCT in treated areas.*
Figure 2B. *Validations of the mean PCV, infection rates using the Ag-ELISA and BCT in untreated areas.*

Table 5. *ELISA test results on samples positive to* T. congolense *by buffy coat technique.*

Parameters	*T. brucei*	*T. congolense*	*T. vivax*

	ILRI	IAEA	ILRI	IAEA	ILRI	IAEA
Sensitivity (%)	0	9	81	46	2.5	14
Specificity (%)	100	100	100	100	100	100
Positive predictive value (%)	0	77	100	100	100	100
Negative predictive value (%)	92	93	98.4	92	92	92

Table 6. *Overall ELISA test performance compared to BCT (positive to* T. congolense).

Parameters	ILRI	IAEA
Sensitivity (%)	81	46
Specificity (%)	99.8	98.1
Positive predictive value (%)	97	67
Negative predictive value (%)	98.4	96

Table 7. *ELISA test results on samples (positive to* T. vivax) *by BCT.*

Parameters	*T. brucei*		*T. congolense*		*T. vivax*	
	ILRI	IAEA	ILRI	IAEA	ILRI	IAEA
Sensitivity (%)	2	2	40	22	34	25
Specificity (%)	100	100	100	100	100	100
Positive predictive value (%)	100	100	100	100	100	100
Negative predictive value (%)	95	94	97	95	97	96

Table 6 gives the results of the overall comparison of the ELISA test to BCT (positive to *T. congolense*) assuming that sera positive to *T. brucei* and *T. vivax* monoclonals were false positives. The overall results showed that the ILRI reagents were more sensitive and had higher predictive values than the IAEA reagents.

ELISA test results on samples positive for *T. vivax* by BCT are shown in Table 7. The results showed very low sensitivities for both reagents for the *T. brucei* monoclonal. The results using *T. congolense* or *T. vivax* monoclonal again indicate low sensitivity of the test for both reagents.

Table 8 gives the results of the comparison of the ELISA test to BCT (positive to *T. vivax*) assuming that sera positive to *T. brucei* and *T. congolense* monoclonals are false positive. The overall results showed that ILRI's reagents are slightly more sensitive.

χ^2 squared analysis was used to compare the performance of ILRI and IAEA kits, which detected 65/80 and 37/80 samples, respectively, positive to *T. congolense* of those detected positive to *T. congolense* by BCT, and 17/50 and 15/60 samples, respectively, positive to *T. vivax* of those detected positive to *T. vivax* by BCTs. The calculated value of $2\ \chi^2 = 19.72$ ($P<0.001$) demonstrated a large difference in performance of the two kits for BCT-detected *T. congolense* infections. The ILRI kit was more sensitive than the IAEA kit. However, there was no difference in performance of the two kits in relation to *T. vivax* ($\chi^2 = 0.68$).

Table 8. *ELISA test performance compared to BCT (positive to* T. vivax).

Parameters	ILRI's reagents	IAEA's reagents
Sensitivity (%)	34	25
Specificity (%)	97.8	98.5
Positive predictive value (%)	45	52
Negative predictive value (%)	97	96

Table 9. *Results of the evaluation of the specificity of Ag-ELISA from samples collected in a trypanosome-free zone.*

Trypanosome species	Cut-off point (%)	Specificity
T. brucei	5	96.1
	10	98.2
	15	99.2
	20	99.3
T. congolense	5	99.8
	10–20	100
T. vivax	5	98.2
	10	99.3
	15–20	99.5

Specificity of the antigen-detection ELISA

The specificity of the ELISA was evaluated in Seibersdorf (Austria) (D. Rebeski, personal communication) based on test results for 596 sera from Nara (Table 9).

High specificity was obtained with a cut-off point of 10% for all three species.

Discussion and conclusions

The estimation of the sensitivity and the specificity of a test requires two types of populations: one completely disease free, and one in which every animal is infected (Burges 1988). To fulfill these conditions in the present study the sera were collected from two different parts of the country: the first a trypanosome-free zone (Nara) and the second a trypanosome-infected area (Niena). One thousand, two hundred and one sera collected in Nara were used to estimate the sensitivity of the test and 596 sera from Niena were tested for the specificity of the assay.

Ease of application, cost and reliability are some of the important factors in the choice of a test. Other important factors are: which test is most sensitive, which test is most specific, are the tests independent? These criteria are important since tests that are high in sensitivity and/or specificity are ideally required (Burges 1988). The Ag-ELISA was evaluated using two sets of reagents: ILRI's and that of IAEA.

The following conclusions were made:

1. The specificity when measured on a negative population from a tsetse-free area was satisfactory for each trypanosomal species: 98–99% at a cut-off point of 10%.
2. Both kits (ILRI and IAEA) suffered from low sensitivity when measured on parasite-positive populations for both *T. congolense* (respectively 81 and 46%) and *T. vivax* (respectively 34 and 25%).
3. There were significantly more antigen-positive cases (using the monoclonal *T. brucei*) in a low PCV population than in a normal PCV population for *T. congolense.*

In conclusion, the use of antigen-ELISA is not recommended for trypanosome monitoring due to its low sensitivity. Further, the prevalence of antigen-positive samples was found to be uncorrelated with the trypanosomal infection rate in cattle as determined by BCT.

References

Diall O., Diarra B. and Sanogo Y. 1993. Evaluation of monoclonal antibody-based antigen-detection immunoassays for the diagnosis of *Trypanosoma congolense* and *Trypanosoma vivax* infections in Mali. IAEA TecDoc 707:65–68.

Nantulya V.M. 1990. Trypanosomiasis in domestic animals: the problem of diagnosis. *Revue Scientifique et Technique de I'Office International des Epizooties* 9:357–367.

Nantulya V.M. and Lindqvist K.J. 1989. Antigen-detection enzyme immunoassays for diagnosis of *Trypanosoma vivax, T. congolense* and *T. brucei* infections in cattle. *Tropical Medicine and Parasitology* 40:267–272.

Masake R.A. and Nantulya V.M. 1991. Sensitivity of an antigen-detection enzyme immunoassay for diagnosis of *Trypanosoma congolense* infections in goats and cattle. *Journal of Parasitology*77:231–236.

Burgess G.W. (ed). 1988. *ELISA Technology in Diagnosis and Research.* Graduate School of Tropical Veterinary Science, James Cook University of North Queensland, Townsville, Australia. 341 pp.

Importance of antigen-detection ELISA in monitoring and implementing control strategies for trypanosome infection in KwaZulu-Natal

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Summary

The early history of trypanosomosis in cattle in KwaZulu-Natal, South Africa, dates back to the latter part of the previous century, when it caused serious epidemics in northern KwaZulu. The advent of organo-chlorine insecticides provided suitable means to remove tsetse, and between 1945 and 1952 *G. pallidipes* was eradicated from northern KwaZulu-Natal by large-scale aerial spraying operations. Since 1954 only occasional and isolated cases of trypanosomosis have been reported. However, in 1990 bovine trypanosomosis re-emerged in this area. Using thick and thin blood smears, 10-15% of these animals in three districts, Ingwavuma, Ubombo and Hlabisa, were found to be infected with either *T. congolense* and/or *T. vivax.* By the end of 1993 more than 115,000 cattle were treated, costing nearly US$ 115,000, in treatment alone. Subsequently a more detailed survey was launched using the antigen detection ELISA to estimate the extent of the problem in the area.

Each of the four districts at risk (Nongoma, Ingwavuma, Ubombo and Hlabisa) was divided into sections (n=137) with one or more assigned to a diptank (n=106). Twenty cattle, selected systematically as they went through the diptank, were sampled (serum, haematocrit for packed cell volume (PCV) and buffy coat smears) per section. A total of 3166 samples were collected. Buffy coat smear examination revealed a *Trypanosoma* prevalence of between 2.4–9.8%. The Ag-ELISA results also showed a high prevalence of *T. brucei.* However, comparison of results of smear examination with Ag-ELISA showed overall rates of infection by the Ag-ELISA to be much higher, and these did not appear to reflect the clinical situation. It was therefore concluded that the cut-off optical density (OD) value of 0.05 for positive cases was not very sensitive and alternative criteria to determine positive cases must be found. Presently the Ag-ELISA cannot be used as a definitive tool to guide intervention studies in KwaZulu-Natal.

Introduction

The early history of trypanosomosis in cattle in KwaZulu-Natal dates back to the previous century, when the then governor of Natal asked Sir David Bruce to investigate the notorious 'tsetse-fly disease' (=nakane, a Zulu word which has developed into the colloquial name nagana) in northern KwaZulu-Natal (then Zululand). He found both *T. brucei* and *T. congolense* in domestic animals in Zululand, the latter being responsible for the vast majority of cases of nagana in cattle while no less than 24% of the game animals examined harboured trypanosomes (Bruce 1895, 1897). Curson (1924, cited by Henning 1956) also found another pathogenic trypanosome, *T. vivax,* in domestic animals in Zululand. Bruce (1895) was the first to prove that nagana was spread by tsetse-fly*Glossina* spp and Jowett (1911) demonstrated that

pathogenic trypanosomes can also be transmitted mechanically by other biting flies. *Glossina* spp was wide-spread in the western and eastern Transvaal *(G. m. morsitans)* and north-eastern KwaZulu-Natal *(G. pallidipes, G. austeni* and *G. brevipalpis)* in the early 1800's (Henning 1956). They disappeared from large parts of these areas with the rinderpest pandemic of 1896–1897.

Soon after the annexation of Natal by the British in 1887 game protection laws were enforced, as a result of which the indigenous antelopes multiplied rapidly. Parts previously devoid of game (due to excessive hunting) now swarmed with antelopes of all species. With this increase and extension of game, tsetse-fly and nagana spread. By 1894 the disease assumed such alarming proportions that its investigation was undertaken by Bruce (Bruce 1895). The rinderpest pandemic destroyed almost entire populations of wild animals. However, in parts of Zululand, including the present-day game reserves, pockets of wild animals and tsetse flies survived and by 1905 nagana was once again threatening livestock. *Glossina pallidipes* dispersed into farming areas causing serious epidemics (Henning 1956). The advent of organochlorine insecticides provided suitable means to remove tsetse flies, and between 1945 and 1952 *G. pallidipes* was eradicated from Zululand by large-scale aerial spraying operations (Henning 1956). From 1952–1987 only isolated cases of nagana were reported from Zululand. Most of the cases occurred around the St Lucia lake system where *G. austeni*and *G. brevipalpis* still occurred. At that time it was assumed that since both these *Glossina* spp favour dense, woody coverts, riverine thickets, or forests often associated with high rainfall or subsoil moisture, their distribution would be much more confined (than savanna tsetse flies) and will therefore never become a serious economic problem (Henning 1956). However, human and animal populations have steadily increased, resulting in closer contact between cattle and the shaded thicket and riverine habitats of the fly and in 1990 bovine trypanosomosis re-emerged in this area (Bosman 1990).

During 1990 an increase in 'redwater' was reported by the local community in areas surrounding the game reserves. On further investigation nagana was diagnosed at a diptank east of the Hluhluwe Game Reserve. A survey was started using thick and thin blood smears from 20 emaciated cattle selected at diptanks in the area. This survey revealed that 5–15% of cattle in three districts (Ingwavuma, Ubombo and Hlabisa) were infected with either *T. congolense* and/or *T. vivax* (Figure 1). As soon as a positive diagnosis was made the whole diptank area was considered infected. The dip compound at the diptank was changed from amitraz (Triatix) to cyhalothrin (Grenade) and animals reported sick by the owner were treated, at first with diminazene (Berenil) and later with homidium bromide (Ethidium). Later blanket treatment with homidium bromide was applied. By the end of 1993 more than 115,000 cattle had been treated, costing nearly US$ 115,000 in treatment alone. The costs increase substantially when other costs like drug administration (equipment, travel, personnel, time, etc), change in dipping compound, etc, are added. Accurate data on production losses and actual mortality are not available but nagana was a major cause of death of animals which were already under severe stress caused by the drought conditions which prevailed in the area at that time. The treatments and change in dipping compound were very effective in rapidly reducing clinical cases. However, pyrethroid resistance developed in ticks and from April 1993 the diptanks reverted back to amitraz and every fifth animal was also treated with deltamethrin (Deca-spot) in order to maintain some level of fly control.

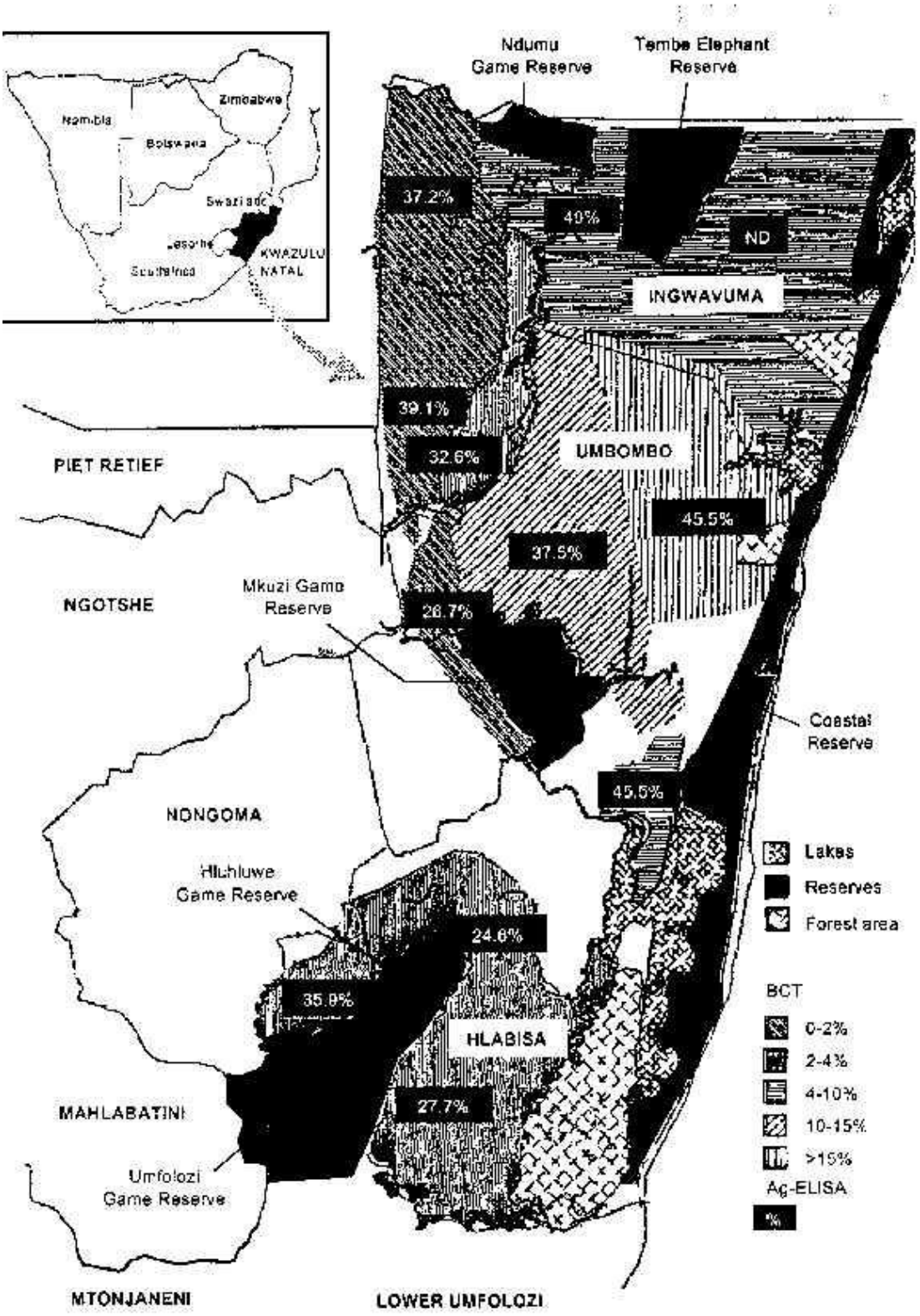

Figure 1. *Prevalence of nagana in KwaZulu-Natal as determined by Ag-ELISA and BCT.*

The nagana problem seems to be confined to some 16,000 km2 of northern KwaZulu-Natal comprising 426,000 people, 300,000 head of cattle and 130,000 small ruminants. Most of the area currently infested with nagana is used for traditional mixed farming and the presence of tsetse and nagana seriously handicaps development. The Food and Agriculture Organization of the United Nations (FAO) has also recently become involved by providing limited assistance under a technical cooperation programme for the long-term control/eradication of trypanosomosis in KwaZulu-Natal.

Survey

Clinically the disease is present in its chronic form suggesting low fly challenge. Even though trypanosome infections were detected, failure to adopt formal random techniques and the low sensitivity of the method employed prevented accurate estimation of the prevalence of trypanosomosis in the region. ILRAD was consulted in 1993 to assist in designing an unbiased cross-sectional study of cattle trypanosomosis in northern KwaZulu-Natal, using a more sensitive and specific diagnostic technique-the antigen detection ELISA (Ag-ELISA)-to estimate the extent of the problem in the area and to help with decisions on control strategies.

Recently specific monoclonal antibodies (MAb) were derived against non-variable trypanosomal antigens from in vitro propagated procyclic forms of *T. congolense, T. vivax* and *T. b. brucei* (Nantulya et al 1987). These antibodies were shown to detect species-specific circulating antigens in sera of infected cattle. The use of these specific MAbs in an antigen-trapping ELISA (Ag-ELISA) resulted in a sensitive and specific diagnostic test enabling many latent infections to be detected (Nantulya and Lindqvist 1989). Circulating antigens can be detected as early as 8–14 days post-infection and persist probably as long as the infection persist in the animal (Masake et al 1995), but are cleared from the circulation within two weeks, following drug cure (Nantulya and Lindqvist 1989). False negative results have been reported early on in infections and may occur due to low levels of circulating antigens (Nantulya and Lindqvist 1989; Masake et al 1995).

Table 1. *Estimated prevalence of* Trypanosoma *spp infection in northern KwaZulu-Natal using Ag-ELISA and BCT.*

District	No. of sections	No. of samples	Ag-ELISA			BCT *Trypanosoma* spp (%)
			T. brucei	*T. congolense*	*T. vivax*	
Ingwavuma	38	886	0.389 [0.336;0.443]†	0.449 [0.403;0.495]†	0.336 [0.271;0.402]†	3.4
Ubombo	43	1008	0.352 [0.292;0.412]	0.455 [0.386;0.524]	0.332 [0.281;0.383]	9.8
Hlabisa	50	1138	0.299 [0.242;0.355]	0.276 [0.223;0.329]	0.296 [0.246;0.346]	2.4
Nongoma	6	134	0.328	0.366	0.299	3.7

†95% confidence interval for the true prevalence.

Sample design

Each of the four districts at risk (Ingwavuma, Ubombo, Hlabisa and Nongoma) were divided into sections with one or more sections assigned to a diptank. There were a total of 137 sections and 106 diptanks (Table 1). A multistage sampling design was planned, with the primary unit being the section and the secondary unit, cattle. Twenty cattle were sampled per section and selected systematically as they went through the diptank.

Samples

Blood samples were collected in 10 ml vacutainer tubes, kept at ambient temperatures for a maximum of 24 hours (except in very hot [>30] areas). After clotting, the clot was removed, the sample centrifuged and the serum decanted into 2 ml plastic tubes and stored at -20°C before being tested with the Ag-ELISA or Latex agglutination test (only on some of the samples).

Blood was also collected in capillary tubes (containing heparin as anticoagulant) and centrifuged to determine the PCV and then used to prepare buffy coat smears and examined by dark ground/phase contrast microscopy (also referred to as the buffy coat technique (BCT) (Murray et al 1977). A questionnaire was completed for each section sampled.

Antigen-ELISA

The Ag-ELISA was performed on all serum samples using monoclonal antibodies capable of capturing species-specific non-variable antigens of *T. congolense, T. vivax* and *T. brucei,* as described by Nantulya and co-workers (Nantulya et al 1987; Nantulya and Lindqvist 1989).

Results

A total of 3166 cattle, out of a population of 253,828 (= 1.2%), were sampled. The average PCV for the samples with a positive BCT result (n=139) was 32.1%, while the average PCV for the animals that tested negative (n=300) was 30.3%. It would therefore appear that the presence of *Trypanosoma* is unrelated to the PCV values in this study.

The results of the BCT and Ag-ELISA are summarised in Table 1. Figure 1 summarises the geographical prevalence of *Trypanosoma* spp as determined with the Ag-ELISA.

The Ag-ELISA revealed that *T. brucei* is also present in the animals and it would appear that *T. congolense* is more prevalent than *T. brucei* or *T. vivax* (although not statistically significant) at most diptanks.

The results of the Ag-ELISA and Latex agglutination tests are compared in Figure 2. It is clear that there are discrepancies between the two tests, the Latex test only identifying ca 10% (for *T. congolense* and *T. vivax*) of Ag-ELISA-positive animals (52% in the case of *T. brucei*), while only 4–8% *(T. congolense* and *T. vivax,* respectively) of Ag-ELISA-negative animals tested positive with the Latex test (46% in the case of *T. brucei)*.

Discussion

The diagnosis of trypanosomosis is notoriously difficult. Clinical signs are non-specific and parasitaemia is usually low and intermittent. Although the examination of blood smears alone is

a relatively insensitive way to detect infection it is simple and of great practical significance as it can also be used to diagnose anaplasmosis, babesiosis and theileriosis. The most sensitive direct method of detecting *T. congolense* and *T. vivax* infections is by examination of wet preparations of the haematocrit buffy coat, under phase contrast illumination (Murray et al 1977).

Alternative laboratory approaches to diagnose trypanosomosis in animal populations are to demonstrate specific antibodies in the serum of infected animals for which several techniques have been described (reviewed by Nantulya 1990). Since antibodies persist for a long time, even after successful elimination of the infection in the animal (self cure or chemotherapy), these antibody detection techniques will only provide a presumptive diagnosis as it is not possible to distinguish between current and latent infections (Luckins et al 1979; Nantulya et al 1984, 1986). Another major problem of using serological tests to detect trypanosome antibodies is the lack of specificity of the antigens used and cross reactions occurring between *Trypanosoma* spp.

Although it is reported that the Ag-ELISA is four to five times more sensitive than conventional techniques (thick and thin blood smear and BCT) (Masake and Nantulya 1991; Masake et al 1995) the estimated prevalence of trypanosomosis in KwaZulu-Natal appeared to be so much higher than expected that it appeared to in no way reflect the clinical position on the ground. Recent tsetse fly surveys in the area only collected *G. austeni* and *G. brevipalpis* and have also shown that the distribution of these fly species are much wider than generally accepted (i.e. distribution not confined only to dense, woody coverts, riverine thickets or forests) (E.M. Nevill, unpublished observations). The cut-off set for the Ag-ELISA (OD>0.05) seems to compromise the sensitivity and specificity (especially for *T. brucei)* of the test. Because population seroepidemiology may vary over time in different geographical regions, the cut-off should be adjusted based on local studies. There also seems to be a significant difference in the results of the Ag-ELISA and Latex agglutination tests. These apparent discrepancies result in severe problems in the interpretation and eventual control strategies. Many trypanosome-infected animals appear to be clinically normal and even if sub-clinical disease is present it may not be economical for treatment to be given.

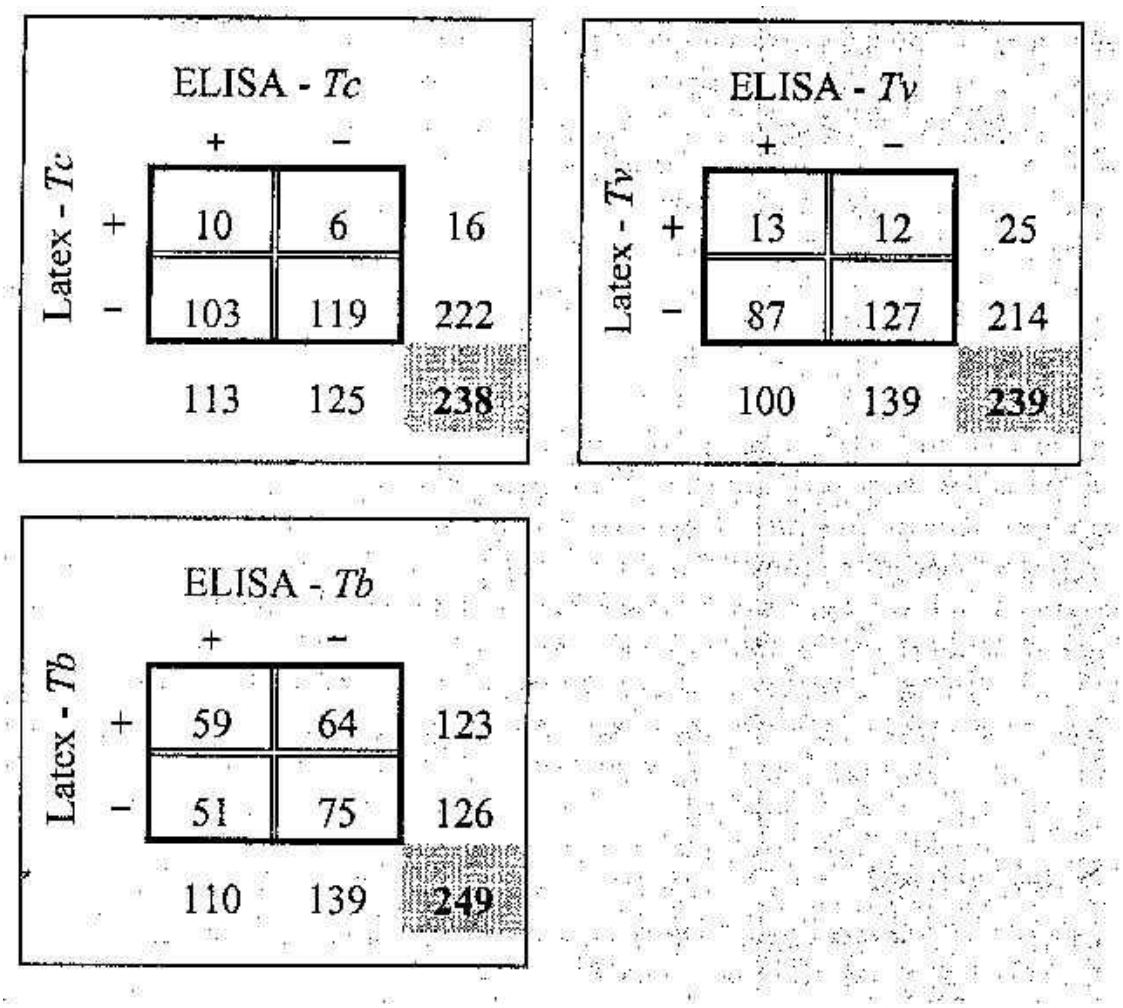

Figure 2. *Comparison of the Latex and ag-ELISA tests for* Trypanosoma *spp.*

Other shortcomings of the Ag-ELISA test are:

- The test was evaluated under ideal laboratory conditions (eg environmental temperatures), but this may not be tenable in other laboratories resulting in varying OD readings that do not fall within specifications. The test should be evaluated at a range of environmental temperatures and conditions.
- The test also seems to be vulnerable to slight alterations in the quality of reagents and buffers, requiring standardisation (titration of reagents to determine optimal dilutions) each time it is performed, especially if the test is not done on a routine basis.
- Each serum sample must be tested separately with each of the three monoclonal antibodies.

On a national level diagnosis is essential for disease surveillance and monitoring and provides essential information for the formulation and execution of disease control programmes. Any surveillance programme should have a high probability of detecting a disease if it is present in a country or region. The techniques used in such a surveillance programme must be sensitive (detect true health), specific (minimum of false identification of a health status), be completed within reasonable time and be simple to perform. Accurate data is essential to place the tsetse

fly and nagana problem in the broader context of socio-economic and agricultural development, natural resource management and land husbandry.

References

Bosman P.P. 1990. Trypanosomiasis *(T. congolense)* in South Africa. *Office International de Epizooties: Disease Information* 3:79–80.

Bruce D. 1895. In: *Preliminary Report on the Tsetse Fly Disease or Nagana in Zululand.* Ubombo, Zululand, December 1895. Durban: Bennett and David, South Africa.

Bruce D. 1897. In: *Further Report on the Tesetse-fly Disease of Nagana in Zululand.* Ubombo, Zululand, May 1896, London.

Henning M.W. 1956. In: *Animal Diseases in South Africa.* 3rd edition. Central News Agency Ltd., Pretoria, South Africa.

Jowett W. 1911. Further notes on cattle trypanosomiasis of Portuguese East Africa. *Journal of Comparative Pathology and Therapeutics* 24: 21–40.

Luckins A.G., Boid R., Rae P., Mahmoud M.M., Elmalik K.H. and Gary A.R. 1979. Serodiagnosis of infection with *Trypanosoma evansi* in camels in the Sudan. *Tropical Animal Health and Production*11:1–12.

Masake R.A. and Nantulya V.M. 1991. Sensitivity of an antigen detection enzyme immunoassay for diagnosis of *Trypanosoma congolense* infections in goats and cattle. *Journal of Parasitology*77:231–236.

Masake R.A., Moloo S.K., Nantulya V.M., Minja S.H., Makau J.M. and Njuguna J.T. 1995. Comparative sensitivity of antigen-detection enzyme immunosorbent assay and the microhaematocrit centrifugation technique in the diagnosis of *Trypanosoma brucei* infections in cattle. *Veterinary Parasitology* 56:37–46.

Murray M., Murray P.K. and McIntyre W.I.M. 1977. An improved parasitological technique for the diagnosis of African trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 71:325–326.

Nantulya V.M. 1990. Trypanosomiasis in domestic animals: the problems of diagnosis. *Revue Scientifique et Technique Office International des Epizooties* 9:357–367.

Nantulya V.M. and Lindqvist K.J. 1989. Antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma vivax, T. congolense* and *T. brucei* infections in cattle. *Tropical Medical Parasitology* 40:267–272.

Nantulya V.M., Musoke A.J., Ruraringwa F.R. and Moloo S.K. 1984. Resistance of cattle to tsetse-transmitted challenge with *Trypanosoma brucei* or *Trypanosoma congolense* after spontaneous recovery from syringe-passaged infections. *Infection and Immunity* 43:735–738.

Nantulya V.M., Musoke A.J. and Moloo S.K. 1986. Apparent exhaustion of the variable antigen repertoires of *Trypanosoma vivax* in infected cattle. *Infection and Immunity* 54:444–447.

Nantulya V.M., Musoke A.J., Rurangirwa F.R., Saigar N. and Minja S.H. 1987. Monoclonal antibodies that distinguish *Trypanosoma congolense, T. vivax* and *T. brucei. Parasite Immunology* 9:421–431.

Use of antigen-ELISA in monitoring and implementing trypanosomosis control strategies in Kenya and Uganda

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Summary

Antigen-ELISA was employed in the diagnosis of trypanosome infection and determination of efficacy of a trypanocidal drug, cymelarsan, in eliminating *Trypanosoma evansi* infection in camels in Kenya. In this first study a polyclonal rabbit anti-*T. evansi* antibodies (RATE) and RATE-peroxidase conjugate were employed in a sandwich ELISA. In a second study in Kenya, anti-trypanosome specific murine monoclonal antibodies were used in a sandwich ELISA to evaluate the efficacy of a commercial Cypermethrin® dip for the control of *Glossina pallidipes* and bovine trypanosomosis on Taita Ranch. In a third study, these monoclonal-based sandwich ELISAs were used to assess the success of the Kenya-Uganda joint border tsetse and trypanosomosis control. Both ILRI and IAEA versions of Ag-ELISA were used for detection of trypanosome antigens in naturally infected cattle in Kenya. Before these various versions of ELISA were used, they were validated and standardised for relative sensitivity and specificity. Results obtained in the camel studies in Kenya showed a relative sensitivity of >74% and a specificity of >79% in diagnosing *T. evansi* infection using either CTVM or KETRI diagnostic kits. The ILRI version of ELISA gave a relative sensitivity of 82 and 90% for bovine trypanosomosis in Kenya and Uganda, respectively. It was hard to determine the specificity of this test in Uganda due to lack of negative controls, but in Kenya the test was 99% specific. On the other hand, the Vienna version of bovine antigen-ELISA was 100% specific in Kenya but only 8.5% sensitive, while in Uganda the test gave specificities of 95, 97 and 75% for *T. brucei, T. congolense* and *T. vivax,* respectively, and relative sensitivity for the three trypanosome species were 75, 30 and 65%, respectively. Based on these results and others from collaborating African countries, it was recommended that the Vienna version of antigen-ELISA be readjusted to improve on its relative sensitivity. The cymelarsen treatment was instituted based on antigenaemia resulting in the reduction of the number of positive cases from 75 to 11% as revealed by Ag-ELISA. This subsequently led to total elimination of trypanosome antigens in camels.

Introduction

Animal trypanosomosis is endemic in more than 90% of Uganda and is the single most important disease constraint to livestock production (COCTU 1987) in that country. In Kenya, tsetse-transmitted trypanosomosis is endemic in 60% of Kenya's rangelands, which constitute 25% of the country (KETRI 1990). Though large-scale eradication of tsetse and trypanosomosis may remain elusive for many years to come, the availability of low-cost technologies and prospects of community participation give optimism for the smaller, and more manageable tsetse and trypanosomosis programmes (Chadenga 1994). In the past, one of the major constraints to the control of trypanosomosis has been the lack of sensitive diagnostic tests. The recent development of tests which detect circulating trypanosomal antigens has given a ray of

hope onto this predicament (Nantulya 1989, 1991). This paper discusses the results of studies carried out to evaluate the success of some of the smaller tsetse and trypanosomosis control programmes in Kenya and Uganda using antigen-ELISA (Nantulya and Lindqvist 1989).

Materials and methods

Antigen-ELISA for *T. evansi* infection in camels

RATE and RATE-peroxidase conjugate were obtained from Edinburgh, UK. The *T. evansi* Ag-ELISA was standardised using serum samples from known parasite-negative and parasite-positive camels. Subsequently, the test was used to study the prevalence of trypanosome infection in five camel herds comprising 641 camels and to evaluate the efficacy of cymelarsan used for treating the KETRI camel herd (61 camels) based at Athi River, Kenya. Anti-*T. evansi* monoclonal antibody and corresponding peroxidase conjugate were prepared at KETRI.

Antigen-ELISA for diagnosis of bovine trypanosomosis caused by *T. brucei, T. congolense* and *T. vivax*

Reagents for bovine antigen-ELISA were either obtained from ILRI or IAEA. In Uganda, the study was conducted on sera collected from cattle herds located near the Kenya-Uganda border where tsetse and trypanosomosis control were being performed using spot-on (deltamethrin), block treatment with diminazene aceturate and deltamethrin impregnated traps or screens. In Kenya Ag-ELISA was conducted on sera collected from cattle subjected to experimental Cypermethrin® (Rhône Mérieux, France) dipping at Taita and Taru ranches. Trypanosome-negative sera were obtained from cattle residing at KETRI (Muguga) and ILRI herd (Kapiti plains), comprising 60 and 200 samples.

Parasitological diagnosis

Buffy coat technique (BCT) was employed for parasitological diagnosis as described by Woo (1970). Trypanosomes were identified by microscopic examination of thin-stained blood smears. For the*T. evansi* studies mouse subinoculation was employed as described by Godfrey and Killick-Kendrick (1962).

Results

Table 1 shows the set-up of antigen-ELISAs which have been used in Kenya and Uganda for trypanosomal studies. Table 2 summarises the specificities and sensitivities of the ELISAs determined at KETRI, Kenya and at Livestock Research Institute (LIRI), Uganda for animal trypanosomosis. The CTVM *T. evansi* polyclonal-based ELISA gave fairly good relative sensitivity and specificity and on that basis was employed for epidemiological studies. In the five camel herds studied, results showed prevalence rates of *T. evansi* infection ranging from 45.5 to 86% compared to 1 to 18% using parasitological tests. Higher ELISA OD values were observed in those herds with the highest rate of patent infection or where chemotherapeutic intervention was not being practised. However, the study revealed evidence of endemic trypanosomosis in all sampled herds and although treatment with quinapyramine sulphate suppressed parasitaemia, the presence of circulating trypanosomal antigens indicated the probable persistence of infection due to resistance to this drug. In experimental camels, the use of Ag-

ELISA in evaluating the efficacy of cymelarsan revealed that more than 80% of the treated camels were apparently cured as evidenced by the disappearance of parasites and trypanosomal antigens from the blood circulation. However, relapses were observed in a few animals. Antigenaemia persisted in a number of the camels throughout the study period. Treatment on the basis of an antigenaemia reduced the proportion of ag-positive animals from 75 to 26% and parasites remained undetectable up to the end of the study.

Table 1. *Types of antigen-ELISA used in Kenya and Uganda.*

Coating antibody	Blocking agent	Conjugate	Chromogen	Institution	Country of origin
RATE	1% BSA	RATE-PO	TMB	CTVM	Kenya
T.e. MAb	None	T.e.-PO	OPD	KERTI	Kenya
T.b. MAb	1% BSA	T.b.-MAb-PO	ABTS	ILRI	Uganda
T.c. MAb	1% BSA	T.c.-MAb-PO	ABTS	ILRI	Uganda
T.v. MAb	0.5% NMS	T.c.-MAb-PO	ABTS	ILRI	Uganda
T.b. MAb	0.5% NMS	T.b.-MAb-PO	TMB	IAEA	Uganda
T.c. MAb	0.5% NMS	T.c.-MAb-PO	TMB	IAEA	Uganda
T.v. MAb	0.5% NMS	T.v.-MAb-PO	TMB	IAEA	Uganda
T.b. MAb	0.5% NMS	T.b.-MAb-PO	TMB	ILRI	Kenya
T.c. MAb	0.5% NMS	T.c.-MAb-PO	TMB	ILRI	Kenya
T.v. MAb	0.5% NMS	T.v.-MAb-PO	TMB	ILRI	Kenya

RATE = rabbit anti *T. evansi;* T.e. MAb = monoclonal antibody against *T. evansi;* T.b. = *T. brucei;* T.c. = *T. congolence;* T.v. = *T. vivax;* BSA = bovine serum albumin; PO = peroxidase; NMS = normal mouse serum; TMB = tetra methyl-benzidine; ABTS = 2,2'-azino di [3-ethylbenzthiazoline] sulphonate; OPD = Othophenylenediamine.

Table 2. *Relative sensitivity and specificity of the antigen-ELISAs used in Kenya and Uganda.*

Species/institution of origin	Relative sensitivity† (%)	Specificity (%)	Country
T. evansi/CTVM	74	79	Kenya
T. evansi/KETRI	92	100	Kenya
T.b.-ILRI	–	99	Kenya
T.c.-ILRI	83	99	Kenya
T.v.-ILRI	80	99	Kenya
T.b.-IAEA	75	95	Uganda
T.c.-IAEA	30	97	Uganda
T.v.-IAEA	65	75	Uganda
T.b.-IAEA	–	98	Kenya
T.c.-IAEA	30	100	Kenya
T.v.-IAEA	18.3	99	Kenya

$$^{*}\text{Relative sensitivity} = \frac{\text{Number of samples positive by ag} - \text{ELISA}}{\text{Number of samples positive by BCT}} \times 100$$

BCT = buffy coat technique.

In another study an IgM trypanosome-specific monoclonal antibody raised against *T. evansi,* MAb TEA1/23.4.6. was used to study *T. evansi* infection in goats and camels. Trypanosomal antigens were detected in infected goats 24 hr after intravenous inoculation of 2 × 106 trypanosomes/goat and although the levels of parasitaemia fluctuated, positivity was maintained throughout the course of infection. Following treatment, antigens concentration dropped to undetectable levels between 7 and 48 days. The same trend was observed in infected camels which were effectively treated. On the contrary there was an elevation of circulating trypanosomal antigens several weeks prior to reappearance of trypanosome in blood circulation.

The ILRI antigen-ELISA used for bovine trypanosomosis in Uganda gave a relative sensitivity of 91% based on 118 parasitologically positive cattle. In sampled herds the test revealed evidence of mixed infection and a higher prevalence of the disease than parasitological tests based on microscopic examination. In areas where 1% deltamethrin (Spot-on®, Cooper, Harare, Zimbabwe) had been applied, there was marked reduction of infection from about 10 to 0% by BCT, and 75 to 15% by antigen-ELISA, while in the areas where tsetse control was not being practised the disease incidence remained elevated.

In a subsequent study in Uganda using IAEA modified antigen-ELISA, a total of 3035 cattle (2733 from a tsetse-infested area and 302 from a tsetse-free area) were screened by both BCT and antigen-ELISA. The observed specificities of antigen-ELISA for *T. brucei, T. congolense* and *T. vivax* were 95, 97 and 75%, respectively, while sensitivities for the same trypanosome species were 75, 30 and 65%, respectively.

In Kenya, the results of the ILRI version of antigen-ELISA used to evaluate the efficacy of Cypermethrin dip for the control of *Glossina pallidipes* gave a specificity of 100% for the three species and sensitivities of 80% for *T. vivax* and 83% for *T. congolense.* Trypanosoma brucei infections were not detected parasitologically. The study showed a marked reduction in trypanosome infection on the ranch where Cypermethrin dip was applied (Taita Ranch) as opposed to the control ranch (Taru Ranch) where there was no fly control. The IAEA version of antigen-ELISA gave specificities of 100, 98 and 99% for *T. congolense, T. brucei* and *T. vivax,* respectively, and relative sensitivities of 1 and 18.5% for *T. congolense* and *T. vivax,* respectively. No *T. brucei* infections were detected parasitologically by BCT.

Discussion and recommendation

Studies carried out on the use of antigen-ELISA in Uganda and Kenya demonstrate the usefulness of combining serological diagnosis with tests based on parasite detection for the control of animal trypanosomosis. Fairly good relative sensitivity and specificity was achieved with most tests except for IAEA version of antigen-ELISA which suffered from low relative sensitivity.

However, in order to achieve internationally accepted standards, these tests could be improved in the following ways. Firstly, the sample sizes used for standardisation and validation were in most cases too small. This means that the sensitivities recorded cannot be relied on. Secondly

there was a problem of obtaining true negatives. Given the fact that antigen-ELISA is more sensitive than the parasite detection tests (Nantulya 1989) there was the definite lack of a 'goldstandard' test for comparison. This shortcoming could be overcome by using PCR in addition to parasitological tests. Before PCR is developed, standardised and validated for routine trypanosomosis diagnosis, the problem of specificity and relative sensitivity might linger on for some time to come. It is apparent that most of the monoclonal antibody probes used in these studies are of IgM class. Since the latter tend to give non-specific reactions, attempts should be made to generate MAbs of IgG class. By paying attention to the analytical requisites, diagnostic performance, standards and quality control, the performance of trypanosomal antigen-ELISA can be greatly improved.

References

Chadenga V. 1994. Epidemiology and control of trypanosomiasis. *Onderstepoort Journal of Veterinary Research* 61:385–390.

COCTU (Coordinating Office of the Control of Trypanosomiasis in Uganda). 1987. *Annual Report.* pp. 1–28.

Godfrey D.G. and Killick-Kendrick R. 1962. *Trypanosoma evansi* in camels in Nigeria: a high incidence demonstrated by inoculation of blood in rats. *Annals of Tropical Medicine and Parasitology*56:14–19.

KETRI (Kenya Trypanosomiasis Research Institute). 1990. *Strategic Plan* 1990–2000. pp. 1–8.

Nantulya V.M. 1989. An antigen detection enzyme-linked with immunoassay for the diagnosis of *rhodesiense* sleeping sickness. *Parasite Immunology* 11:69–75.

Nantulya V.M. 1991. Molecular diagnosis of parasites (review). *Experimentia* 47:143–145.

Nantulya V.M. and Lindqvist K.J.Q. 1989. Antigen detection enzyme immunoassays for the diagnosis of *Trypanosoma vivax, T. congolense* and *T. brucei* infections. *Tropical Medicine and Parasitology*40:267–272.

Nantulya V.M. 1990. Trypanosomiasis in domestic animals: the problem of diagnosis. *Revue Scientique et Technique de la Office International des Epizooties* 9:357–367.

Woo P.T.K. 1970. The haematocrit centrifugation technique for the diagnosis of African trypanosomiasis. *Acta Tropica* 27:384–386.

Application of the antigen-detection ELISA test in the diagnosis of trypanosomal infections in ruminants in Burkina Faso

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Summary

The antigen-detection ELISA (Ag-ELISA) test proposed by ILRI for the diagnosis of trypanosomosis has been evaluated in a comparative study of the susceptibility to trypanosomal infections in experimentally infected sheep and goats, and in an observational study implemented in Pays Lobi in Burkina Faso, a trypanosomosis endemic area, and in Markoye, a tsetse-free zone located in the north. The Ag-ELISA, according to the protocol of IAEA, was evaluated in a cross-sectional study in south western and northern Burkina Faso and during the monitoring of a trypanosomosis-control programme in the pastoral zone of Yalé. Low sensitivities were found for both kits, and for *Trypanosoma vivax* were as low as 10% across the different studies. Different batches of the IAEA kit were also compared and showed inconsistent results.

Introduction

The antigen-detection ELISA (Ag-ELISA) test, proposed by Nantulya and Lindqvist (1989), was described to be a useful tool for the diagnosis of trypanosomosis, particularly in chronic infections (Masake and Nantulya 1991) and in monitoring the effectiveness of a trypanocidal treatment (Nantulya and Lindqvist 1989). From 1990 to 1996 the two different designs of the test proposed by ILRI and IAEA have been evaluated within epidemiological studies implemented by Centre International de Recherche-Dévéloppement sur l'Elevage en zone Subhumide (CIRDES). This is a summary of the results.

Materials and methods

Evaluation of the kits from ILRI

This exercise has been implemented in a comparative study of the susceptibility to trypanosomal infection of experimentally infected sheep and goats, and in an observational study carried out in Pays Lobi, a trypanosomosis-endemic area. The methodology consisted of comparing the Ag-ELISA and buffy coat/contrast phase (BCT) tests (Murray et al 1977) by a systematic application of both methods to the same samples. Three hundred and thirteen samples were collected and investigated in the experimental study and 1633 in the field study. Fifty sera from a tsetse-free area in the north of Burkina Faso and 49 sera from Germany, respectively, were also tested.

Evaluation of the kits from IAEA

The kit design proposed by IAEA has been evaluated in 2362 samples collected in south western and 320 in northern Burkina Faso systematically applying the Ag-ELISA, BCT and the thin smear technique (TST) to the same samples. Nine hundred and sixty one samples were investigated in a study that monitored the dynamics of infection status in cattle following treatment with diminazene aceturate in a tsetse-control operation with deltametrin in the pastoral zone of Yalé. Forty sera have also been tested using different IAEA Ag-ELISA kits.

Statistical analysis

For studies using ILRI's kit statistical analysis consisted of determining the co-association, sensitivity and specificity of results from the Ag-ELISA in comparison with the BCT following the procedure described by Masake and Nantulya (1991). For IAEA's design, the Ag-ELISA test was compared to BCT and TST by means of kappa statistics.

Results and discussion

ILRI kits

In the experiment with *T. congolense*-infected small ruminants, antigens were detected following parasitaemia, showing a peak following a decrease. However, in *T. vivax*-infected groups antigenaemia remained below the threshold. The apparent sensitivity was 63% for *T. congolense* infection and only 10% for *T. vivax;* sensitivities for BCT were 55 and 49% for *T. congolense* and *T. vivax,* respectively.

Of the 1633 samples collected in Pays Lobi, BCT detected 8.8% positive samples. Of these 144 samples Ag-ELISA detected only 76% of the 51 *T. congolense*-BCT-positive and only 17% of the 93 *T. vivax*-BCT-positive. The predominant trypanosomal species identified by BCT was *T. vivax* followed by *T. congolense* whereas Ag-ELISA identified mostly *T. congolense* followed by *T. brucei.*

The analyses of the 50 samples from a tsetse-free area in the north of the country gave an apparent specificity of 98%. None of the 49 sera from Germany was positive. The low sensitivity of the kits from ILRI for *T. vivax* in experimental infections has been previously reported (Kanwé et al 1993). Further investigations in naturally occurring infections have confirmed these observations (Bengaly et al 1995).

Table 1. *Values of kappa statistics between ILRI Ag-ELISA test and parasitological (buffy coat and thin smear) techniques.*

Ag-ELISA	Trypanosome species	Buffy coat technique	Thin smear technique
Overall	*T. brucei*	0.00	0.00
	T. congolense	0.06	0.01
	T. vivax	0.04	0.00
Kit A	*T. congolense*	0.07	–0.06

	T. vivax	0.08	0.03
Kit B	*T. congolense*	0.07	0.06
	T. vivax	0.03	–0.01

Table 2. *Mean PCV by trypanosomal infection status as determined by different diagnostic methods in cattle in Burkina Faso.*

Diagnose method	Infection status	
	Negative	Positive
BCT	31.7 ± 5.2[†]	25.6 ± 6.4
TST	31.1 ± 5.4	26.7 ± 6.2
Ag-ELISA	31.9 ± 5.2	30.2 ± 5.9

[†]Standard deviation.

IAEA kits

Kappa statistics computed between Ag-ELISA and BCT or TST applied to the 2362 samples collected in south western Burkina Faso were below 0.07 for the different species. These data showed a lack of agreement between the Ag-ELISA and microscopic methods.

Further analyses which took into account kit batch did not improve agreement between the methods (Table 1). Indeed, values of kappa statistic of -0.25, 0.19 and 0.0 for *T. brucei, T. congolense* and*T. vivax,* respectively, were recorded between two batches of kits, applied to the same 40 sera. The apparent sensitivities were 37 and 33% for *T. congolense* and *T. vivax* infections, respectively. The apparent specificity, resulting from analyses of 320 samples from a tsetse-free area in northern Burkina Faso, was 100% for *T. brucei* and *T. congolense* and 99.7% for *T. vivax.*

In the agropastoral zone of Yalé, the monitoring of animal infection status by BCT showed a marked decrease in prevalence of *T. congolense* and *T. vivax* which was contrary to levels obtained by Ag-ELISA (Figure 1). Buffy coat technique and TST results were also more closely correlated with packed cell volume (PCV) than were the results of the Ag-ELISA test (Table 2).

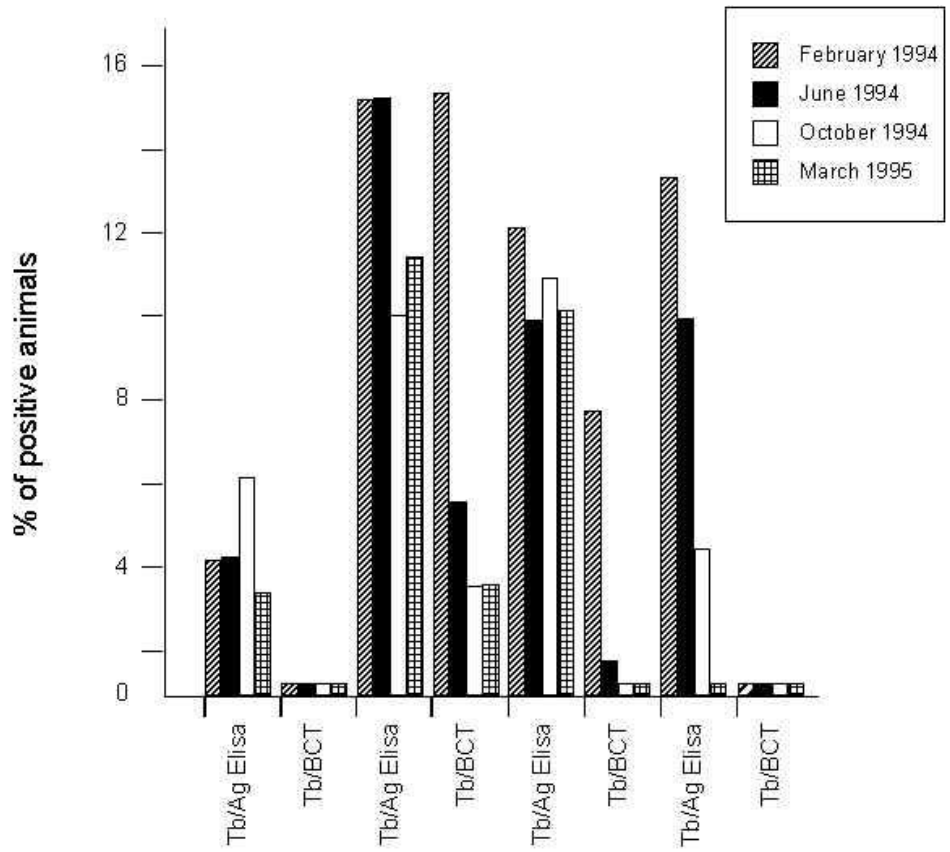

Figure 1. *Kinetic of the prevalence based on Ag-ELISA and buffy coat techniques in groups of animals infected with different trypanosome species.*

Conclusion

Epidemiological surveys carried out by CIRDES during the last five years have shown inconsistencies between ILRI and IAEA designs of the Ag-ELISA test when compared with BCT and TST. The infection status of samples negative by BCT or TST but positive by Ag-ELISA test is unclear. The hypothesis that lymphoid nodes and cephalo-rachidien liquid in such samples may contain trypanosomes needs further study. The polymerase chain reaction (PCR) technology applied to such samples might also clarify their infection status.

References

Bengaly Z., Kanwe A.B. and Duvallet G. 1995. Evaluation of an antigen detection-ELISA test for the diagnosis of trypanosomiasis in naturally infected cattle. *Tropical Medical Parasitology* 46:284–286.

Kanwé A.B., Bengaly Z., Saulnier D., Duvallet G. 1993. Evaluation du test de détection des antigènes circulants de trypanosomes à l'aide d'anticorps monoclonaux: Infections expérimentales et naturelles. *Revue d'Elevage et de Medecine Vétérinaire des Pays Tropicaux* 45 (3):265–271.

Masake R.A., Nantulya V.M. 1991. Sensitivity of an antigen detection enzyme immunoassay for diagnosis of *Trypanosoma congolense* infection in goats and cattle. *Journal of Parasitology* 77 (2):231–236.

Murray M., Murray P.K., McIntyre W.I.M. 1977. An improved parasitological technique for the diagnosis of African trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*71:325–326.

Nantulya V.M. and Lindqvist K.J. 1989. Antigen detection enzyme immunoassays for the diagnosis of *Trypanosoma vivax, T. congolense* and *T. brucei* infection in cattle. *Tropical Medical Parasitology*40 (3):267–272.

The use of enzyme-linked immunosorbent assays (ELISAs) for trypanosomosis surveys and surveillance

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Summary

The performance of a trypanosomal antigen-detection ELISA (Ag-ELISA) and a trypanosomal antibody-detection ELISA (Ab-ELISA) was evaluated. The Ag-ELISA had low sensitivity. The Ag-ELISA using dried blood spots as antibody source, on the other hand, performed well and is believed to have potential as a practical diagnostic test in trypanosomosis surveys. Nevertheless, more development work will have to be done to standardise the trypanosomal Ab-detecting ELISA and determine its performance parameters.

Introduction

In Zimbabwe, tsetse-transmitted trypanosomosis is a severe threat to the livestock industry. For the past decades, intensive tsetse control operations have pushed the tsetse front back to Zimbabwe's eastern and north-eastern borders (Figures 1and 2). Current tsetse-control strategies involve the use of insecticide-treated, odour-baited targets and, insecticide-treated mobile baits (Shereni 1989). Nevertheless, Zimbabwe's impressive achievement in controlling tsetse is continuously threatened by tsetse invading from neighbouring countries. In view of this, a Regional Tsetse and Trypanosomosis Control Programme (RTTCP) was initiated with the aim to control tsetse in the fly-belt common to Malawi, Mozambique, Zambia and Zimbabwe.

Various methodologies have been used to monitor the success of tsetse control operations in the RTTCP region. Tsetse surveillance methods are not very sensitive, especially for *Glossina morsitans morsitans.* This makes an objective evaluation of the tsetse situation difficult. To complement entomological surveys, when low-density vector populations are present, trypanosomosis surveys can be conducted. In most cases use is made of direct parasitological diagnostic tests (Paris et al 1982). The clear limitation, from a diagnostic point of view, of parasitological tests to detect infected animals promoted the development of indirect methods, mainly immunodiagnostic techniques. The development of enzyme-linked immunosorbent assays (ELISA) for diagnosing tsetse-transmitted trypanosomosis has shifted the emphasis even more on immunodiagnosis rather than parasitological diagnosis. Currently, ELISAs have been developed for the detection of either trypanosomal antigens or trypanosomal antibodies. Both tests could be very useful diagnostic tests in trypanosomosis surveys and/or surveillance. However, their performance still needs to be evaluated before their routine use can be promoted. This paper describes the work conducted in Zimbabwe on the evaluation of both diagnostic tests.

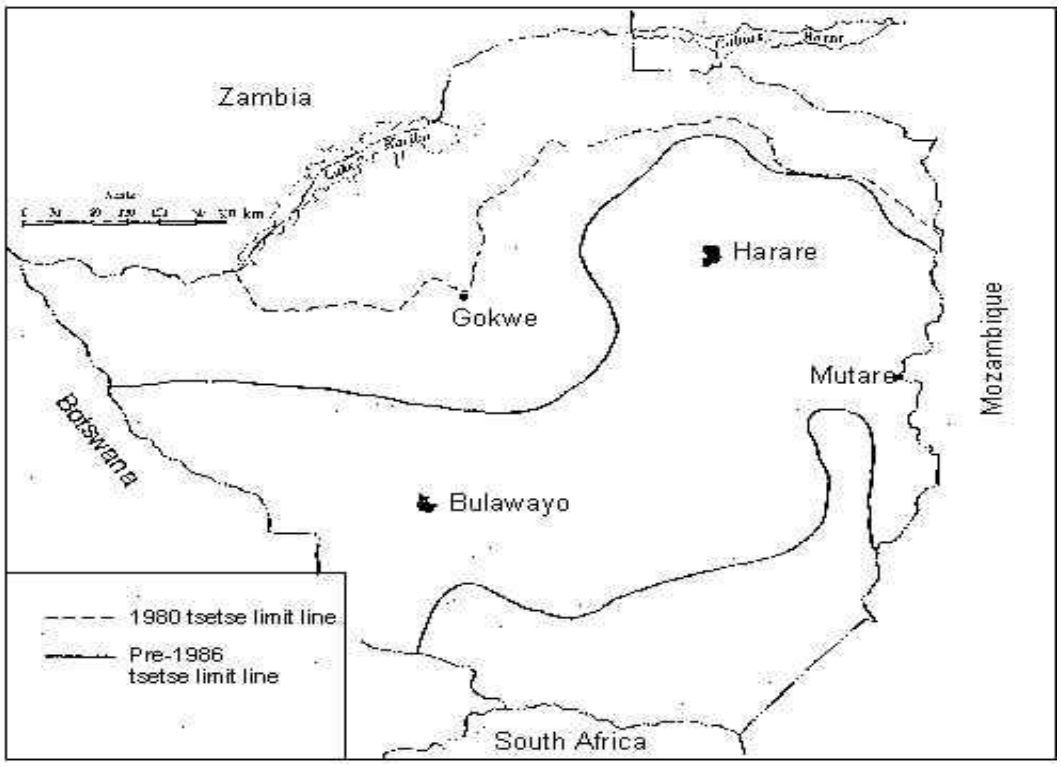

Figure 1. *Tsetse limit in the past.*

Materials and methods

The trypanosomal antigen-detection ELISA (Ag-ELISA)

The Ag-ELISA was evaluated using reagents and protocols from both the International Livestock Research Institute (ILRI) and the International Atomic Energy Agency (IAEA).

Blood was collected from cattle in tsetse-free and tsetse-infested areas of Zimbabwe. All samples were examined for the presence of trypanosomes using parasitological methods (buffy coat method). The sera were stored at -20°C until use. The circulating trypanosomal antigens were detected in the sera using a sandwich enzyme-linked immunosorbent assay as described by Nantulya and Lindqvist (1989). Mouse monoclonal antibodies specific for *Trypanosoma brucei, T. congolense* and *T. vivax* antigens were used. Bench protocols were strictly followed.

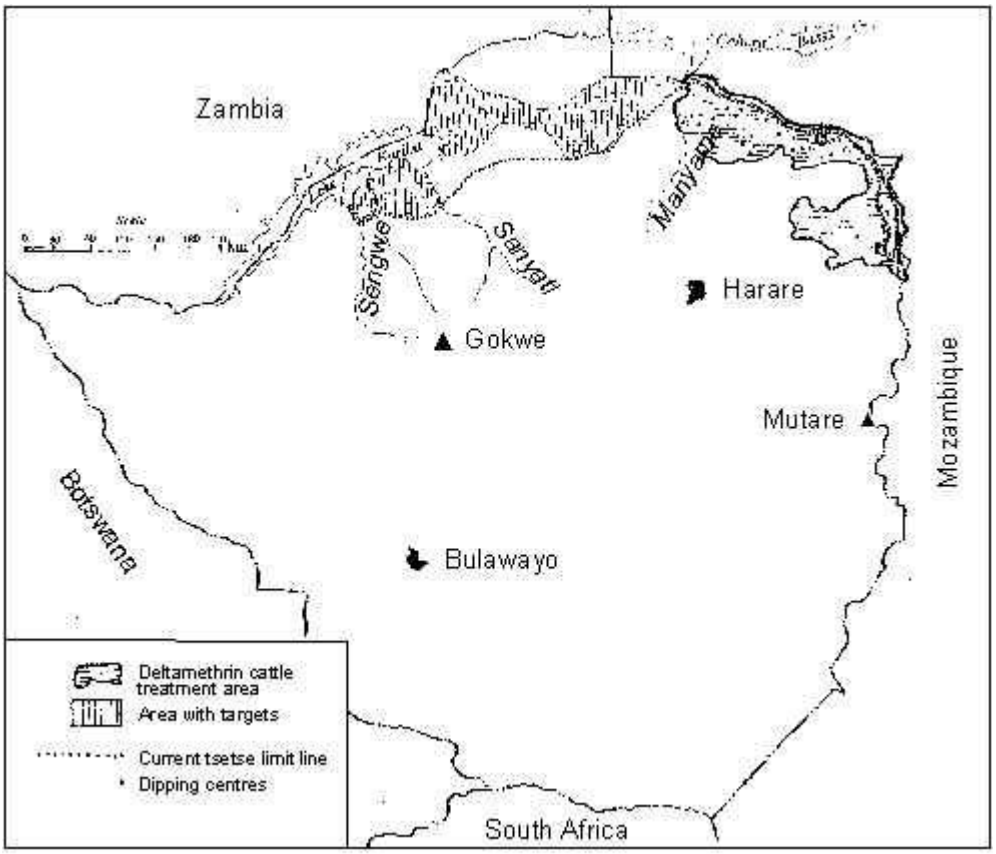

Figure 2. *Current tsetse limit and control areas.*

The trypanosomal antibody-detection ELISA (Ab-ELISA)

Bovine blood was collected in tsetse-infested areas in Zambia and tsetse-free areas in Zimbabwe. Blood was collected from an ear vein into heparinised microhaematocrit centrifuge capillary tubes. Blood contained in one heparinised microhaematocrit centrifuge capillary tube was extruded onto a filter paper (Whatman No. 4, Whatman®). Samples were air-dried out of direct sunlight, placed in a plastic bag with self-indicating silica gel desiccant; the bag was sealed and kept as cool as possible until specimens were refrigerated or frozen.

Table 1. *Results of negative population testing using IAEA test.*

Area	No. of cattle tested	No. of negatives	Negative %	T.c.	T.b./T.c.	T.v.
Nyaguwe	46	35	76	0	1	10
Koyoyo	50	43	86	1	0	6
Nora	50	43	86	1	1	5
Commercial farm	94	91	97	0	0	3

Eluted blood spots were screened for the presence of anti-trypanosomal antibodies using an indirect anti-trypanosomal Ag-detection ELISA (Luckins 1977). Use was made of a *T. congolense* (IL 3000) invariable antigen batch prepared by the Parasitology Laboratory of the Department of Paraclinical Studies of the School of Veterinary Medicine, University of Zambia. A rigorous system of quality assurance was adopted. Hence, each ELISA-microplate was run with strong positive, weak positive and negative reference sera that were required to comply with pre-set values. The optical density (OD) of each ELISA sample tested was expressed as a percentage (percentage positivity, PP) of the strong positive reference standard.

Results

The Ag-ELISA

The prevalence of *T. brucei* infections was high in samples analysed using both Ag-ELISAs. This high prevalence of *T. brucei* infections in areas where tsetse has never been and never will be present is incompatible with the epidemiology of tsetse-transmitted trypanosomosis. The majority of those infections were, therefore, considered false positives. Moreover, the tests had low sensitivity resulting in serological negative results of parasitologically positive *(T. vivax* and *T. congolense)* samples.

Modification of the IAEA Ag-ELISA resulted in improved specificity. The seroprevalence of trypanosomosis in sera collected from 240 cattle from several tsetse-free areas in Zimbabwe (communal areas and a commercial farm) varied between 3 and 24% (Table 1).

According to the above results the specificity of the test was:

T. brucei	99.2%
T. congolense	98.3%
T. vivax	90.9%

The main problem encountered with the IAEA antigen-ELISA was the instability of the conjugate. This was especially the case for the *T. vivax* conjugate whose potency decreased with time (Figure 3).

Moreover, in order to obtain repeatable results freshly distilled water had to be used.

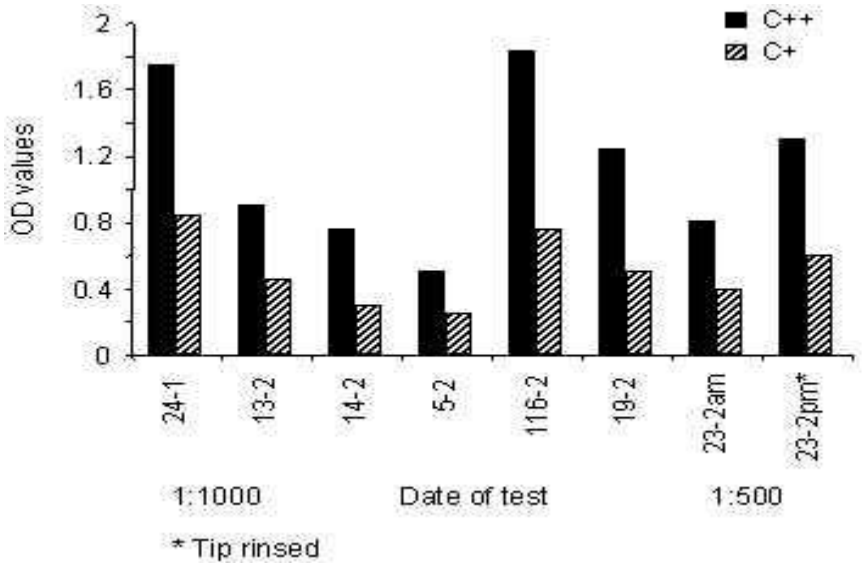

Figure 3. *OD values for* T. vivax *controls using antigen ELISA test four times for each of two conjugate dilutions.*

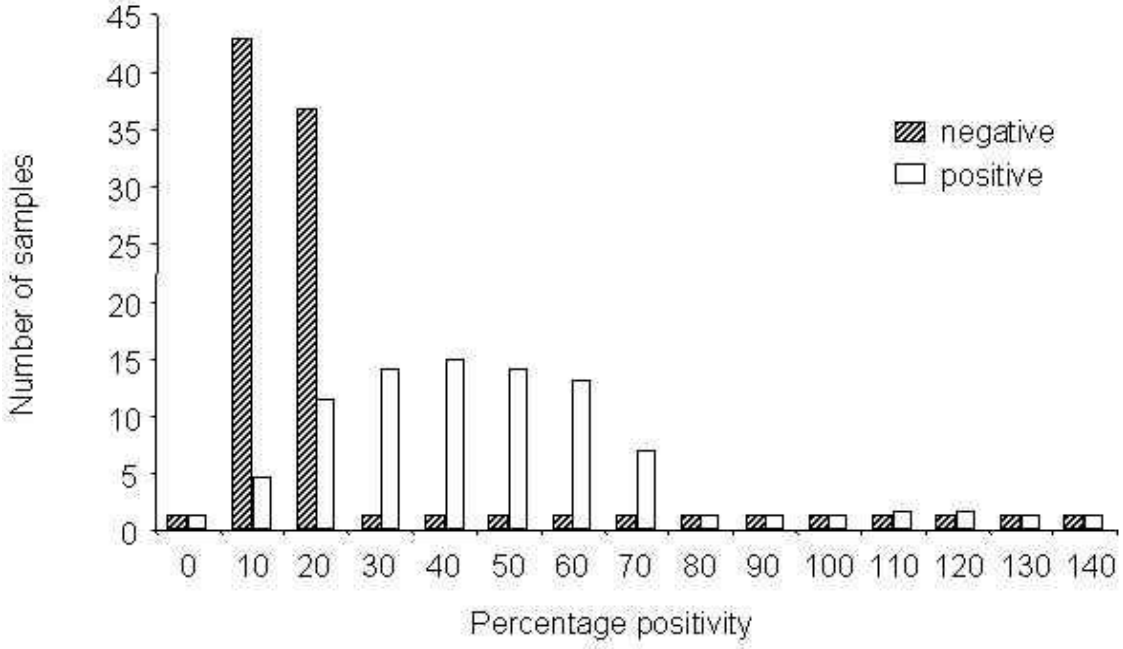

Figure 4. *Frequency distribution in percentage positivity of antibody ELISA values of 160 known positive and negative blood spot samples.*

Table 2. *Mean and standard deviations of ELISA values of known positive and negative samples.*

	OD values		Percentage positivity	
	Negative	Positive	Negative	Positive

Mean	0.107	0.589	9.94	43.15
SD	0.028	0.370	2.79	27.93

The antibody-ELISA

Preliminary work with the antibody-ELISA indicated a clear discrimination between the distribution of the OD values of approximately 50 parasitological positive and approximately 50 parasitological negative samples (Figure 4). Table 2 summarises the average OD values and PP of these samples.

The test's performance largely depended on the quality of the water used. As is the case for the Ag-ELISA, freshly distilled and deionised water should be used. This results in the inconvenience of making fresh buffers almost on a daily basis.

To standardise the test, use was made of a batch of antigen of which large quantities are available. Moreover, large batches of strong positive and negative sera have been prepared and will continue to be used as reference sera.

Conclusion and discussion

Our observations confirm the low sensitivity of the Ag-ELISA. Its value in detecting tsetse-transmitted trypanosomal infections during trypanosomosis surveys/surveillance is, therefore, questionable.

Although the Ab-ELISA has not yet been used for testing large numbers of field samples, it is believed to have potential in trypanosomosis surveys to determine the spread of the disease. Its use as a tool to evaluate the effect of tsetse control operations on the transmission of trypanosomosis will need more research into the dynamics and persistence of trypanosomal antibodies. The use of eluted blood spots as an antibody source has certainly improved the sustainability of Ab-ELISA. This particular modification has the significant advantage that samples do not have to be kept at low temperatures facilitating sample storage and sample transportation. Nevertheless, more development work will have to be done to standardise the test and determine its performance parameters.

Acknowledgements

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References

Luckins A.G. 1977. Detection of antibodies in *Trypanosoma*-infected cattle by means of a microplate enzyme-linked immunosorbant assay. *Tropical Animal Health and Production* 9:53–62.

Nantulya V.M. and Lindqvist K.J. 1989. Antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma vivax, T.congolense* and *T.brucei* infections in cattle. *Tropical Medecine and Parasitology* 40:267–272.

Paris J., Murray M. and McOdimba F. 1982. A comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomiasis in cattle. *Acta Tropica* 39:3070–316.

Shereni W. 1989. Strategic and tactical developments in tsetse control in Zimbabwe (1981–1989). *Insect Science and its Application* 11:399–409.

Validation and use of Ag-ELISAs in the diagnosis of *Trypanosoma evansi:* infections in buffalo in Indonesia

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Summary

Two antigen detection ELISAs (Ag-ELISA) for *Trypanosoma evansi* were extensively evaluated in Indonesia in experimental animals and in field trials to determine their sensitivity and specificity. In experimentally infected buffalo, the development of antigenaemia varied according to the strain of trypanosome used for infection, the individual animal, the period of infection and the particular epitope recognised by the monoclonal trapping antibody. In a primary infection, antigens were detected as early as 7 days after infection or as late as 42 days. Following treatment with trypanocidal drug, antigens declined in some buffalo within 12–47 days. A similar pattern of responses was seen when the buffalo were infected with a heterologous strain of *T. evansi.* Serum antibody assays were carried out using IgM- and IgG-ELISAs and card agglutination tests (CATT). Antibodies were detected by 7–42 days after infection and disappeared slowly after treatment. Estimates of the diagnostic sensitivity of the different tests during the course of infection in experimentally infected animals showed considerable variation, with maximum sensitivity of the Ag-ELISAs being achieved about 42 days after primary infection and between 50 and 64 days after secondary infection. A similar pattern of sensitivity was shown by IgM- and IgG-ELISA and CATT tests. For field use, estimations of sensitivity were based on the results obtained from sera collected from naturally infected buffalo and specificity on the results of assays on sera from Australian buffalo. For the Ag-ELISAs, sensitivity estimates varied between 67 and 82% and diagnostic specificity was between 75 and 78%.

Introduction

Several MAbs have been developed at CTVM and ILRI, which specifically recognise antigenic determinants present only in trypanosomes of the sub-genus *Trypanozoon* and while monoclonal antibodies have been used in Ag-ELISAs for the diagnosis of African trypanosomoses for a number of years (Rae and Luckins 1984; Nantulya and Linquvist 1989; Nantulya et al 1987; Masake and Nantulya 1991) there has been little attempt at their proper validation in terms of quality assurance and determination of diagnostic sensitivity and specificity for use in diagnosis of *Trypanosoma evansi*in S.E. Asia. The aim of the present study therefore was to evaluate the two Ag-ELISAs for the detection of *T. evansi* infections in individual Indonesian buffaloes and the ability of the assays to monitor parasite clearance after chemotherapy. In order to achieve this, buffaloes were experimentally infected with *T. evansi* and treated with a trypanocide to: 1) obtain profiles of antigenaemia of individual buffaloes, 2) determine when antigenaemia is first detectable post-infection, 3) determine the frequency with which individual buffaloes and groups of buffaloes are positive by weekly Ag-

ELISAs compared with other diagnostic tests, 4) monitor changes in the level of antigenaemia after chemotherapy and 5) determine the diagnostic sensitivity and specificity of the tests in field studies in Indonesia.

Materials and methods

Two MAbs (2G6 MAb and Tbr7 MAb) were chosen for use in the *T. evansi* Ag-ELISA; 2G6 MAb from CTVM is an IgG fraction derived from MAbs against *T. evansi* which recognises a 70 kDa antigen; Tbr7 MAb from ILRI is an IgM fraction derived from MAbs against *T. b. rhodesiense* which recognises a 15 kDa antigen. Assays were standardised using high binding, 96-well microtitre ELISA plates (Immulon 2, Dynatech M129B). Plates were coated with trapping MAb diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6 shaken at ambient temperature for 30 seconds to ensure even distribution of the MAb and then stored overnight at 4°C. The following day, plates were washed with phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS/0.05T). Test samples and control sera were diluted with PBS containing PBS/0.50T; test serum samples were added in duplicate, and high and low positive (C++, C+), negative (C-) and conjugate (Cc) controls (diluent buffer only) were included on each plate. After incubation at 37°C for 30 minutes, the plates were washed as before. The monoclonal antibodies conjugated with horseradish peroxidase were diluted with PBS/0.50T and added to the plates which were shaken at 37°C for 30 minutes. After washing, the substrate/chromogen solution, tetramethylbenzidine dihydrochloride/H2O2 was added, the plate incubated for 10 minutes at 37°C, the enzymatic reaction stopped by addition of 2 M sulphuric acid and the OD read at 450 nm. Absorbance values were expressed as percent positive values (PP). Upper (UCL) and lower (LCL) control limits for the Ag-ELISA control sera were determined by assaying multiple replicates of the different control sera; the 10 and 90% percentile values were chosen as the lower and upper control limits respectively because the data did not have a normal distribution.

Two isolates of *T. evansi* were used for the experimental infections: an isolate of low pathogenicity, *T. evansi* Bakit 259 from East Java, was used for a primary infection and a more pathogenic isolate,*T. evansi* Bakit 362 from Madura Island, for secondary infection. Each buffalo was intravenously injected with 2 × 107 trypanosomes. Thirty five buffaloes were infected with *T. evansi* Bakit 259 and monitored for 105 days before treatment with the trypanocidal drug, cymelarsen. Following chemotherapy, all buffaloes were injected intravenously with *T. evansi* Bakit 362 and monitored for 64 days before treatment. Weekly serum samples were tested by Ag-ELISA, IgM-ELISA, IgG-ELISA and CATT and blood samples by the microhaematocrit centrifugation test (MHCT), mouse inoculation (MI) and wet blood film examination.

For the field studies, five visits to northern Central Java were made over a 24-month period and during each visit approximately 100 buffaloes were sampled from each of five districts (Batang, Pekalongan, Pemalang, Tegal and Brebes). To obtain estimates of diagnostic specificity for the serological tests, sera were assayed from 263 Australian buffalo and 80 Australian cattle which had never been exposed to trypanosomosis risk, whilst for diagnostic sensitivity, sera from naturally infected Indonesian buffalo were used.

Results and discussion

Diagnostic specificity

The diagnostic specificity estimates obtained from buffalo and cattle sera and calculated with associated 95% confidence intervals for both Ag-ELISAs and for Ab-ELISAs are shown in Table 1. The specificity of 2G6 Ag-ELISA was similar to the specificity of Tbr7 Ag-ELISA using buffalo sera but was significantly lower using cattle sera ($p<0.001$), at both cut-off values. Specificity estimates were significantly higher for Tbr7 Ag-ELISA ($p<0.01$) and for 2G6 Ag-ELISA ($p<0.05$) at the 30% cut-off value. The underlying cause of the false positive results obtained with the two ELISAs is not known. The specificity of the CATT was 100% (97, 100) which was significantly higher than the estimates obtained for the IgM- and IgG-ELISAs ($p<0.001$) and the IgG-ELISA specificity was significantly higher than the IgM-ELISA specificity ($p<0.001$) (Table 1). The specificity estimates of both the IgM- and IgG-ELISAs were significantly higher using the higher cut-off value ($p<0.001$).

Diagnostic sensitivity—experimental studies

Ag-ELISAs

There were three major types of antigen response: 1) development of antigenaemia, 2) no antigen detectable or 3) persistence of pre-infection antigenaemia. Antigenaemia was first detected 7 to 42 days after the primary and secondary infections by both the 2G6 Ag-ELISA and Tbr7 Ag-ELISA. Some buffaloes had similar 2G6-specific and Tbr7-specific profiles of antigenaemia whereas in other buffaloes the two antigens appeared at different stages of infection, with differences in the pattern of development of antigenaemia. Both Ag-ELISAs detected peaks of antigenaemia in many buffaloes from 42 days post-infection and declining antigen levels thereafter. Estimates of sensitivity of the 2G6 Ag-ELISA and Tbr7 Ag-ELISA calculated for different stages of the secondary *T. evansi* Bakit 362 showed the sensitivity of the Tbr7 Ag-ELISA was 83% at 15 days post-infection and was significantly higher compared with the 2G6 Ag-ELISA at 15 to 36 days post-infection ($p<0.05$). For both Ag-ELISAs, the highest sensitivity estimates were obtained 57 to 64 days post-infection. The observed differences in sensitivity reflect the underlying fluctuations in serum trypanosomal antigens that occur in buffaloes. Previous studies have reported clearance of *T. vivax* and *T. congolense* antigens within two weeks and of *T. brucei* antigens within four weeks of treatment with a trypanocidal drug (Nantulya and Lindqvist 1989). However in the present study, antigen clearance was much more variable and in some buffaloes antigenaemia persisted after treatment for up to 74 days after the primary infection and up to 8 months after the secondary infection, suggesting that these Ag-ELISAs may have limitations for the differentiation of active *T. evansi* infections from previous exposure to the parasite.

Table 1. *Estimates of diagnostic specificity (%), with 95% confidence intervals (CI), for (A) the 2G6 Ag-ELISA and Tbr7 Ag-ELISA and (B) for the IgM-ELISA and IgG-ELISA, using sera from Australian buffaloes (n=263) and cattle (n=80).*

(A)

Ag-ELISA	Species	20% PP cut-off		30% PP cut-off	
		Specificity (%)	95% CI	Specificity (%)	95% CI
2G6	Buffalo	75	(70, 80)	83	(79, 88)
Tr7	Buffalo	78	(73, 83)	87	(83, 91)
2G6	Cattle	68	(56, 78)	70	(59, 80)
Tbr7	Cattle	100	(96, 100)	100	(96, 100)

(B)

Ag-ELISA	20% PP cut-off		30% PP cut-off	
	Specificity	95% CI	Specificity (%)	95% CI
IgM	55	(46, 64)	89	(83, 94)
IgG	92	(86, 96)	98	(94, 100)

Antibody assays

There was a wide range of serum antibody responses in individual buffaloes. Buffaloes were positive by antibody-detection tests by 7 to 42 days post-infection, but by contrast with the Ag-ELISAs usually remained positive, particularly by the IgG-ELISA, throughout the remainder of the monitoring period. In some buffaloes antibody responses were low and not all antigenaemic buffaloes had antibody responses. In the primary infection, the sensitivity of the IgM-ELISA was higher compared with the IgG-ELISA and CATT on most sampling dates post-infection. The IgM-ELISA had a sensitivity of 50% on day 7 post-infection whereas the sensitivity of the IgG-ELISA increased more slowly but was higher in the later stages of infection. The sensitivity of the CATT increased slowly, but unlike the IgG-ELISA, declined after 56 days post-infection. With the IgM-ELISA and IgG-ELISA, the highest sensitivity estimates were obtained 50 to 64 days post-infection. The CATT had maximal sensitivity 15 to 29 days post-infection. In the primary infection, the ranking of the different diagnostic tests in descending order of frequency of positive results was CATT>MI>IgG-ELISA> IgM-ELISA>2G6 Ag-ELISA>Tbr7 Ag-ELISA>MHCT for buffaloes shown to be parasitaemic. In this group of buffaloes, 25% (19, 30) of all weekly samples were positive by 2G6 Ag-ELISA compared with 15% (11, 20) by Tbr7 Ag-ELISA. By antibody-detection tests, 34% (28, 41) of all weekly samples of parasitaemic buffaloes were positive by IgM-ELISA and IgG-ELISA, and 55% (48, 61) were positive by CATT. In the secondary infection, 54% (47, 62) of all weekly samples from parasitaemic buffaloes were positive by 2G6 Ag-ELISA compared with 76% (69, 82) by Tbr7 Ag-ELISA, 54% (47, 62) by IgM-ELISA, 82% (76, 87) by IgG-ELISA and 76% (70, 82) by CATT. The ranking of the tests in descending order of frequency of positive tests for secondary infection was IgG-ELISA>CATT>Tbr7 Ag-ELISA>MI>2G6 Ag-ELISA>IgM-ELISA>MHCT.

Diagnostic sensitivity—field studies

During the field work in Central Java, 139 buffaloes were found to be naturally infected with *T. evansi* by MHCT (n=39) and by MI (n=100). All these sera were tested by the 2G6 and Tbr7 Ag-ELISAs as well as IgM-ELISA, IgG-ELISA and CATT tests and estimates of their sensitivity calculated. The diagnostic sensitivity of the 2G6 Ag-ELISA and Tbr7 Ag-ELISA were estimated based on results of MHCT, or MI, or a combination of these tests, as shown in Table 2. Using buffaloes positive by MHCT alone, the Tbr7 Ag-ELISA had a significantly higher sensitivity than the 2G6 Ag-ELISA using a 20% cut-off value ($p<0.05$) and a 30% cut-off value ($p=0.01$); the sensitivity of the 2G6 Ag-ELISA was higher at the 20% cut-off value ($p<0.05$) but the sensitivity of the Tbr7 Ag-ELISA did not differ significantly. The diagnostic sensitivities of the IgM-ELISA, IgG-ELISA and CATT were also estimated on the basis of being positive by MHCT, MI or a combination of these tests (Table 3). For these different buffalo groups, the sensitivity estimates of the CATT were 79 (70, 87), 77 (61, 89) and 78% (72, 85), respectively. Using MHCT-positive buffaloes alone, the IgG-ELISA had a significantly higher sensitivity ($p<0.05$) compared with the CATT and there was a significant reduction in the sensitivity of the IgM-ELISA ($p<0.05$) but not of the IgG-ELISA, by using the higher cut-off value. Using MI-positive buffaloes, there was no significant difference between the sensitivity estimates obtained. Using MHCT- and MI-positive buffaloes, both IgM and IgG-ELISAs had a sensitivity significantly higher than the CATT ($p<0.001$) and the sensitivity of the IgM-ELISA was significantly lower at the higher cut-off value ($p<0.001$) but not for the IgG-ELISA. The results suggest that these Ag-ELISAs could be used as screening tests for groups of buffaloes in trypanosomosis endemic areas but would not be suitable for the diagnosis of *T. evansi* infections in individual buffaloes.

Table 2. *Estimates of diagnostic sensitivity (%), with associated 95% confidence intervals (CI), for the 2G6 Ag-ELISA and Tbr7 Ag-ELISA using sera from Indonesian buffaloes shown to be naturally infected with Trypanosoma evansi by (A) microhaematocrit technique (n=100), (B) mouse inoculation (39) and (C) either microhaematocrit technique or mouse inoculation (139).*

	20% PP cut-off		30% PP cut-off	
Test	Sensitivity (%)	95% CI	Sensitivity (%)	95% CI
(A)				
2G6 Ag-ELISA	67	(57, 76)	51	(41, 61)
Tbr7 Ag-ELISA	80	(71, 87)	69	(59, 78)
(B)				
2G6 Ag-ELISA	79	(64, 91)	67	(50, 81)
Tbr7 Ag-ELISA	82	(67, 93)	77	(61, 89)
(C)				
2G6 Ag-ELISA	71	(63, 79)	55	(46, 63)
Tbr7 Ag-ELISA	81	(75, 88)	71	(64, 79)

Table 3. *Estimates of diagnostic sensitivity (%), with associated 95% confidence intervals, (CI) for the IgM-ELISA and IgG-ELISA using sera from Indonesian buffaloes shown to be naturally infected with Trypanosoma evansi by (A) microhaematocrit technique (n = 100), (B) by mouse inoculation (n = 39) and (C) by either microhaematocrit technique or mouse inoculation (n = 139).*

Test	20% PP cut-off		30% PP cut-off	
	Sensitivity	95% CI	Sensitivity	95% CI
A				
IgM-ELISA	86	(78, 92)	75	(65, 83)
IgG-ELISA	90	(82, 95)	85	(77, 91)
B				
IgM-ELISA	92	(79, 98)	87	(73, 96)
IgG-ELISA	87	(73, 96)	77	(61, 89)
C				
IgM-ELISA	88	(82, 93)	78	(72, 85)
IgG-ELISA	89	(84, 94)	83	(77, 89)

Predictive values

Positive and negative predictive values for the different tests are shown in Table 4. Using a 20% cut-off value, the ranges of positive predictive values calculated for theoretical true prevalence values of 0.10 to 0.90 were 0.24 to 0.96 (2G6 Ag-ELISA) and 0.29 to 0.97 (Tbr7 Ag-ELISA). Positive and negative predictive values of the Tbr7 Ag-ELISA were higher compared to the 2G6 Ag-ELISA values, and were higher at a 30% cut-off value, particularly at low true prevalence values. With a true prevalence of 0.50 and a 20% cut-off value, the positive predictive values of the 2G6 Ag-ELISA and Tbr7 Ag-ELISA were 0.74 and 0.79, respectively. The usefulness of the Ag-ELISAs in areas with a low trypanosomosis prevalence was questionable because the probability that a test-positive buffalo is truly *T. evansi*-infected was no greater than by chance alone as indicated by a positive predictive value less than 0.50. The results for the antibody assays showed that as true prevalence values increased, the positive predictive values of the IgM-ELISA and IgG-ELISA increased and the negative predictive values decreased. At a theoretical test prevalence of 0.50, the positive predictive values of the IgM-ELISA, IgG-ELISA and CATT were 0.88, 0.92 and 1.0, respectively. With all theoretical test prevalence values, the CATT had the highest positive predictive value (1.0) and the IgG-ELISA had highest negative predictive values compared with the other tests. The positive predictive value of the antigen assays is not sufficiently high to warrant their application in eradication programmes nor for pre-exportation checks without other *T. evansi* tests. After importation of naive buffaloes, from Australia for example and in outbreaks of trypanosomosis, the diagnosis of *T. evansi* infections in clinical cases and the monitoring for subclinical and new infections could include MHCT tests in parallel with Ag-ELISAs. To increase the probability of detecting *T. evansi*-infected animals, parallel testing could be used, for example with the MHCT to detect early infections and the IgG-ELISA to detect later infections. Serial testing using an antibody-detection ELISA and then an antigen-detection ELISA for all positive samples would increase the overall specificity which would be necessary in the final stages of an eradication campaign but there would be an associated decrease in overall sensitivity.

Table 4. *Positive predictive values (PPV) and negative predictive values (NPV) calculated for various theoretical true prevalence values (P) for (A) two Ag-ELISAs and (B) for different antibody detection assays.*

A

P	2G6 Ag-ELISA				Tbr7 Ag-ELISA			
	20% PP		30% PP		20% PP		30% PP	
	PPV	NPV	PPV	NPV	PPV	NPV	PPV	NPV
0.10	0.24	0.96	0.26	0.94	0.29	0.97	0.38	0.96
0.30	0.55	0.86	0.58	0.81	0.61	0.91	0.70	0.88
0.50	0.74	0.72	0.76	0.65	0.79	0.80	0.85	0.75
0.70	0.87	0.53	0.88	0.44	0.90	0.63	0.93	0.56
0.90	0.96	0.22	0.97	0.17	0.97	0.31	0.98	0.25

B

P	IgM-ELISA		IgG-ELISA		CATT	
	PPV	NPV	PPV	NPV	PPV	NPV
0.10	0.44	0.97	0.55	0.99	1.0	0.98
0.30	0.75	0.90	0.83	0.95	1.0	0.91
0.50	0.88	0.80	0.92	0.89	1.0	0.82
0.70	0.94	0.63	0.96	0.78	1.0	0.66
0.90	0.98	0.31	0.99	0.48	1.0	0.34

References

Masake R.A. and Nantulya V.M. 1991. Sensitivity of an antigen detection enzyme immunoassay for diagnosis of *Trypanosoma congolense* infections in goats and cattle. *Journal of Parasitology*77:231–236.

Nantulya V.M. and Lindqvist K.J. 1989. Antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma vivax, T. congolense* and *T. brucei* infections in cattle. *Tropical Medicine and Parasitology* 40:267–272.

Nantulya V.M., Musoke A.J., Rurangirwa F.R., Saigar N. and Minja S.H. 1987. Monoclonal antibodies that distinguish *Trypanosoma congolense, T.vivax* and *T.brucei. Parasite Immunology* 9:421–431.

Rae P.F. and Luckins A.G. 1984. Detection of circulating trypanosomal antigens by enzyme immunoassay. *Annals of Tropical Medicine and Parasitology* 77:587–596.

Other diagnostic assays AB-ELISA and DNA-based test

Antibody detection tests for diagnosis of African trypanosomosis

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African trypanosomes have a complex antigenic structure consisting of variable surface glycoprotein antigens (VSGs) and invariant antigens.

The strongly immunogenic VSG defines the variable antigen type (VAT) of an individual trypanosome and represents about 10% of its total protein content. In the course of an infection, different VAT populations succeed each other in a loosely defined hierarchical order, starting with a series of so-called predominant VATs. Trypanosomes of the same taxon may express distinct VAT repertoires, with varying degrees of overlap due to cross-reacting IsoVATs.

At the outer surface of an intact live trypanosome, only a few `external VSG epitopes' are exposed. These epitopes elicit VAT- or IsoVAT-specific trypanocidal antibodies with lytic, agglutinating and opsonising properties. After cell death or detachment of the surface coat, however, a large number of `internal, cryptic VSG epitopes' become exposed. Some of these epitopes are common to different VSGs and may be exploited in antibody detection tests.

Trypanosomes probably contain hundreds or thousands of invariant antigens but most of them are minor components. As compared with VSGs, most invariant antigens are weak immunogens. In buffer extracts a few tens of major components can be revealed by immunoelectrophoresis with hyperimmune antisera (Le Ray 1975). In addition, various membrane-bound, non-hydrosoluble antigens have been reported, including cytoskeleton elements, invariant surface glycoproteins (ISGs), enzymes and receptors. Within a given subgenus all the trypanosome species have essentially the same major invariant antigens and there is also considerable overlap between subgenera. Whereas several subgenus specific antigens have been identified, species- and subspecies-specific antigens have not yet been recorded.

The serum of an infected host thus contains a broad spectrum of antibodies to variable and invariant antigens. Which kind of antibodies are detectable depends on the antigen preparation and the test system. Tests with live bloodstream form trypanosomes, such as immune trypanolysis, only detect VAT-specific antibodies. Direct agglutination tests with fixed bloodstream forms (e.g. CATT) detect antibodies to external and internal VSG epitopes, and probably to some invariant surface antigens as well. Immunofluorescence tests on trypanosome-infected blood smear preparations detect antibodies to various VSG epitopes and invariant antigens. When the trypanosomes have previously been fixed in suspension with 1–2% of formaldehyde, surface antigens become the predominant reactant. To minimise the interference of VSG, a mild acetone-formaldehyde fixation procedure has been proposed by Katende et al (1987). Tests using total buffer or detergent extracts of bloodstream from trypanosomes (ELISA, indirect agglutination, dot blot, Western blot, etc.) detect antibodies to variable and solubilised invariant antigens. In all these instances a minimal requirement to

standardise the antigenic preparation is to start with trypanosome-clone populations of well defined VAT.

Trypanosoma brucei gambiense, a causative agent of sleeping sickness, displays a limited diversity in VAT repertoires. Several predominant IsoVATs of this parasite have been identified by immune trypanolysis tests and most of the current antibody detection tests are based on the use of selected VATs (CATT, IFAT) or VSGs (Latex agglutination, indirect haemagglutination, ELISA). A similar approach seems possible for *T. evansi.*

For the salivarian trypanosomes in general, further evaluation and selection of invariant antigens is required to develop more reliable antibody detection assays. Tests combining the crude antigen cocktail present in whole trypanosomes or their extracts with the cocktail of antibodies present in test sera have limited subgenus specificity and cross-reactions also occur with non-trypanosomosis samples. By increasing the serum dilution, specificity may be improved at the expense of sensitivity. The indirect immunofluorescence and immunoenzyme assays can be optimised by using anti-IgG (gamma-chain) specific conjugates. Several possibilities can be explored to develop more selective test systems. Direct agglutination tests with procyclic trypanosomes only detect antibodies to invariant surface antigens. Immunodiffusion tests allow the visualisation of individual immune complexes. Trypanosomes contain some highly specific enzymes and corresponding antibodies can be detected in host serum by the assay of Borowy et al 1991. Various non-hydrosoluble trypanosomal antigens, including cytoskeleton components, remain to be evaluated as serodiagnostic reagents.

Since it is quite possible that the best serodiagnostic antigens are to be found among some minor components of the trypanosomal cell, recombinant and synthetic antigens might bring the final solution.

Reference

Katende J.M., Musoke A.J., Nantulya V.M. and Goddeeris B.M. 1987. A new method for fixation and preservation of trypanosome antigens for use in the indirect immunofluorescence antibody test for diagnosis of bovine trypanosomiasis. *Tropical Medicine and Parasitology* 38:4144.

Borowy N.K., Schell D., Schäfer C. and Overath P. 1991. Diagnosis of human African trypanosomiasis and visceral leishmaniasis based on the detection of anti-parasite-enzyme antibodies. *The Journal of Infectious Diseases* 164:422–425.

Le Ray D. 1975. Structures antigéniques de *Trypanosoma brucei. Annales de la Société belge de Médecine tropicale* 55:131–311.

Nucleic acid-based systems for detection of trypanosomes

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Summary

Diagnosis is an essential requirement in the management of disease both at the level of the individual, when a decision has to be made whether to treat or not, and at the epidemiological level for evaluating the performance of disease control strategies. An ideal diagnostic test should be rapid, specific, highly sensitive and amenable to automation. The test should distinguish closely related species of infectious organisms if the disease syndromes they cause require different management approaches. Furthermore, it should be of use in studies that seek to elucidate dynamics of the interaction of the infectious organism with its hosts, vectors and the environment. Recombinant DNA technology offers new approaches to the development of novel assays for diagnosis of African trypanosomosis, with the potential to fulfil a majority of these requirements. Developments made in the general area of DNA-based systems for detection of trypanosomes in mammalian hosts and the tsetse vector are summarised and discussed here, in the context of their potential contribution to epidemiology of livestock infective trypanosomes.

Introduction

Parasite detection and specific identification are central to the study and eventual understanding of the epidemiology of the diseases they cause. Accurate identification of the parasites is necessary for several reasons, but more particularly because of the parasite variability. African trypanosomes is one of the parasites with an incredible potential for variation. The best-studied variation in these organisms is antigenic variation involving the variant surface glycoprotein (VSG). In addition to antigenic variation, there are other forms of probably more subtle variations but which are relevant to the epidemiology of the diseases caused by the trypanosomes. These include mode of transmission, host and vector specificity, virulence and response to therapeutic drugs. Because of these variations, the identification and characterisation of pathogenic organisms, like the parasitic protozoa, will increasingly rely on the use of reagents derived from recombinant DNA (Caskey 1987; Hide and Tait 1991; Majiwa et al 1993).

Recombinant DNA-based reagents are efficient, reliable and can be automated. They can be used to follow an infectious agent over time and space, to investigate disease incidence and transmission dynamics due to the infectious agents and to perform accurate diagnosis in hosts and vectors (Wilson 1991).

Recombinant plasmids for detection of trypanosomes

Over the past few years, several DNA sequences have been identified and shown to be useful in the specific identification of different species of the African trypanosomes infective to man and livestock (Majiwa et al 1985; Gibson et al 1988). These sequences identify the parasites in both the mammalian host blood and sera, and in the vector gut and proboscidae (Kukla et al 1987;

Majiwa and Otieno, 1990; Masiga et al 1992). These DNA diagnostics can not only detect, but also distinguish the parasites at species, sub-species, antigenic repertoire and antigenic type levels.

In Table 1 is a list of the recombinant plasmids containing different repetitive DNA sequences used as probes in the identification of different trypanosome species or types. They are all repetitive, being arranged either in tandem arrays or as dispersed repeats in the genome. They hybridise specifically with DNA from the respective trypanosome species. Their capacity to detect trypanosomes present in naturally infected livestock or tsetse flies has been documented (Majiwa and Otieno 1990; Nyeko et al 1990; McNamara and Snow 1991). When labelled to high specific activity, the probes can detect the parasites, in single or mixed infections, in both the mouth parts and guts of the tsetse vector. These probes have ordinarily been used in radioactive format; however, with the availability of systems for non-radioactive labelling and detection of DNA, they have been converted to this format (Majiwa et al 1994).

Table 1. *Recombinant plasmids containing repetitive DNA sequences specific for different species of the African trypanosomes.*

Parasite specificity	Recombinant plasmid
Trypanozoon	pgDR1
Trypanozoon	177repeat
T. evansi, type A	pKT420
T. congolense (s)[†]	pgNRE-372
T. congolense (k)	pgNIK-450
T. congolense (t)	pgNgulia-11
T. congolense (war/f)	TSW103
T. simiae	pgNS-600
T. vivax	lgDIL-10k
T. vivax	Tv47
T. vivax	pkDIL900
T. vivax	pkDIL900WA
T. vivax	pgDSIL 800/3

[†]s = savannah-type *T. congolense;* k = Kilifi-type *T. congolense;* t = Tsavo-type *T. congolense;* war/f = West African forest/riverine type *T. congolense.*

Sensitive detection of trypanosomes by polymerase chain reaction (PCR)

The nucleotide sequence composition has been determined for a majority of the DNA probes. It has therefore been possible to design oligonucleotide primers (Table 2) for PCR (Saiki et al 1988) amplification of DNA to detect low parasite numbers. Although the PCR can detect an extremely low number of parasites (Moser et al 1989; Solano et al 1995), the sensitivity of this detection can be increased by hybridisation of the PCR products with a specific probe. An additional advantage of this approach is that primers specific for different trypanosome species can be placed in a single reaction tube to perform multiplex PCR, and the products obtained

hybridised separately with the different specific probes. These reagents have made it possible to perform investigations which were impossible to do in the past, leading to a more accurate description of species prevalence (McNamara and Snow 1991; Nyeko et al 1990), the discovery of new trypanosome genotypes (Majiwa et al 1993; McNamara et al 1994) and the demonstration of the presence of trypanosomes in cattle in which such parasites could not be detected by microscopy or xenodiagnosis (Majiwa et al 1994).

Future prospects

Further refinements and improvements of these DNA-based diagnostics will have to include conversion of the assays to ELISA format (Nutman et al 1994), and the quantification of the parasites in infected hosts/vectors by quantitative PCR.

These refinements will lead to their wider application, for better understanding of the relative contribution of different trypanosome species to the epidemiology of trypanosomosis, monitoring control programmes, i.e. tsetse control or livestock treatment campaigns, and conservation of wildlife biodiversity (Mihok et al 1992).

It is envisaged that with the advent of genome analyses efforts, DNA markers will be found that are linked to specific parasite phenotypes, like drug resistance and virulence. Nucleic acid diagnostics based upon such markers will be most useful in investigating the prevalence and direct consequences of these phenotypes.

As more information is gained regarding ways in which different parasites cause pathology in their respective hosts, it will be possible to device nucleic acid-based diagnostics which can provide an index of pathology or host morbidity resulting directly from the infection by a particular parasite type.

Because of future prospects of DNA diagnostics, many commercial companies have invested in the emerging field of genomics, with a hope of exploiting the information coming from the different genome analyses projects, to develop novel diagnostic kits for detection of genetic mutations and infectious organisms. Although there are no kits produced so far for the detection of parasitic protozoa, they will be relatively easy to develop once appropriate markers have been identified. The primary task remains that of identifying DNA markers, genes or gene mutations linked to specific phenotypes. Once such markers are in hand, they can be incorporated in novel diagnostic assays such as: (a) oligonucleotide ligation assay (OLA; Tobe et al 1996), employed in the detection and typing of polymorphisms arising from small deletions or insertions, often found in diallelic variants; (b) nucleic acid sequence-based amplification (NASBATM; van der Vliet et al 1993) which involves alternate steps in synthesis of DNA from RNA template and synthesis of RNA from the DNA template in a homogenous isothermal amplification process; and (c) systematic evolution of ligands by exponential enrichment (SELEX; Tuerk 1997), an assay system that exploits combinatorial chemistry to identify an oligonucleotide with a high specificity to a particular molecule. Once an oligonucleotide with the desired binding specificity has been identified, it is used in the enzyme-linked oligonucleotide assay (ELONA; Drolet et al 1996) to quantify levels of the analyte molecule in the test sera. SELEX-derived ligands are precise, accurate and specific.

Table 2. *Oligonucleotide primers for specific amplification of DNA from different trypanosomes.*

Primer number or designation	Sequence of the primer	PCR product size (bp)	Trypanosome specificity	Reference
ILO342 IL0343	GAT CCG CAG CCG GGC CTG CCG CGG TGG CTC CTT CCC	1500	Trypanozoon	Majiwa et al 1994
ILO344 ILO345	CGA GCG AGA ACG GGC AC GGG ACA AAC AAA TCC CGC	370	*T. congolense*(s[†])	Majiwa et al 1994
ILO892 ILO893	CGA GCA TGC AGG ATG GCC G GTC CTG CCA CCG AGT ATG C	400	*T. congolense*(t)	Majiwa et al 1994
ILO963 ILO968	GCT GCA GGT CGA CGG ATC CCC TCG AGA ACG AGC A	450	*T. congolense*(k)	Majiwa et al 1994
TCF1 TCF2	GGA CAC GCC AGA AGG TAC TT GTT CTC GCA CCA AAT CCA AC	350	*T. congolense*(war/f)	Masiga et al 1992
TSM1 TSM2	CCG GTC AAA AAC GCA TT AGT CGC CCG GAG TCG AT	437	*T. simiae*	Masiga et al 1992
TVW1 TVW2	CTG AGT GCT CCA TGT GCC AC CCA CCA GAA CAC CAA CCT GA	150	*T. vivax*	Masiga et al 1992
ILO1264 ILO1265	CAG CTC GCC GAA GGC CAC TTG GCT GGG CG CTA CCA CAG TCG CAA TCG TCG TCT CAA GG	400	*T. vivax*	Masake et al 1997

[†]s = Savannah-type *T. congolense;* k = Kilifi-type *T. congolense;* t = Tsavo-type *T. congolense;* war/f = West African riverine/forest type *T. congolense.*

Conclusion

In general, DNA-based diagnostic reagents require no animals and are therefore not subject to animal variation during synthesis; they or detailed information about them are most often available in the public domain databases and from this they can be consistently re-synthesised or re-cloned anywhere in the world. Additionally, the target molecule upon which they are based need not be immunogenic since the detection system will most often be based on invariant segments of the genome. The reagents are normally stable during storage and the assays can be automated.

References

Caskey C.T. 1987. Disease diagnosis by recombinant DNA methods. *Science* 236:1223–1229.

Drolet W.R., Moon-McDermott L. and Romig T.S. 1996. An enzyme-linked oligonucleotide assay. *Nature Biotechnology* 14:1021–1025.

Gibson W.C., Dukes P. and Gashumba J.K. 1988. Species-specific DNA probes for the identification of African trypanosomes in tsetse flies. *Parasitology* 97:63–73.

Hide G. and Tait A. 1991. The molecular epidemiology of parasites. *Experientia* 47:128–142.

Hill S.M., Urwin R. and Crampton J.M. 1992. A simplified, non-radioactive DNA probe protocol for field identification of insect vector specimens. *Transactions of the Royal Society for Tropical Medicine and Hygiene* 86:213–215.

Höltke H.J., Sagner G., Kessler C. and Schmitz G. 1992. Sensitive chemiluminescent detection of digoxigenin-labelled nucleic acids: a fast and simple protocol and its applications. *BioTechniques*12:104–113.

Kimmel B.E., ole-MoiYoi O.K. and Young J.R. 1987. *Ingi*, a 5.2 kb dispersed sequence element from *Trypanosoma brucei* that carries half of a smaller mobile element at either end and has homology with mammalian lines. *Molecular and Cellular Biology* 7:1465–1475.

Kukla B.A., Majiwa P.A.O., Young J.R., Moloo S.K. and ole-MoiYoi O.K. 1987. Use of species-specific DNA probes for detection and identification of trypanosome infection in tsetse flies. *Parasitology*95:1–16.

Majiwa P.A.O., Maina M., Waitumbi J.N., Mihok S. and Zweygarth E. 1993. *Trypanosoma (Nannomonas) congolense:* molecular characterization of a new genotype from Tsavo, Kenya. *Parasitology*106:151–162.

Majiwa P.A.O., Masake R.A., Nantulya V.M., Hamers R. and Matthyssens G. 1985. *Trypanosoma (Nannomonas) congolense:* identification of two karyotypic groups. *European Molecular Biology Organization Journal* 4:3307–3313.

Majiwa P.A.O., ole-MoiYoi O.K. and Nantulya V.M. 1993. New techniques for diagnosis of the African trypanosomiases. *AgBiotech News and Information* 5:115N–120N.

Majiwa P.A.O. and Otieno L.H. 1990. Recombinant DNA probes reveal simultaneous infection of tsetse flies with different trypanosome species. *Molecular and Biochemical Parasitology* 40:245–254.

Majiwa P.A.O., Thatthi R., Moloo S.K., Nyeko J.H.P., Otieno L.H. and Maloo S. 1994. Detection of trypanosome infections in the saliva of tsetse flies and buffy coat samples from antigenaemic but aparasitaemic cattle. *Parasitology* 108:313–322.

Masake R.A., Majiwa P.A.O., Moloo S.K., Makau J.M., Njuguna J.T., Maina M., Kabata J., ole-MoiYoi O.K. and Nantulya V.M. 1997. Sensitive and specific detection of Trypanosoma vivax using the polymerase chain reaction. *Experimental Parasitology* 85:193–205.

Masiga D.K., Smyth A.J., Hayes P., Bromidge T.J. and Gibson W.C. 1992. Sensitive detection of trypanosomes in tsetse flies by DNA amplification. *International Journal for Parsitology* 22:909–918.

McNamara J.J. and Snow W.F. 1991. Improved identification of *Nannomonas* infections in tsetse flies from the Gambia. *Acta Tropica* 48:127–139.

McNamara J.J., Mohammed G. and Gibson W.C. 1994. *Trypanosoma (Nannomonas) godfreyi* sp.nov. from tsetse flies in the Gambia: biological and biochemical characterization. *Parasitology*109:497–509.

Mihok S., Munyoki E., Brett R.A., Jonyo J.F., Röttcher D., Majiwa P.A.O., Kang'ethe E.K., Kaburia H.F.A. and Zweygarth E. 1992. Trypanosomiasis and the conservation of black rhinoceros *(Diceros biconis)* at the Ngulia Rhino Sanctuary, Tsavo West National Park, Kenya. *African Journal of Ecology* 30:103–115.

Moser D.R., Cook G.A., Ochs D.E., Bailey C.P., McKane M.R. and Donelson J.E. 1989. Detection of *Trypanosoma congolense* and *T . brucei* subspecies by DNA amplification using the polymerase chain reaction. *Parasitology* 99:57–66.

Nutman T.B., Zimmerman P.A., Kubofcik J. and Kostyu D.D. 1994. A universally applicable diagnostic approach to filarial and other infections. *Parasitology Today* 10:239–243.

Nyeko J.H.P., ole-MoiYoi O.K., Majiwa P.A.O., Otieno L.H. and Ociba P.M. 1990. Characterization of trypanosome isolates from Uganda using species-specific DNA probes reveals predominance of mixed infections. *Insect Science and its Applications* 11:271–280.

Saiki R.K., Gelfand D.H., Stoffel S., Scharf S.J., Higuchi R., Horn G.T., Mullis K.B.A. and Erlich H. 1988. Primer-directed enzyme amplification of DNA with thermostable DNA polymerase. *Science*239:487–491.

Solano P., Argiro L., Reifenberg J.M., Yao Yao and Duvallet G. 1995. Field application of the polymerase chain reaction (PCR) to the detection and characterization of trypanosomes in *Glossina longipalpis (Diptera: Glossinida)* in Cote d'Ivoire. *Molecular Ecology* 4:781–785.

Stone T. and Durrant I. 1992. Enhanced chemiluminescence for the detection of membrane-bound nucleic acid sequences: advantages of the Amersham system. *Genetic Analysis Techniques and Applications* 8:230–237.

Tobe V.O., Taylor S.L. and Nickerson D.A. 1996. Single-well genotyping of diallelic sequence variations by two-color ELISA-based oligonucleotide ligation assay. *Nucleic Acids Research* 24:3728–3732.

Tuerk C. 1997. Using the SELEX combinatorial chemistry to find high affinity nucleic acid ligands to target molecules. *Methods in Molecular Biology* 67:219–230.

Van der Vliet G.M., Schukkink R.A., van Gemen B., Schepers P. and Klatser P.R. 1993. Nucleic acid sequence-based amplification (NASBA) for identification of mycobacteria. *Journal of General Microbiology* 139:2423–2429.

Wilson S.M. 1991. Nucleic acid techniques and the detection of parasitic diseases. *Parasitology Today* 7:255–259.

Summary and conclusions

Summary of discussion groups

Participants were divided into four discussion groups, covering two discussion topics. One group discussed the shortcomings of the Ag-ELISA test and possible solutions. The other three groups discussed the more general priorities and technical requirements for trypanosomosis diagnosis. Their discussions are summarised below.

Topic I. Ag-ELISA: current problems and potential solutions

Problems

- Both diagnostic sensitivity and specificity are lower than desired and highly variable.
- The three species-specific tests often do not give species-specific results.
- The reasons for this variable performance are unknown; thus, the assays cannot be sufficiently improved by simple modifications. The actual working principle underlying the assay remains obscure.

Potential solutions

Identify which substances are detected or interfere with the assay

- The actual substances detected by the assay need to be determined. These could be target antigens either unbound or bound in the form of immune complexes.
- A number of substances may interfere with the assay causing either false positives or false negatives. Some potential interfering substances include:
    - anti-Ig antibodies
    - heterophile antibodies
    - anti-idiotype anitbodies
    - complement components.
- Once interfering substances are identified, specific measures (e.g. heat inactivation, additives, etc.) could be taken to counteract their effects and thus improve the sensitivity and specificity of the assay.

Better positive and negative controls in the test kit

- *Positive controls:* freeze-dried trypanosome extract or purified antigen should be included.
- *Negative controls:* a dummy mouse IgG would be of benefit.

Revise the level of discrimination to which trypanosomes are identified

- The target level of specificity of test kits for differentiating trypanosomes could be revised from the species to the subgenus level as follows:
    - Tryapanozoon *(T. brucei, T. evansi, T. equiperdum)*
    - Nannomonas *(T. congolense)*
    - Dutonella *(T. vivax)*

- In addition, a salivarian kit might be envisaged.

Improve selection of candidate target antigens and antibodies

- The theoretically optimal target antigens should have the following characteristics:
    1. subgenus or salivarian specific
    2. present in high concentrations in trypanosomes
    3. weakly immunogenic (to minimise elimination or interference by immune complexes)

- The capturing antibodies should be of the IgG isotype. Either a combination of monoclonal antibodies or even the IgG fraction of a polyclonal antiserum should be used in preference to a single monoclonal.
- A number of immunisation procedures should be considered for generating appropriate capturing antibodies. Possible immunogens include purified native antigens, recombinant or denatured antigens and infected host serum.
- The revealing monoclonal antibodies should also be of the IgG isotype but they should recognise different epitopes than the capturing antibodies.

Catalogue existing analytes

- An inventory of existing monoclonal antibodies and well-studied antigens should be assembled along with their bibliographic references.

Topic II. Trypanosomosis diagnosis: priorities and technical requirements

General diagnostic needs

All groups defined the needs for further test development in terms of different target domains. Two target domains were defined, the farm or animal level and the area level. Areas were defined to be either agro-ecological zones or other regional or national subdivision. Consideration of trypanosome target species was also thought to be potentially important. The diagnostic needs for trypanosome detection in the field for each of the two target domains were considered as follows.

Animal/farm level

- Diagnosis of presence or absence of infection is essential.
- Differentiation between parasite species is less important.
- A crush side test is necessary for timely diagnosis and treatment.
- Analytic sensitivity is more important for chronic than acute infections.
- It is important to be able to assess the efficacy of treatment, particularly for group treatments (tsetse control or block chemo-prophylaxis).

Area level

- The basic need is to estimate the prevalence in livestock rather than the incidence of new infections.

- The determination of tsetse density and vector infection rate is also important in order to match tsetse challenge to trypanosome infection rates in hosts.
- The differentiation of trypanosomes by species or sub-genus will be required for epidemiological research into infection transmission dynamics but not for general surveillance, for which broad spectrum diagnostic tests would be adequate.

Potential applications for different diagnostic tests

- For the determination of prevalence or incidence of current infections at both farm or area level, all diagnostic tests except the Ab-ELISA test were considered appropriate.
- For differention to species level, DNA methods are likely to be the most appropriate.
- The Ab-ELISA test, however, could be a satisfactory alternative for assessing the spatial distribution of prevalence of trypanasomosis at the area level.
- The Ab-ELISA might also be a suitable alternative to the Ag-ELISA in longitudinal monitoring of the impact of a trypanasomosis control programme, particularly for comparing the prevalence of trypanosomes in animals born following the intervention between treatment and control areas.

Current and desired sensitivity and specificity requirements

Using ratings from 1 (low) to 5 (high) for sensitivity and specificity, one group produced the following table.

Table 1. *Summary of current and desired specificities and sensitivities for different trypanosomosis diagnostic tests.*

	Analytical specificity		Analytical sensitivity		Diagnostic specificity		Diagnostic sensitivity	
	Current	desired	Current	desired	Current	desired	Current	desired
Ab-ELISA	1	3	3	3	–	–	–	–
Ag-ELISA	2	4	1	5	3	5	1	5
DNA	5	5	5	5	?	5	?	5
Buffy coat	4	4	1	2	5	5	1	2

On current test performance the following points were made:

- The only definitive test at present is the buffy coat test.
- The currently available versions of the Ag-ELISA test fall short of the overall desired requirements for both specificity and sensitivity. It does not discriminate well between positive and negative populations.
- The lack of correlation between parasitaemia and antigenaemia in various studies is also a concern with respect to the performance of the Ag-ELISA test.
- The relative robustness of the different tests were discussed. It was considered that major improvements in quality assurance in terms of consistency of sensitivity, specificity and robustness, both within and between laboratories, were essential for each of the Ab-ELISA, Ag-ELISA and DNA tests.
- It was felt that a higher diagnostic sensitivity could be achieved with any of the Ab-ELISA, Ag-ELISA or DNA tests than was possible by the buffy coat test. However, the

current low levels of diagnostic sensitivity in Table 2 demonstrate that major efforts are needed to improve test performance.
- Future test development must include careful validation in both the laboratory and the field.

Conclusions

General

A panel of invited participants (T. de Waal, J. McDermott, P. Wright and P. van Meirvenne) met and summarised the contributions of the discussion groups with respect to the main diagnostic requirements and potential applications of diagnostic tests for trypanosomosis. The main summary points were:

1. Participants felt that there were applications for all the detection systems that are either available now or under development. These include antigen, antibody, nucleic acid and parasite detection systems.
2. The only definitive tests at present are the parasite detection methods.
3. Parasite detection methods define the minimum diagnostic standard for sensitivity, which new tests must achieve.
4. Broad spectrum tests for pathogenic trypanosomes (salivarian tryps; 3 sub-genera) would be sufficient for routine use in the field.
5. The antibody and antigen-detection tests have potential broad-spectrum application.
6. There is a need for subgenera-specific tests. Tests based on the detection of nucleic acids have the best potential for this purpose.

These summary points were then presented to all participants and a general discussion on the diagnostic testing needs and options took place. These are summarised below.

Diagnostic needs

Diagnostic needs were identified by discussion groups for various situations. These were further discussed and five major situations were identified.

1. *Spatial distribution of trypanosomosis:* consistent test required; lack of sensitivity could be overcome by increased sample size.
2. *Impact of control programmes:* consistent test required; greater sensitivity required, particularly if short-term impact to be assessed; improved tests could be useful.
3. *Declaration of eradication:* higher sensitivity and specificity required; currently available tests were inadequate.
4. *Diagnosis for herd-level chemoprophylaxis:* decision will be based on a herd prevalence threshold; parasite detection probably adequate for this.
5. *Diagnosis for animal-level chemotherapy:* current infection needs to be detected; better combination of sensitivity and specificity required.

These general needs were then summarised in tabular form (Table 2) into three domains and the potential test systems for each domain agreed upon.

Table 2. *Summary of main diagnostic needs for trypanosomosis in the field.*

Application	Level	Location	Analyte	Broad	Specific	Technology
Survey (prevalence, monitoring)	Regional, National Subnational	Laboratory Farm	Ab Pa Na	x	 x x	Ab-ELISA Buffy coat or slide
Surveillance	National Subnational Farm	Laboratory Farm	Pa Ag Ab Na	 x x	x x	Buffy coat Ag-ELISA Ab-ELISA Nucleic acid test
Diagnosis	Farm	Farm Laboratory	Pa Na Ag	x x	x x	Buffy coat Nucleic acid test Ag-ELISA

Ab = antibody; Na = nucleic acid; Pa = parasite; Ag = antigen

Field experience with available diagnostic tests

1. The Regional Tsetse and Trypanosomosis Control Programme (RTTCP) in southern Africa was satisfied with the performance of the buffy coat and Ab-ELISA test for surveys. They use buffy coat for surveillance but would prefer a more sensitive test if available. They felt a pen-side test for individual animal chemotherapy decisions was required.
2. For trypanotolerance selection in the field, Dr Guy d'Ieteren considered that the antigen test provided further information beyond parasite detection for the identification of trypanotolerant animals. He thought that the Ag-ELISA test performed well for studies of trypanotolerance.

State of development of antigen, antibody and nucleic acid diagnostic tests

The status of resources and reagents available for each main diagnostic test category was discussed and a tabular summary (Table 3) agreed upon.

Table 3. *Test systems being developed for trypanosomosis: current availability of main test development requirements.*

Developmental requirements	Antigen-detection		Antibody-detection		Nucleic acid tests	
	Now	To develop	Now	To develop	Now	To develop
Available expertise						
Standard analytes						+/–
Quality assurance	+/–		+/–			
Reagents	+/–					+/–
Protocols	+/–		+/–			

Recommendations

Recommendations for immediate action in diagnostic test development for trypanosomosis

1. Emphasis should be given to the development of a broad-spectrum test(s), either antigen or antibody detection, which has consistent performance across different regions and disease states.
2. Species identification will be important for more detailed epidemiological studies. Identification to the sub-genus level would be sufficient for most applications. Nucleic acid tests were agreed to be the most promising route for the differentiation of sub-genera and species.
3. Criteria need to be developed for the standardised comparison of the analytical performance of all tests at the same time. This needs to be done with samples from different sites and different disease states.
4. In the immediate future, the standardised comparison should focus on broad-spectrum tests for pathogenic trypanosomes. All tests (e.g. antibody, antigen) developed for this purpose should be evaluated for analytical performance together.
5. Critical evaluation of the feasibility of larger scale development of a broad-spectrum test should only be performed when the evaluation of analytical performance has been made.

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