ANIMAL TRYPANOSOMOSIS: DIAGNOSIS AND EPIDEMIOLOGY

Results of a FAO/IAEA Co-ordinated Research Programme on the use of immunoassay methods for improved diagnosis of trypanosomosis and monitoring tsetse and trypanosomosis control programmes

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Frontcover: Milking of a N'Dama cow in The Gambia (R.H. Dwinger).
Backcover (above): Close-up photograph of a tsetse fly (D. Elsworth, ILRI).
Backcover (below): Bloodstream form of *Trypanosoma brucei* (ILRI).

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SUMMARY

1. THE CO-ORDINATED RESEARCH PROGRAMME

An initial FAO/IAEA Co-ordinated Research Programme (CRP) was organised from 1987-1993 with funding from the Government of the Netherlands to validate an antigen-detection ELISA developed by the International Laboratory for Research on Animal Diseases (ILRAD). The test had shown promising results in laboratory investigations detecting the percentage of trypanosome infected animals. The ELISA was validated in ten countries in Africa and the results were extensively reported in IAEA-TECDOC-707 [1]. Although some difficulties in the interpretation of results were encountered, the test was considered reliable enough to request additional funding for a second CRP in order to apply the assay as a tool for monitoring disease control programmes in Africa. To bridge the gap of two years between the two CRPs, funding was obtained from the Overseas Development Agency of the United Kingdom and the IAEA Department of Technical Co-operation. The results of these two years have been reported in IAEA-TECDOC-925 [2]. In September 1995 the second CRP, entitled "Use of immunoassay methods for improved diagnosis of trypanosomosis and monitoring tsetse and trypanosomosis control programmes" was initiated. The background, activities, results, conclusions and recommendations are reported below.

A total of 20 research institutes were actively participating in the CRP of which 15 are located in Africa.

TABLE I. DETAILS OF SCIENTISTS AND RESEARCH INSTITUTES INVOLVED IN THE CRP

Country	Chief Scientific Investigators	Research Institutes
Burkina Faso	Zakaria Bengaly	Centre International de Recherche Développement sur l'Elevage en zone Sub-Humide (CIRDES)
Cameroon	Christian N. Ndamkou	Laboratoire National Vétérinaire de Boklé
Côte d'Ivoire	Ahouty E. N'Depo	Laboratoire Central de Pathologie Animale (LCPA)
Ethiopia	Assefa Regassa	National Animal Health Research Center Department of Parasitology, Protozoology Team
Ethiopia	Nega Tewelde Tikue	National Tsetse and Trypanosomiasis Investigation and Control Coordination Centre (NTTICC)
France	Gérard Duvallet	CIRAD-IEMVT, Unité de Recherche Pathotrop
Ghana	Christopher K. Doku	Tsetse and Trypanosomiasis Unit, Central Veterinary Lab.
Kenya	John McDermott	International Livestock Research Institute
Kenya	Johnson Ouma Odera	Kenya Trypanosomiasis Research Institute, KETRI
Mali	Oumar Diall	Laboratoire Central Vétérinaire du Mali
Nigeria	Samuel Ayo Ajayi	National Veterinary Research Institute, Parasitology Division
Senegal	Mamadou Seye	Institut Sénégalais de Recherches Agricoles Laboratoire National de l'Elevage et de Recherches Vétérinaires - ISRA/LNERV
Sudan	Mohamed Fadl	Dept. of Veterinary Parasitology, University of Khartoum
The Gambia	Joseph Axel Faye	International Trypanotolerance Centre (ITC), Disease Research Unit of the ITC
Uganda	Joseph W. Magona	Livestock Health Research Institute (LIRI)
United Kingdom	Antony G. Luckins	Centre for Tropical Veterinary Medicine (CTVM) The University of Edinburgh
United Kingdom	Mark Eisler	University of Glasgow, c/o ILRI
United Republic of Tanzania	Heriel A. Mbwambo	Animal Disease Research Institute (ADRI)
Zambia	Noreen Machila-Eisler	Central Veterinary Research Institute
Zimbabwe	Wonderful Shumba	RTTCP

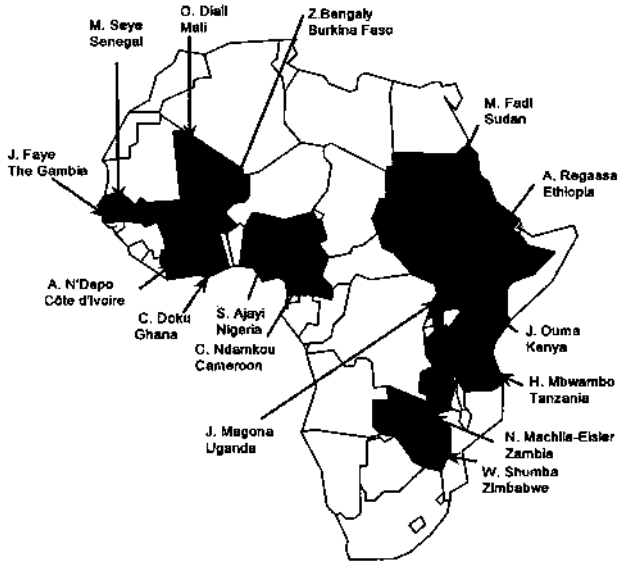

FIG 1. FAO/IAEA Co-ordinated Research Programme to improve the diagnosis and control of animal trypanosomosis in Africa through the application of enzyme linked immunosorbent assays.

The four Research Co-ordination Meetings (RCMs) during the past five years are listed in Table II.

TABLE II. DETAILS OF THE RESEARCH CO-ORDINATION MEETINGS

Meeting	Location	Date	Number of participants	Comments
First	Dakar, Senegal	9-13 September, 1996	16	introduction of EQAP
Second	Onderstepoort, South Africa	6-9 October, 1997	20	practical workshop participation in 24th ISCTRC conference
Third	Kampala, Uganda	5-9 October, 1998	29	practical workshop and concerted action workshop
Fourth	Addis Ababa, Ethiopia	6-10 September, 1999	28	instruction in GPS and GIS

EQAP = external quality assurance programme; ISCTRC = International Scientific Council for Trypanosomiasis Research and Control; GPS = global positioning system; GIS = geographic information system.

2. RESEARCH ACTIVITIES

2.1. Antigen ELISA

Initially research activities and applications in the field concerned the antigen-detection ELISA developed by scientists at the International Laboratory for Research on Animal Diseases (ILRAD) and transferred to the FAO/IAEA Agriculture and Biotechnology Laboratory in Seibersdorf. Results showed high proportions of false negatives and false positives, which were thought to be due to low levels of antibodies early during infection, due to antigen-antibody complexes and due to competition between the monoclonals used for coating and the monoclonals used for detection, since they were

raised against the same antigenic epitopes. Subsequently, double blind studies using the antigen-detection ELISA and serum samples from experimentally infected cattle showed that the test was not suitable for a reliable diagnosis of the disease (see Eisler *et al.*, this publication). Moreover, studies on goats experimentally infected with *T. vivax* showed similar results (see Rebeski *et al.*, this publication). Initially, attempts were made to improve the test (see IAEA-TECDOC-925, pages 11-13), but either sensitivity or specificity proved to be unsatisfactory. Consequently, it was decided to stop the application of the antigen-detection ELISA in the field and replace the test with an improved version of an existing antibody-detection ELISA.

2.2. Antibody ELISA

The antibody-detection ELISA was described by Luckins in 1977, but not applied widely due to lack of standardised reagents. Research at the FAO/IAEA Agriculture and Biotechnology Laboratory in Seibersdorf resulted in the standardisation of antigen preparation, antigen coating of ELISA plates, quality control aspects and data analysis (see Rebeski *et al.*, this publication).

3. APPLICATION OF THE TEST

The validation and application of the antibody-detection test using ELISA plates pre-coated with denatured *T. congolense* or *T. vivax* antigen is described by the Research Contract holders in this publication. The test has been used for the detection of antibody levels in cattle as part of disease control programmes in Ghana, Burkina Faso, Kenya, Zimbabwe and Bolivia.

The test has been used in Zanzibar following the eradication of tsetse flies using the sterile insect technique (see Dwinger *et al.*, this publication). The absence of antibodies in young stock born after the removal of tsetse flies was verified. A similar tsetse eradication project has been initiated in the Southern Rift valley of Ethiopia. The serology laboratory at Sebetha will be equipped and personnel sufficiently trained to enable the use of the ELISA for monitoring progress in disease eradication and for the detection of remaining foci.

4. QUALITY ASSURANCE PROGRAMME

A total 16 laboratories participated in the external quality assurance programme (EQAP) for the FAO/IAEA trypanosomosis direct sandwich ELISA for the detection of antigens of *T. brucei*, *T. congolense* and *T. vivax*. The EQAP consisted of three components: a questionnaire, internal quality control (IQC) data and an external quality control test panel. All laboratories were provided with a computer software program EDI (ELISA data interchange), which facilitates automatic reading of optical density figures and the calculation of test results. The EDI program automatically created a file containing the IQC data of each plate tested. The file containing the IQC data of the last 30-40 plates were sent to Vienna for analysis. The EQC test panels consisted of 5 serum samples per trypanosome species (3 positive samples and 2 negative samples). The results have been reported in a separate report [3].

5. TRAINING ACTIVITIES

Training was organised using a variety of different modalities. The various possibilities for training were the following:

- Training course
- Scientific visit (usually 3 weeks visiting 1-3 institutes for an exchange of scientific ideas)
- Fellowship (tailor-made instruction lasting 1-6 months)
  Expert visits (usually part of a Technical Co-operation Project)
- Research Coordination Meeting
- Workshop
- Associate Professional Officer

A total number of 23 scientists and technicians involved in the CRP or in related subjects dealing with the diagnosis and epidemiology of trypanosomosis were trained during the past five years. In addition, five workshops were organised.

6. GEOGRAPHIC INFORMATION SYSTEMS (GIS) FACILITY

From August 1996 an Associate Professional Officer (APO) was posted at the Animal Production & Health Section for a period of two years. The APO, Mr. T. Ndegwa from Kenya, was funded by the Government of the Netherlands through the programme to employ multilateral associate experts from developing countries (MAD-OL programme). He installed a GIS facility at the Joint FAO/IAEA Division in Vienna and prepared a data base for storage and analysis of field data. In close collaboration with the University of Oxford, FAO and ILRI he designed disease distribution maps for several countries (Niger, Cameroon, Tanzania, Sudan). The APO moved to another posting in Harare, Zimbabwe, and was replaced by a second APO, Mrs. A. Erkelens, from the Netherlands. The APO prepared a GIS training manual for disease mapping on the application of the following software programs: ARC/INFO, ArcView, Cartalinx and IDRISI for computer assisted geographic image analysis, mapping and presentation.

A model for the determination of priority areas for tsetse control in Africa using GIS was developed during a two year period. The model is based on different (external) factors, e.g. habitat of tsetse (vegetation, climate, elevation, etc.), population pressure (cattle and human), livestock distribution and land use. The criteria for the prioritisation were established with a team of experts from different disciplines (e.g. health, environment, economy, agriculture). The identification of priority areas was done in close collaboration with FAO and the University of Oxford and focused initially on a tsetse infested area in Ethiopia (see Erkelens *et al.*, this publication).

7. COST-BENEFIT ANALYSIS

A consultant from the Department of Farm Management, Animal Health and Management Economics Unit, Wageningen Agricultural University, was contracted to assess the costs and benefits of the use of improved diagnostic methods, in particular the ELISA tests for detecting trypanosomal antigens and antibodies. The results have been analysed under supervision of Professor A. Dijkhuizen and Professor R. Huirne (see Van Binsbergen *et al.*, this publication).

8. SUSTAINABILITY OF KIT PRODUCTION

In order to ensure sustainability of the ELISA test, efforts have been made to transfer the technology. Initial contacts with the Onderstepoort Veterinary Institute (OVI) in South Africa, indicated the interest of this institute to act as a regional production and distribution center for the test. Subsequently, permission was obtained from the institutes of origin of the trypanosome clones used for *in vitro* cultivation and antigen preparation (ILRI and the Swiss Tropical Institute) for transfer of biological materials. As a first step in the transfer of the technology a scientist from OVI was trained in various aspects of kit production and distribution at the FAO/IAEA Agriculture and Biotechnology Laboratory (see De Waal, this publication). Contacts with other African institutes interested in producing and distributing the ELISA kits will be pursued.

9. INTERNATIONAL COLLABORATION

At various stages during the programme collaboration was sought with other institutes working on trypanosomosis, such as ILRI, Nairobi (see McDermott, this publication), University of Edinburgh (see Luckins, this publication; Jones, this publication), University of Glasgow (see Eisler *et al.*, this publication), CIRAD/EMVT, Montpellier (see Solano *et al.*, this publication), Prince Leopold Institute, Antwerp Freie Universität, Berlin, Justus-Liebig University, Giessen (see publications listed under 1.3.2), FAO, Rome (see Dwinger *et al.*, this publication), Oxford University (see Erkelens *et al.*, this publication).

The "Programme to clarify and solve the problem of African trypanosomosis" (PAAT) aims to bring together all those concerned with and affected by this disease: ranging from rural communities to governments, international organisations, research institutes, donors and development agencies. As a result FAO, IAEA, OAU/IBAR and WHO have agreed to combine forces in the fight against trypanosomosis in Africa by forming a joint Programme Secretariat. The Secretariat assists the Programme Committee in decision making and directing the programme. The programme is based on concerted international planning and action, prioritised and problem driven research, focused

investments and interventions, integrated vector and disease control and participation of local communities. Two advisory modules, one on research and development, the other one on policy, planning and implementation provide information on specific fields of expertise to the Programme Committee. The project leader assisted in the initiation of PAAT, representing at times the IAEA in the Secretariat, acted as a co-ordinator of one of seventeen Advisory Groups, organised the 4th Programme Committee meeting in Vienna in November, 1998 and assisted the Secretariat in running other PAAT meetings.

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11. OVERALL CONCLUSIONS AND *RECOMMENDATIONS*

A large number of conclusions and recommendations can be found in the individual articles contained in this publication.

1. During the last RCM the group concluded that the application of the antibody-detection ELISA is a powerful tool for characterising trypanosomosis risk areas and monitoring the impact of control programmes. It is not appropriate for individual diagnosis or for detailed transmission studies.

1a. Different diagnostic tests may be required for specific purposes and no single test will be suitable for all applications as related to the diagnosis of trypanosomosis. It is felt that more research should be promoted to develop new diagnostic techniques such as PCR-ELISA that will be useful for purposes for which the antibody-detection ELISA is not suitable, such as individual diagnosis and transmission studies.

2. The distribution of ELISA plates pre-coated with antigen has many advantages over existing formats used for the diagnosis of trypanosomosis including storage, shelf-life, transport and test reproducibility.

3. Field use in the different countries confirmed that plates coated with denatured antigen of *T. congolense* and *T. vivax* were more reliable and robust than those coated with native antigen.

3a. It is recommended that plates coated with denatured antigen of T. congolense *and* T. vivax *and should become the standard format for use in antibody-ELISA.*

4. The introduction of quality control including the routine use of internal quality control charts is seen as an important improvement. The use by the RCH's of their own internal quality controls enabled them to assess the assay performance locally without external support. This allowed the identification and correction of problems in all testing components, for example, water quality, test control performance and sample handling.

4a. The antibody-detection ELISA for bovine trypanosomosis should be used routinely in all major African animal trypanosomosis (AAT) control programmes. In order to make it readily available for such application and for use in laboratories in Africa, a suitable laboratory for production and distribution should be identified in Africa.

5. The FAO/IAEA antibody-detection ELISA has been used in other countries outside Africa and could be useful in situations where non-tsetse transmitted pathogenic trypanosomes are prevalent.

6. The programme has improved the infrastructure in many African laboratories and facilitated the training of personnel in the diagnosis of trypanosomosis. Moreover, human resource development has enabled some RCH to act as regional experts. The programme has promoted successfully ELISA as a generic technology in these institutes.

7. It is felt very useful that during RCM's the scientists are given the opportunity to present their results, to exchange views and learn from each others experiences. In addition, progress was noted during the project in the interpretation of data and the quality of the presentation of results by the scientists.

8. The emphasis on RCH estimating sensitivity and specificity of the antibody-detection ELISA restored their confidence in the value of ELISA and reinforced the role serological diagnosis can play in the control of AAT.

9. The successful application of a similar antibody-detection ELISA developed and standardised in laboratories in Zimbabwe, Zambia and neighbouring countries has confirmed the success of technology transfer as part of the CRP and increased the confidence of the users in enabling their own programmes without IAEA support. National laboratories should be aware that they have the option of the current standardised diagnostic ELISA test and of a protocol for the production of antigen locally and the purchase of other reagents commercially. Thus, they can choose what is most appropriate for their circumstances.

ADDITIONAL RECOMMENDATIONS

10. It is recommended that all data from RCH concerning sensitivity, specificity and cut-off point should be analysed at a central location (e.g. Vienna).

11. Progress on quality control of tests needs to be continued. It is recommended that all laboratories follow the procedures as described elsewhere [4] with regard to internal quality control, cut-off values, etc.

12. The cost of the antibody-ELISA should be kept as low as possible (on a cost-recovery basis) by the distributor in view of the economic constraints of the end users and in recognition of the role they have played in its field validation. Feedback of data from scientists using the test to the distributor should be a way of continuing validation at low cost.

13. For the diagnosis of trypanosomosis it is not as important to have universal international standardisation of tests as it is for diseases of trade. However, in regional control programmes diagnostic tests should be standardised across all participating countries for comparative purposes.

14. Further efforts to simplify and increase robustness of the antibody-detection ELISA is encouraged. Efforts should include the investigation and promotion of conjugate and control sera in a lyophilised form and the use of filter paper for sample collection. It is recommended to research the use of recombinant antigen as a possibility for improving antigen production.

15. Sample sizes need to be appropriate to estimate test sensitivity and specificity in each country. For characterising risk areas approximately 200 positive and 200 negative samples are reasonable. For other applications (e.g. assessment of control) larger sample sizes will be required.

16. In view of the encouraging results obtained by the RCH using the denatured antigen on pre-coated ELISA plates, this workshop recognises the need for continuation of further improvements on the diagnostics of trypanosomosis.

12. ACHIEVEMENTS OF THE PROGRAMME

The achievements of the programme have been outlined above and are presented in the reports collected in the present publication. In summary the achievements are:

- the establishment of a network of co-ordinated research
- an improved laboratory infrastructure and laboratories equipped to perform ELISA
- the development of a standardised antibody-detection ELISA
- the development of an ELISA test that can be used to monitor tsetse control programmes
- the development of an ELISA test that can be used for verification of disease eradication
- the introduction of quality assurance aspects (EQAP, SOP, GLP)
- the training of a large number of African scientists and technicians
- the introduction of computers and software for data analysis
- the assistance with data analysis
- the introduction of the PCR technique for the detection of trypanosomal DNA
- the development of a model to identify priority areas for tsetse control
- the involvement in the initiation and development of PAAT
- the transfer of ELISA kit production and research capabilities.

13. FUTURE RESEARCH DIRECTIONS

The antibody-detection tests have a high specificity and sensitivity, but interpretation of positive results is difficult since antibodies persist for several months following treatment with trypanocidal drugs. Consequently, antibody detection tests can not be used to identify infected animals, although a change in antibody titers can be used to assess the effectiveness of tsetse control programmes.

On the other hand, detection of parasites does indicate infection and would be a valuable method if the rather low sensitivity of the existing methods could be increased. The polymerase chain reaction (PCR) is known to be a very sensitive test for detecting (genetic material of) parasites. For trypanosomosis in particular this test would be ideally suited as the "gold standard". It would have to verify doubtful samples, which have been detected positive by ELISA, but have not been found positive parasitologically in order to distinguish the true from the false positives. At the same time it would be useful if the PCR technique could be employed to detect infected animals that have tested negative in the ELISA and standard parasitological techniques due to insufficient sensitivity of these latter two tests (in other words to detect the false negatives).

A combination of ELISA, which can assess large numbers of samples and novel molecular techniques such as the PCR to detect parasite DNA might be the answer to the need for a reliable and accurate diagnosis of the disease. The practical significance of such a test would be in disease eradication programmes. In such cases it is of great importance to detect remaining foci of infection (to detect the false negatives). It is equally important to unmask the false positives, which would assist in indicating when to stop eradication efforts. However, it should be noted that the PCR technique will show false positives if insufficient controls are being used during the sampling and testing procedures.

Another activity for future development could be to design a simple pen-side test to detect trypanosomes pathogenic to livestock. However, it is doubtful that following the investments to develop such a test, the sale price of the test would be affordable to the individual livestock owner especially since it will not negate the cost of treatment in case of a positive result.

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DEVELOPMENT, STANDARDISATION AND VALIDATION OF ELISA METHODS TO IMPROVE THE CONTROL OF TRYPANOSOMOSIS

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Abstract

DEVELOPMENT, STANDARDISATION AND VALIDATION OF ELISA METHODS TO IMPROVE THE CONTROL OF TRYPANOSOMOSIS.

During the period from 1995 to 2000, comprehensive laboratory and field studies on enzyme-linked immunosorbent assay (ELISA) methods for detection of trypanosomal antibodies and antigens were undertaken to improve the proficiency of diagnostic laboratories involved in control of trypanosomosis in the tropics. The work was initiated by the FAO/IAEA through the Coordinated Research Programme D3.20.13 and undertaken in close collaboration with Research Agreement Holders and Research Contract Holders.

Initially, the CRP facilitated the field evaluation of three direct sandwich antigen detection ELISAs based on monoclonal antibodies. Diagnostic laboratories were supported with ELISA equipment, disposables, and training. ELISA reagents were produced in sufficient quantities and distributed in a standardised kit format. As a result of the laboratory and field evaluation studies, the assays were found unreliable for trypanosomosis control and rejected for routine use in diagnostic laboratories.

At that time, no standardised ELISA system was available for trypanosomosis that was considered suitable for distribution and use under tropical conditions. Through the CRP, a new generation of standardised antibody ELISAs were developed and established using a novel approach, namely the use of antigen-precoated ELISA plates. In addition, the potential of native and denatured trypanosomal antigens as diagnostic candidates was examined. In-house and field evaluation studies in the tropics demonstrated that a reasonable robustness with an acceptable diagnostic assay proficiency was achieved by means of utilising plates precoated with denatured antigens. Moreover, a data charting method for continuous monitoring of the operational performance of the ELISAs was developed and established. It was routinely used as remote control and follow up tool saving the need for costly expert missions to the diagnostic laboratories during the assay validation period. In parallel, preliminary studies also focused on various antigen detection ELISA configurations in support of the development and establishment of the antibody ELISAs. It is concluded that the approach presented has provided an improvement for developing robust and standardised serological methods for trypanosomosis control in tropical countries. Priorities of future research activities are discussed.

1. INTRODUCTION

The complementary use of direct parasitological and indirect serological methods is routine practice in diagnostic laboratories monitoring control against African trypanosomosis. The disease in ruminant livestock is caused by *Trypanosoma congolense* subgenus *Nanomonas*, *T. vivax*, subgenus *Duttonella*, and *T. brucei brucei* subgenus *Trypanozoon*. The very specific but low sensitive standard parasitological methods had necessitated the introduction of immunoassay methods which aim to improve the diagnostic sensitivity at acceptable specificity. Primary binding assays, which measure directly the antibody-antigen reaction, proved more reliable for serological analysis of bovine trypanosomosis than secondary binding assays, which produce results based on a reaction consequent upon the initial antibody-antigen interaction such as complement fixation test or indirect haemagglutination.

Among the primary binding assays such as the enzyme-linked immunosorbent assay (ELISA), card agglutination test (CATT) and the indirect fluorescence antibody (IFA) test, the ELISA method is considered superior because it offers a simple, fast, objective, standardisable, versatile, robust, safe, portable, and cost-effective screening tool for large number of test samples [1].

In 1977, the use of ELISA method was introduced for detection of trypanosomal antibodies, exploiting crude antigens from rodent-propagated trypanosomes [2], which stimulated further studies [3, 4, 5, 6, 7]. For detection of circulating trypanosomal antigens in serum samples, direct sandwich ELISAs were developed [8] exploiting monoclonal antibodies (MABs) [9].

The wide application of ELISA method for trypanosomosis in laboratories in developing countries was facilitated by the establishment of the technique *per se*, as well as through intensive training of laboratory staff in the assay procedure, data analysis, interpretation and assay troubleshooting aspects, which was initiated by a Coordinated Research Programme of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture [10, 11].

From a retrospective judgement of two decades of routine ELISA application in trypanosomosis serology, it became evident that the value of ELISA method has been frequently hampered because ELISA techniques have lacked: i) the acceptable robustness after transfer from the research and development laboratories to routine diagnostic laboratories in tropical countries, ii) the comparability of diagnostic proficiencies because ELISAs were used in a non-standardised format with respect to the exploited biological reagents, and iii) the continuity of research and assay modification following field release. Consequently, the true diagnostic proficiency of the assays was never established.

A report is presented here in chronological order, commencing with a summary of the standardisation and evaluation activities (1995-1996) on the FAO/IAEA kit format of the MAB-based ELISAs for antigen detection which were originally developed at the International Livestock Research Institute (ILRI) [8]. Then, the main topic of the report outlines the research and development studies (1997-1999) on new ELISA methods to detect trypanosomal antibodies and antigens.

The bench work was carried out at the Animal Production Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria, under the Animal Production and Health Subprogramme of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, in close collaboration with international institutes, and diagnostic laboratories in the tropics participating in the Coordinated Research Programme D3.20.13.

2. PERIOD 1995-1996

2.1. Evaluation of the performance of monoclonal antibody-based antigen ELISAs

The FAO/IAEA ELISA kit formats of the MAB-based direct sandwich ELISAs [8] were critically evaluated under laboratory and field conditions in collaboration with the Research Agreement Holders and Research Contract Holders [10, 11, 12, 13, 14]. It was demonstrated that these assays were found unreliable when compared with standard parasitological techniques such as the dark ground/phase contrast buffy coat technique [15, 16, 17]. The results were presented and comprehensively discussed at the workshop "Evaluation of the Performance of Antigen ELISAs for the Detection of Trypanosomes" held at ILRI, 9-11 December 1996. As a consequence of the discussions, these ELISA configurations were rejected for routine use in diagnostic laboratories [18].

2.2. Establishment of in vitro conditions for serum-free batch production of monoclonal antibodies to trypanosomal antigens

The batch to batch production of MABs in mouse ascites derived from three cloned hybridoma cell lines (*T. brucei* 7/47.34.25; *T. congolense* 39/30.38.11; *T. vivax* 27/9.45) [9] was adapted to the use of serum-free cell culture medium by means of an automated Cell Pharm Mini Biorector System 500 (Unisyn Fibertec, USA) mounted with hollow fiber cartridges (molecular weight cut-off 30 kilo daltons). The hybridoma cell lines were cultured in 5 cell culture media (Promocell HGM-af, C-25030; Dipro Cytoferr-Hybridoma Medium, 9950; Gibco Hybridoma-SFM cat. no. 061-02045; RPMI 640, 10% FCS; Hyclone B-6001-D). Cell growth was controlled monitoring the pH (MultiLab 540, WTW, FRG) and glucose metabolism (Reflolux® S, Boehringer Mannheim, FRG) in 2 to 3 day intervals over a period of 6 weeks. The cell number was estimated using a Neubauer hemocytometer. The cell viability was determined by means of the trypan blue dye-exclusion procedure. The total protein content of the supernatant was measured using Coomassie Plus Protein Assay Reagent® (Pierce, USA). Routinely, every 2 to 3 days, 20-30 ml of supernatant was collected from the cartridge. The immunoglobulins of pooled supernatant were transferred into phosphate buffered saline (PBS, pH 7.4) and concentrated to 1 mg/ml using the ultrafiltration cell and membranes with molecular weight cut-off ≤ 30 kilo daltons (Amicon, USA). The isotype of purified immunoglobulins was determined using isotyping ELISA kit (Gibco, UK). The functional activity of the MABs was assessed by screening defined reference serum from cattle infected with trypanosomes using the ELISA method. As a result, Promocell HGM-af cell culture medium was found best suited for in vitro production of

all cell lines. It also provided best in vitro metabolism conditions for the cell lines to produce monoclonal antibodies. The use of cell culture medium other than Promocell HGM-af either failed to maintain the cell lines over time or lacked biological activity, i.e., no antibodies were found in the supernatant. In conclusion, a protocol was established for routine in vitro rather than in vivo (mouse ascites) batch to batch production of monoclonal antibodies resulting in defined, standardised and quality assured reagents used for immunoassays.

2.3. External quality assurance programme

A round of the external quality assurance programme (EQAP) for the FAO/IAEA version of the MAB-based, direct sandwich antigen-detection ELISAs started in August 1996. The results were presented in the EQAP interim report 1996, which can be requested from the FAO/IAEA Animal Production and Health Subprogramme, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

3. PERIOD 1997-1999

In response to some of the conclusions and recommendations made at the workshop at ILRI in 1996 [18], the FAO/IAEA Animal Production and Health Subprogramme initiated research activities focusing on development and validation of a new generation of enzyme-linked immunosorbent assays (ELISAs) for detection of *T. congolense* and *T. vivax* antibodies and antigens.

3.1. Standardisation and quality assurance of ELISA methods

Filtered (0.2 µm) deionized and distilled water was used throughout for all ELISA work (conductivity: 1.32 µS/cm ± 0.02 standard error, pH 6.3 ± 0.05 standard error).

Technical aspects of the preparation and titration of reference sera and defined reagents, data normalisation, data processing, assay acceptance, and the preparation of standardized assay protocols have been described elsewhere [19, 20, 21].

The brand of microtitre immunoassay plates chosen for the new generation of ELISAs was selected after comprehensive evaluation of plates for their capacity to prevent non-specific protein binding under blocking conditions and their specific protein binding characteristics with respect to trypanosomal antigens. The study contributed to a better understanding of the unexpected background colour in solid phase immunoassays, and defined an additional method for quality control of immunoassay plates [22].

For legal importation of serum samples from non-member states to the European Union, serum was sterilised using gamma-irradiation procedure in compliance with the appropriate rules and guidelines of Austrian authorities responsible for animal disease and trade control and consumer protection. The treatment effect was found negatively biased for samples approaching the threshold value. For these samples, the readjustment of the diagnostic negative/positive threshold of the ELISA [8] was recommended using defined irradiated samples [23].

The preparation and treatment of reagents (polyclonal antibodies, goat-serum saturated enzyme-labelled antibodies, propagation of trypanosomes in vitro, antigen treatment) and of chemicals used for ELISA systems have been described elsewhere [24].

3.2. ELISA systems

A variety of ELISA configurations for detection of trypanosomal antibodies and antigens were investigated (Table I). Four prototypes of indirect trypanosomosis antibody ELISAs were developed and adopted to a kit format for comprehensive field evaluation studies. In addition, ELISA prototypes for antigen detection were developed and subjected to laboratory rather than field evaluation studies. The antigens exploited in the ELISA systems were derived from *T. congolense* and *T. vivax in vitro* cultures [24].

TABLE I. ELISA SYSTEMS DEVELOPED AND INVESTIGATED FOR DETECTION OF TRYPANOSOMAL ANTIBODY (AB) AND/OR ANTIGEN (AG)

ELISA System	Fresh coating of reagents	Test sample	Detecting AB (unlabeled)	Detecting AB (enzyme-labeled)	Diagnostic target	Assay validation
I-ELISA	*T. c.* AG_n or AG_d	Serum, blood on filter paper	Not applied	Commercial product	*T. c.* AB	Animal Production Unit and diagnostic laboratories in the tropics
I-ELISA	*T. v.* AG_n or AG_d	Serum	Not applied	Commercial product	*T. v.* AB	"
D-C-ELISA	*T. c.* AG_n or AG_d	Serum	Not applied	Polyclonal ABs (bovine)	*T. c.* AG/AB?	Animal Production Unit
I-C-ELISA	*T. c.* AG_n or AG_d	Serum	Polyclonal ABs (rabbit, guinea pig)	Commercial product	*T. c.* AG/AB?	"
I-C-ELISA	*T. v.* AG_n or AG_d	Serum	Polyclonal ABs (rabbit, guinea pig)	Commercial product	*T. v.* AG/AB?	"
I-Inh-ELISA	*T. c.* AG_n or AG_d	Serum	Polyclonal ABs (rabbit, guinea pig)	Commercial product	*T. c.* AG/AB?	"
I-S-ELISA	Polyclonal ABs (rabbit, guinea pig)	Serum	Polyclonal ABs (rabbit, guinea pig)	Commercial product	*T. c.* AG	"

I-ELISA: indirect ELISA; D-C-ELISA: direct competitive ELISA; I-C-ELISA: indirect competitive ELISA; I-Inh-ELISA: indirect inhibition ELISA; I-S-ELISA: indirect sandwich ELISA; AG_n: native antigen; AG_d: detergent/heat treated antigen; *T. c.* : *Trypanosoma congolense*; *T. v.*: *Trypanosoma vivax.*

3.3. Assay robustness of antibody detection ELISAs

The value of using ELISA has been frequently hampered because assays have lacked acceptable robustness to assure successful transfer to laboratories in tropical countries and long-term storage with respect to the stability of the biological reagents used. While antibodies supplied in reference sera or conjugated to enzymes were relatively stable, the quality of trypanosomal crude antigens was seriously affected by temperature. Thus, reduced absorbance was consistently observed in ELISA when plates were coated with trypanosomal antigens from a stock solution, which was stored in polypropylene tubes or siliconized glass vials at + 4 °C overnight at our laboratory. These empirical findings were confirmed by a field trial when antigens in polypropylene tubes were distributed on ice in vacuum flasks to 15 diagnostic laboratories in Africa [25, 26].

Various attempts have been made to improve the quality of antigens from crude trypanosomal lysates using protease inhibitors and stored at -70°C [27] or the use of equal part of glycerol and storage at -30°C [28]. Reduced absorbance in ELISA was shown to be caused by less protein in specified volumes for coating due to adsorption of the antigens to polypropylene surfaces as demonstrated by Coomassie blue staining of proteins (Rebeski and Winger, unpublished data). Consequently, a novel approach was applied to improve the robustness of the indirect trypanosomosis antibody-detection ELISA method using polystyrene 96-well plates precoated and air dried with crude trypanosomal antigens. From the data obtained from laboratory and field evaluation studies, it was concluded that the routine use of antigen precoated polystyrene plates for the enzyme immunoassay technique will contribute to improved assay robustness at an acceptable diagnostic proficiency. The precoating procedure will also provide an improved quality assurance and standardization procedure for the assay, which is required to allow the reliable detection of trypanosomal antibodies and comparison of data between laboratories [29, 30].

3.4. Evaluation of standardized ELISAs using antigen-precoated plates – experimental *T. congolense*-infection

The assay robustness and diagnostic proficiency was examined analysing sera from cattle experimentally infected with *T. congolense*. Four AB-ELISA systems were standardized exploiting native (AGn) or detergent-heat treated antigen preparations (AGd): two *T.c.* AG-based indirect ELISAs (I-TAB ELISA (*T.c.*AGn) and I-TAB ELISA (*T.c.*AGd)), as well as two *T.v.* AG-based indirect ELISAs (I-TAB ELISA (*T.v.*AGn) and I-TAB ELISA (*T.v.*AGd)). The ELISAs were meant to detect bovine antibodies crossreacting to *T. congolense*, *T. vivax* and *T. brucei*. The plates were precoated with native or heat/detergent denatured antigens from *T. congolense* and *T. vivax*, and stored for between 1 to 206 days at +37°C.

The data demonstrated improved robustness of the ELISAs exploiting precoated plates with AGn or AGd preparations. The use of the *T.c.*AG-based ELISAs produced acceptable diagnostic proficiency results. In particular, the *T.c.* AGd-based ELISA proved useful for analyzing *T.congolense* immune sera from chronic infections. The *T.v.*AG-based ELISAs lacked crossreactiviy to *T.congolense* antibodies at cut-off values of 22 % (iTAB ELISA (*T.v.*AGn)), and 13 % (iTAB ELISA (*T.v.*AGd)) [31].

3.5. Evaluation of standardized ELISAs using antigen-precoated plates – field validation

The robustness and diagnostic proficiency of the I-TAB ELISAs were also examined after shipment to 17 laboratories. Four laboratories (Belgium, Burkina Faso, Cameroon, and Kenya) evaluated ELISA tests utilizing microtitre plates which were precoated with native and denatured antigens from *T. congolense*, and those from *T. vivax*. The remaining 13 laboratories (Côte d'Ivoire, Ethiopia, Ghana, Mali, Nigeria, Senegal, Sudan, Tanzania, The Gambia, Uganda, Zambia, Zanzibar, and Zimbabwe) in Africa evaluated I-TAB ELISAs based on *T. congolense* and *T. vivax* denatured antigen preparations. The precoated plates were packed in plastic bags with silica gel desiccant packets and shipped without any specific storage conditions. The frozen reagents were distributed on ice in a vacuum flask.

ELISA evaluation reports were compiled by the Contract holders describing the particular study design and results. Briefly, serum samples, verified positive for trypanosomes by traditional

parasitological techniques, were collected from cattle populations kept under natural tsetse-challenge conditions. Serum samples were also collected from cattle from non or low tsetse-infested areas. Under these circumstances, it was occasionally difficult to estimate the diagnostic assay proficiency correctly of samples, which were found trypanosome-negative by microscopy and serologically positive. Similar difficulties were encountered when the potential cross-reactivity of the antigen preparations to non-trypanosomal antibodies was estimated analyzing serologically positive samples which were also positive for blood parasites other than trypanosomes. For ELISA evaluation, serum samples were also derived from experimental infection studies (The Gambia).

Upon completion of the testing period, the Contract holders reported the results of the internal quality controls (IQCs) [21] of all plates to the Animal Production Unit. The absolute signal and the variation of each IQC, expressed in raw absorbance values and percent positivity (PP) values was computed, to estimate the assay robustness. Irrespective of the factors which could interfere with the assay performance during transport to the tropics, it was demonstrated that the assays performed reasonably within the tentative upper and lower control limits as previously determined at the Animal Production Unit (mean ± 3 STD). By means of modified Youden plot analysis plotting the overall intralaboratory mean PP values of the internal moderate positive serum control (C+) against the PPs of the negative serum control (C-), which showed most inter-laboratory variation, random and systematic errors were found in 1 of 5 laboratories (I-TAB ELISA (*T.c.*AGn), 3 of 14 laboratories (I-TAB ELISA (*T.c.*AGd), 2 of 5 laboratories (I-TAB ELISA (*T.v.*AGn), and 4 of 15 laboratories (I-TAB ELISA (*T.v.*AGd). The complete analysis on the operational assay performance has been reported in detail elsewhere [32].

The estimates of the diagnostic proficiency of the assays varied with the reporting laboratories. For instance, studies in Kenya, Mali, Burkina Faso, and Tanzania reported sensitivity and specificity estimates of 82 %-100 %. Lower estimates were found in, e.g., Cameroon, Sudan and Zambia (70% to 90 %). Uganda reported 81 % sensitivity of the I-TAB ELISA (*T.c.*AGd) for homogenous infections and a sensitivity of 52.5 % for infections due to all trypanosome species. Several laboratories (e.g., Kenya, Uganda, Burkina Faso, The Gambia) observed that *T. vivax* antigen preparations appeared to be more suitable diagnostic antigens than the *T. congolense* derived antigen preparation which was the reverse in Mali and Zambia. Studies from Cameroon, Burkina Faso and Kenya demonstrated that the assay exploiting denatured antigen preparations provided similar diagnostic proficiency in comparison to ELISAs based on native antigen preparations. Further detailed information can be obtained from the country reports.

In summary, the results showed that the distribution of ELISA plates precoated with antigen had many advantages over existing formats used for the diagnosis of trypanosomosis including improvements in storage, shelf-life, transport and test reproducibility. Moreover, application in the different countries demonstrated that plates precoated with AGd of *T. congolense* and *T. vivax* are equally or more reliable and robust as those precoated with AGn.

3.6. Continuous control and monitoring of ELISA performance

For the operational control and troubleshooting of routinely used standardized ELISAs, the preparation of control charts was established such as i) daily summary and single plate data chart; ii) daily precision chart, and iii) daily proficiency chart. The charts were meant to assist laboratories in the monitoring of internal quality controls (IQCs) supplied with the assay and to evaluate the operational ELISA performance within tentative control limits, which were established at the Animal Production Unit. In addition, a comparative explorative analysis on the IQC data has been executed to evaluate the ELISA performance under different conditions [32].

4. DISCUSSION

The use of AG-precoated plates contributed successfully to an improved ELISA robustness required for diagnostic laboratories in developing countries. The laboratory and field evaluation studies demonstrated that precoated plates were superior to freshly coated plates with respect to assay proficiency and precision. The use of precoated plates will certainly contribute to improved assay robustness in the tropics where liquid antigen preparations lacked stability [25, 26]. This was confirmed when assistance was requested by a laboratory in South Africa utilizing liquid antigen

preparations supplied by an other laboratory in Africa (Rebeski, unpublished data). However, the successful use of precoated plates for ELISA can be seriously affected by a variety of inappropriate laboratories conditions, such as the use of distilled rather than deionized water, poorly calibrated pipettes or a malfunctioning shaker-incubator. Moreover, attention needs to be paid to uncertainties such as varying quality of polystyrene batches used for plate manufacturing which could potentially affect the quality of antigen-precoated ELISA plates. Therefore, continuous critical assessment of the plate quality is pivotal to successful and reliable application of this novel approach to detect trypanosomal antibodies.

ELISA data charting methods for continuous evaluation and monitoring of the operational ELISA performance with respect to pre-established limits will also contribute to improved ELISA procedure in developing countries. The charts will assist the operator in data acceptance and interpretation. Moreover, remote troubleshooting of ELISAs as is being done by the Animal Production Unit will be more efficient when data charts are available.

Further improvements of ELISA method for trypanosomosis require continuity of assay standardization aspects and the production of defined reference control serum samples derived from experimental infection. The operator should be advised to incorporate defined reference standards, which represent the local cattle population, in addition to the IQCs.

ELISA research and assay modifications need to be continued irrespective of the availability of ELISA method in a particular kit format for field use. Attention should be paid to the variation in assay proficiency due to the varying antigen/antibody specificities related to the geographical distribution of trypanosomes. It should be noted that the antigen preparations were chosen for their availability rather than their diagnostic potential as diagnostic candidates. Therefore, antigen preparations, derived from crude, purified or recombinant antigen sources, should be evaluated in order to identify a diagnostic candidate as an universal analytical target for both antigen and antibody detection in ELISA systems. Finally, the effect of the sample nature, i.e., serum, plasma, or blood, on the accessibility of the analytical target should be investigated. In this respect, the prototype ELISAs for antigen detection are considered a useful tool and should be subjected to further evaluation studies.

In conclusion, the results of the laboratory research and field work assisted laboratories in developing countries to apply serological methods for the control of animal trypanosomosis in an appropriate and standardised way.

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EVALUATION OF ANTIGEN AND ANTIBODY ELISA's FOR EPIDEMIOLOGICAL SURVEYS OF TSETSE-TRANSMITTED TRYPANOSOMOSIS IN CATTLE

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EVALUATION OF ANTIGEN AND ANTIBODY ELISA's FOR EPIDEMIOLOGICAL SURVEYS OF TSETSE-TRANSMITTED TRYPANOSOMOSIS IN CATTLE.

Sensitivity and specificity of the FAO/IAEA antigen-detection enzyme-linked immunosorbent assay (ELISA) kits for diagnosis of bovine trypanosomosis were investigated using sera from experimental cattle infected by tsetse challenge with cloned populations of *Trypanosoma congolense* (3 populations) or *T. vivax* (1 population). The kits are based on monoclonal antibodies that recognise internal antigens of tsetse-transmitted trypanosomes. Ten cattle were infected with each trypanosome population for at least 60 days, and in combination with uninfected cohorts (n = 16) were used in a double-blind study design.

Sensitivity and specificity of the tests depended on the choice of positive-negative thresholds expressed as percent positivity with respect to the median OD of 4 replicates of the strong positive reference serum provided with the kit. In general, while overall specificities were high, sensitivities of the antigen-ELISA's were poor. For example, at a cut-off of 5% positivity, the sensitivities of the antigen-ELISA's 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. There were no values of the positive-negative threshold at which both sensitivity and specificity were satisfactory. Trypanosome species-specificities of the antigen-ELISA's were also poor. Sensitivity and species-specificity of the antigen-ELISA for *T. brucei* infections were not investigated.

The indirect ELISA for the detection of anti-trypanosomal antibodies in bovine serum was adapted for use with dried blood spots on filter paper, and standardised using a strong positive reference serum and the percent positivity system of data expression.

The antibody-ELISA was evaluated in Zambia for use in epidemiological surveys of the prevalence of tsetse-transmitted bovine trypanosomosis. Known negative samples (sera, n = 209; blood spots, n = 466) were obtained from cattle from closed herds in tsetse-free areas close to Lusaka. Known positive samples (sera, n = 367; blood spots, n = 278) were obtained from cattle in Zambia's Central, Lusaka and Eastern Provinces, diagnosed as being infected with *T. brucei*, *T. congolense*, or *T. vivax* using the phase-contrast buffy-coat technique or Giemsa-stained thick and thin blood smears. For sera, at a cut-off value of 23.0% positivity, sensitivity and specificity were 86.1% and 95.2% respectively. For bloodspots, at a cut-off value of 18.8% positivity, sensitivity and specificity were 96.8% and 95.7% respectively.

It was concluded that the Ab-ELISA could be a useful tool for characterising areas in terms of trypanosomosis risk, and for prioritising control activities. It is not however appropriate for individual animal diagnosis.

1. INTRODUCTION

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Antigen detection ELISA's for species-specific diagnosis of bovine trypanosomosis [5] were developed at the International Livestock Research Institute (ILRI, formerly the International Laboratory for Research on Animal Diseases [ILRAD]), and subsequently adapted as a diagnostic kit by scientists at the Animal Production and Health Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria. The most significant of the adaptations to the original method [5] are the use of:

a) normal mouse serum (0.5%) as a blocking agent in the sample diluent buffer;
b) the chromogen 3,3',5,5' tetramethylbenzidine (TMB) with a timed 1M phosphoric acid quench;
c) the percentage positivity data expression system [6]
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Despite modification, the assays retain the original reagents and configuration; the anti-trypanosomal monoclonal antibodies are from hybridoma cell lines derived at ILRI and are used in a standard direct sandwich ELISA technique. The anti-trypanosomal monoclonal antibodies used are as follows: *T. brucei*, T.b.r.7 (IgM) [7]; *T. congolense*, T.c.39 (IgM) [8]; *T. vivax*, T.v.27 (IgG_1) [9].

The sensitivity and specificity of the FAO/IAEA Trypanosomosis Direct Antigen-ELISA kits were assessed in relation to well-defined experimental trypanosome infections in cattle. ELISA responses were measured in a double blind trial using sera of cattle following primary infection with cloned populations of *Trypanosoma congolense* and *T. vivax* originating from geographically distinct locations in East and West Africa, and sera of uninfected cohorts.

In contrast to the antigen ELISA, the indirect antibody-ELISA (Ab-ELISA) for bovine trypanosomosis saw little practical application in tsetse and trypanosomosis control for almost 20 years since its original description [10]. However an adapted version of the Ab-ELISA was recently adopted by the Regional Tsetse and Trypanosomosis Control Programme (RTTCP) in Southern Africa for epidemiological studies of the disease in cattle. The adaptations included use of either serum samples or dried blood spots on filter paper, and the incorporation of rigorous quality assurance. The sensitivity and specificity of this test were also assessed using of both types of samples collected in the field from cattle of known trypanosome infection status.

2. MATERIALS AND METHODS

2.1. Antigen-detection ELISA

2.1.1. Experimental animals

Boran cattle (*Bos indicus*), aged 12 to 18 months, were obtained from a closed herd at Kapiti Ranch on the Athi Plains, a trypanosomosis non-endemic area of Kenya. The animals 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.

East African Maasai cross Galla goats were maintained in a similar manner to the cattle.

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Fourteen days prior to trypanosome infection, sera were collected from the cattle and screened by serological methods (IFAT) for antibodies to *Babesia bigemina*, *Anaplasma marginale* and *Theileria mutans*. EDTA blood samples were also examined for the presence of trypanosomes using the phase-contrast buffy-coat technique. Packed red blood cell volumes (PCV) were also recorded.

2.1.3. Trypanosomes

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These trypanosome populations have previously been shown to be tsetse transmissible and to produce sub-acute/chronic infections in cattle.

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Cattle were infected by allowing five infective *Glossina morsitans centralis* to engorge on the flank of each animal. Six weeks before infection of cattle, goats were inoculated intramuscularly with one of the four trypanosome populations. Twelve to 14 days later, trypanosomes were detected in peripheral blood. Commencing a further seven days later, teneral *G. m. centralis* were fed daily on the infected goats for 25 days. Flies were then starved for 2 days, and monitored for infection by microscopic examination of salivary probes [17]. Tsetse flies with metacyclic trypanosomes in their saliva were used to challenge cattle.

2.1.5. Parasitological monitoring

Cattle were examined for the presence of trypanosomes in the circulation by the phase-contrast buffy-coat technique [2], using blood collected into EDTA vacutainers (Becton-Dickinson), and an SM Lux microscope (Leitz, Wetzlar, Germany) with a 25x magnification phase-contrast objective. Care was taken to ensure that every microscopic field of the buffy-coats was examined thoroughly.

2.1.6. 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 1200g for 15 minutes at 4°C, and serum aspirated. This procedure resulted in the maximum yield of serum, and no visible haemolysis.

2.1.7. Experimental design

Experiments were conducted using a double-blind system: neither those collecting the samples nor those conducting subsequent laboratory analyses knew beforehand which animals were infected or which parasites they were infected with.

In each of four groups of 14 cattle, ten animals were infected with trypanosomes, while the remaining four cattle remained uninfected as 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 then bi-weekly 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 experiment if either the PCV fell below 14%, or their clinical condition warranted removal on welfare grounds. Consequently, there was variation in the experimental period for the different trypanosome populations.

2.1.8. Serum sample encryption and storage

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 identification of the serum samples; the cryovials were labelled with only their unique code numbers. Thus, the identities of infected and control cattle were not revealed to individuals responsible for testing of experimental samples.

2.1.9. Antigen-ELISA

Trypanosome Ag-ELISA's were conducted using the FAO/IAEA Trypanosome Antigen-ELISA kits (serial numbers TRP95/GLA/01, TRP95/GLA/01, TRP95/06/18), kindly 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 (IgG_1)

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.

2.1.10. Quality assurance and data analysis

The quality assurance (QA) aspects of the FAO/IAEA Trypanosome Antigen-ELISA kit protocol were strictly adhered to. The protocol broadly follows the recommendations of Wright et al. (1993). For each of the three trypanosome species, specific strong positive (C++) and moderate positive (C+) control sera were provided in the kits. Negative control serum (C-) was also provided. Hence, appropriate C++, C+, 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.

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Cattle were infected by allowing five infective *Glossina morsitans centralis* to engorge on the flank of each animal. Six weeks before infection of cattle, goats were inoculated intramuscularly with one of the four trypanosome populations. Twelve to 14 days later, trypanosomes were detected in peripheral blood. Commencing a further seven days later, teneral *G. m. centralis* were fed daily on the infected goats for 25 days. Flies were then starved for 2 days, and monitored for infection by microscopic examination of salivary probes [17]. Tsetse flies with metacyclic trypanosomes in their saliva were used to challenge cattle.

2.1.5. Parasitological monitoring

Cattle were examined for the presence of trypanosomes in the circulation by the phase-contrast buffy-coat technique [2], using blood collected into EDTA vacutainers (Becton-Dickinson), and an SM Lux microscope (Leitz, Wetzlar, Germany) with a 25x magnification phase-contrast objective. Care was taken to ensure that every microscopic field of the buffy-coats was examined thoroughly.

2.1.6. 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 1200g for 15 minutes at 4°C, and serum aspirated. This procedure resulted in the maximum yield of serum, and no visible haemolysis.

2.1.7. Experimental design

Experiments were conducted using a double-blind system: neither those collecting the samples nor those conducting subsequent laboratory analyses knew beforehand which animals were infected or which parasites they were infected with.

In each of four groups of 14 cattle, ten animals were infected with trypanosomes, while the remaining four cattle remained uninfected as 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 then bi-weekly 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 experiment if either the PCV fell below 14%, or their clinical condition warranted removal on welfare grounds. Consequently, there was variation in the experimental period for the different trypanosome populations.

2.1.8. Serum sample encryption and storage

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 identification of the serum samples; the cryovials were labelled with only their unique code numbers. Thus, the identities of infected and control cattle were not revealed to individuals responsible for testing of experimental samples.

2.1.9. Antigen-ELISA

Trypanosome Ag-ELISA's were conducted using the FAO/IAEA Trypanosome Antigen-ELISA kits (serial numbers TRP95/GLA/01, TRP95/GLA/01, TRP95/06/18), kindly 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 (IgG_1)

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.

2.1.10. Quality assurance and data analysis

The quality assurance (QA) aspects of the FAO/IAEA Trypanosome Antigen-ELISA kit protocol were strictly adhered to. The protocol broadly follows the recommendations of Wright et al. (1993). For each of the three trypanosome species, specific strong positive (C++) and moderate positive (C+) control sera were provided in the kits. Negative control serum (C-) was also provided. Hence, appropriate C++, C+, 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. QA calculations were performed using proprietary software (Eisler, unpublished results), specifically designed for sensitivity and specificity calculations.

2.1.11. Sensitivity and specificity calculations

Sensitivity (*Se*) of the phase-contrast buffy-coat technique and the Ag-ELISA for each trypanosome species was calculated separately for each *T. congolense*-infected and *T. vivax*-infected animal using $Se_i = \frac{n_{ip}}{n_i}.100\%$, where n_{ip} is the number of samples from the i^{th} infected animal having percentage positivity values greater or equal to the cut-off, from a total of n_i samples from that animal. Similarly, specificity (*Sp*) of the Ag-ELISA for each trypanosome species was calculated separately for each non-infected control animal using $Sp_i = \frac{n_{in}}{n_i}.100\%$, where n_{in} is the number of samples from the i^{th} non-infected animal having percentage positivity values less than the cut-off, from a total of n_i samples from that animal. Combined estimates of sensitivity (CE_{Se}) and specificity (CE_{Sp}) were obtained as weighted means for groups of N_p infected or N_u uninfected animals, respectively, using:

$$CE_{Se} = \frac{\sum_{i=0}^{N_p}(Se_i.n_i)}{\sum_{i=0}^{N_p}(n_i)}, \qquad CE_{Sp} = \frac{\sum_{i=0}^{N_u}(Sp_i.n_i)}{\sum_{i=0}^{N_u}(n_i)}.$$

Finally 95% confidence intervals for combined sensitivity and specificity estimates were calculated using $CE_{Se} \pm t\,.\,SE_{Se}$ and $CE_{Sp} \pm t\,.\,SE_{Sp}$ respectively, where t is the value of Student's T distribution at $p = 0.05$, and $N_i - 1$ degrees of freedom, and SE_{Se} and SE_{Sp} are the weighted standard errors for sensitivity and specificity respectively, calculated using:

$$SE_{Se} = \sqrt{\frac{\sum_{i=0}^{N_p}\left[(Se_i - CE_{Se})^2.n_i\right]}{\sum_{i=0}^{N_p}[n_i](N_p - 1)}}, \qquad SE_{Sp} = \sqrt{\frac{\sum_{i=0}^{N_u}\left[(Sp_i - CE_{Sp})^2.n_i\right]}{\sum_{i=0}^{N_u}[n_i](N_u - 1)}}.$$

For each of the three Ag-ELISA's, cross-reactivity for heterologous salivarian trypanosome infections was considered equivalent to sensitivity for such infections.

2.2. Antibody-detection ELISA

2.2.1. Sample collection and management

Blood samples were collected from cattle either by jugular or caudal venipuncture using plain vacutainers (Becton-Dickinson) without anticoagulant or from a marginal ear vein using heparinised microhaematocrit centrifuge capillary tubes. Serum was separated from clotted blood in vacutainers by conventional centrifugation and decanting, whereas blood collected in capillary tubes was immediately spotted onto filter paper (Whatman, no. 4) and air dried. Dried bloodspots on filter paper were stored in plastic bags with silica gel at ambient temperature for short periods under field conditions, at +4°C wherever possible, and at –20°C for long-term storage in the laboratory.

2.2.2. Bovine samples for establishment of test parameters

Known negative samples (sera, n = 209; bloodspots, n = 466) were available from non-infected cattle in the tsetse-free area around Lusaka. Paired serum and bloodspot were available from 189 of these animals.

Known positive samples (sera, n = 367; bloodspots, n = 278) were available from naturally infected cattle parasitologically positive for *Trypanosoma brucei*, *T. congolense* or *T. vivax*, from the

Central, Lusaka and Eastern Provinces of Zambia. Paired serum and bloodspot were available from 266 of these animals.

Parasitological examination used the dark ground buffy-coat technique and thick and thin Giemsa-stained blood films.

2.2.3. Antibody-detection ELISA

The simple indirect Ab-ELISA used was as described by Luckins [10], with minor modifications. The antigen for coating ELISA plates was prepared as a soluble fraction of *T. congolense* IL3000 [18] purified by DEAE anion-exchange chromatography of parasites from whole blood of infected rats, with lysis using 7 freeze-thaw cycles, and centrifugation at 10,000g for 30 min. Ninety-six well microtitre plates (Immulon 1: M129A, Dynatech) were coated overnight at +4°C with antigen diluted optimally in 50 mM carbonate-bicarbonate buffer, pH 9.6. One hundred microlitre well volumes were used throughout the ELISA.

Sera were diluted 1/400 in phosphate-buffered saline (PBS), 10 mM, pH 7.4, containing 0.05% Tween 20 (PBST). Six millimetre disks punched from dried bloodspots on filter papers were eluted in 2.0 ml PBST, for 2.5 h or 5h at room temperature, or overnight at +4°C. Earlier studies (data not shown) revealed no significant differences between Ab-ELISA results obtained using these different elution times. Antibody detection used affinity-purified rabbit anti-bovine IgG (whole molecule) peroxidase conjugate (A-7414, Sigma) optimally diluted in PBST.

Incubation of diluted sera and bloodspot eluates, and anti-bovine IgG peroxidase conjugate in microtitre plate wells was at 37°C with orbital shaking for 45 and 30 min respectively. Microtitre plates were washed in 2 mM PBS, pH 7.4, containing 0.05% Tween 20, four times following the coating and serum/bloodspot eluate incubations, and five times following the conjugate incubation. For development of colour, a solution of 3,3',5,5'tetramethylbenzidine dihydrochloride, was prepared by dissolving one tablet (T-3405, Sigma) in 10 ml phosphate-citrate buffer with sodium perborate (P-4922, Sigma). This was incubated in microtitre plate wells at 37°C with orbital shaking, followed after 10 min by an H_2SO_4 (2M) quench. Absorbances were read at 450nm using a multichannel photometer (Multiskan Plus Mk II, Titertek) connected to the serial port of an IBM-compatible personal computer via an RS232C cable.

2.2.4. Quality assurance and data analysis

The percentage positivity (PP) method of data expression and quality assurance [6] was used, as described for the Ag-ELISA. Strong-positive reference serum (C++) was obtained by pooling sera from two bovines following repeated experimental infection with stabilates of *T. congolense*. Negative reference serum (C-) was obtained by pooling sera obtained from cattle in the tsetse-free area around Lusaka. A moderate-positive reference serum (C+) was produced by diluting strong positive reference serum 1/23 in negative reference serum. Conjugate control (CC) comprised PBST only.

Four replicates of internal quality controls (ICQ) at the four levels (C++, C+, C- and CC) were included in every ELISA plate. For acceptance of results from individual microtitre plates, the median OD of the four C++ replicates, and the PPs of at least three of the four replicates at all four levels of IQC had to fall within prescribed target ranges.

ELISA data collation, quality assurance, and calculation of PP values for unknown samples were performed using a data management system written specifically for this purpose using FoxPro® for Windows.

2.2.5. Probability distributions

Parameters of normal distributions, i.e. mean and standard deviation, were calculated using non-transformed percentage positivity data. Parameters of log-normal distributions were calculated similarly, using natural log-transformed percentage positivity data.

2.2.6. Calculation of sensitivity and specificity

Sensitivity and specificity were calculated over a range of positive-negative threshold (cut-off) values from 0 to 100 percent positivity, for both sera and bloodspots. At any given cut-off value, sensitivity and specificity were calculated respectively as the ELISA-positive proportion of samples from parasitologically-positive cattle, and the ELISA-negative proportion of samples from cattle from

14 following tsetse challenge did not appreciably affect the overall sensitivity estimate, which was 12.4% (95% C.I. 5.4% to 19.5%).

If the cut-off was reduced 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. 18.0% 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 II. 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.

3.1.4. Cross-reactivity of sera from 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%) (Table III). Restricting the analysis (based on a 5% positivity cut-off) to those samples collected from day 14 following tsetse challenge slightly increased the overall cross-reactivity estimate to 14.2% (95% C.I. 8.6% to 19.8%) (Table III).

TABLE III. 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	1059	28.5%	24.0%	33.0%
		T. vivax	10	283	23.0%	16.9%	29.0%
	5%	*T. congolense*	30	1059	9.0%	5.3%	12.6%
		T. vivax	10	283	5.3%	0.6%	10.0%
	10%	*T. congolense*	30	1059	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.

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

The cross-reactivity 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%) (Table IV). Excluding data from samples collected within 14 days of tsetse challenge from the analysis had little effect on the cross-reactivity estimate, which was 13.3% (95% C.I. 9.3% to 17.3%) (Table IV).

TABLE IV. 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.

3.1.6. Sensitivity of the T. vivax *Ag-ELISA*

Sensitivity estimates for the *T. vivax* Ag-ELISA are presented in Table IV. 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%). Excluding data from samples collected within 14 days of tsetse challenge from the analysis (based on a 5% positivity cut-off) for *T. vivax*-infected cattle 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%).

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

The cross-reactivity 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 III). Excluding data from samples collected within 14 days of tsetse challenge from the analysis (based on a 5% positivity cut-off) for *T. vivax* IL Dat 1.2-infected cattle moderately increased the overall cross-reactivity estimate to 9.2% (95% C.I. 1.4% to 16.9%) (Table III).

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

The cross-reactivity 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*-infected cattle (n = 10) was 22.3% (95% C.I. 10.9% to 33.6%) (Table II). Excluding data from samples collected within 14 days of tsetse challenge from the analysis (based on a 5% positivity cut-off) for *T. vivax* IL Dat 1.2-infected cattle moderately increased the overall cross-reactivity estimate to 37.9% (95% C.I. 22.2% to 53.7%) (Table II).

3.1.9. Specificities of the trypanosome Ag-ELISA's

The specificities of the trypanosome antigen-ELISA's based on results from uninfected control cattle (n = 16) are presented in Table V.

TABLE V: SPECIFICITY ESTIMATES FOR THE TRYPANOSOME AG ELISA's

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%

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

3.1.10. 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%).

3.1.11. 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. 79.8% 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%).

3.1.12. 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%).

3.1.13. Relationship between cut-off value, sensitivity, specificity and diagnostic efficiencies of the T. congolense *and* T. vivax *Ag-ELISA's*

The relationships between cut-off value, sensitivity and specificity of the *T. congolense* and *T. vivax* Ag-ELISA's are shown in Figs.1 and 2. The optimal diagnostic performance for the *T. congolense* ELISA occurred at a cut-off of approximately 1% positivity, at which both sensitivity and specificity were approximately 60% (Fig. 1). For the *T. vivax* ELISA there was no value for the cut-off at which both sensitivity and specificity were above 50% (Fig. 2).

In terms of trypanosome species differentiation, the *T. congolense* Ag-ELISA was consistently less sensitive for infections with the homologous trypanosome species than for infections with *T. vivax* (Table II). The *T. vivax* Ag-ELISA had consistently better sensitivity for infections with the homologous trypanosome species than for infections with *T. congolense* (Table IV). This Ag-ELISA had better relative sensitivity for *T. vivax* infections than for *T. congolense* infections at higher values of the cut-off.

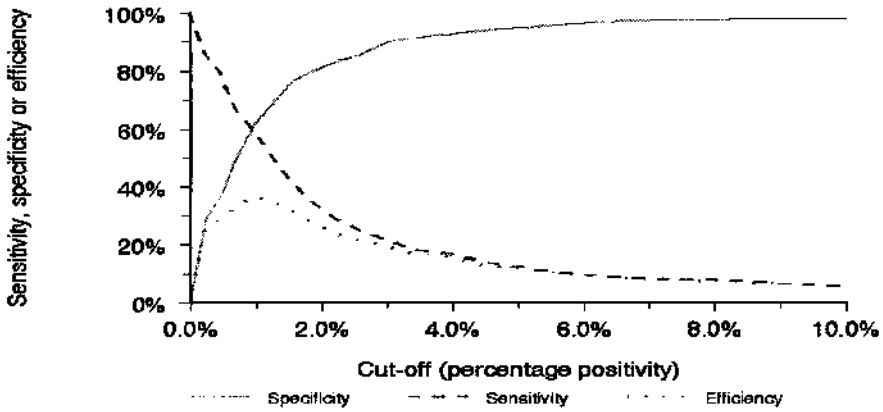

FIG. 1. Sensitivity and specificity of the T. congolense *Ag-ELISA in relation to cut-off. Analyses include samples from day 14 following tsetse challenge.*

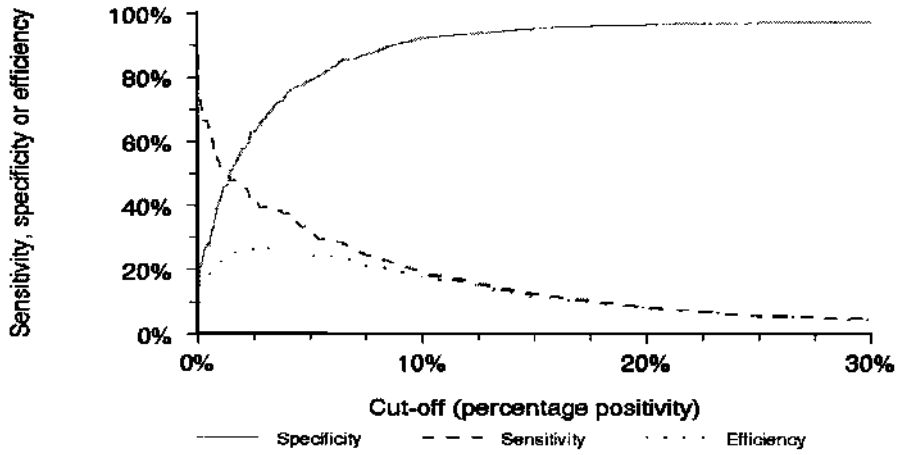

FIG. 2. Sensitivity and specificity of the T. vivax *Ag-ELISA in relation to cut-off. Analyses include samples from day 14 following tsetse challenge.*

3.2. Antibody-detection ELISA

3.2.1. Distributions of Ab-ELISA responses of known negative and positive cattle populations

Distributions of Ab-ELISA responses of sera and bloodspots from negative (n = 209 and n = 466, respectively) and positive (n = 367 and n = 278, respectively) cattle are shown in Figs 3 and 4. For both sera and bloodspots, Ab-ELISA responses of positive cattle closely fitted a cumulative normal distribution (sera: mean = 56.1 PP, SD = 28.1 PP; bloodspots: mean = 61.2 PP, SD = 26.2 PP) whereas responses of negative cattle closely fitted a cumulative log-normal distribution (sera: mean = -2.58, SD = 0.498; bloodspots: mean = -2.11, SD = 0.414). In all cases, values of R^2 adjusted for degrees of freedom were greater than 0.99.

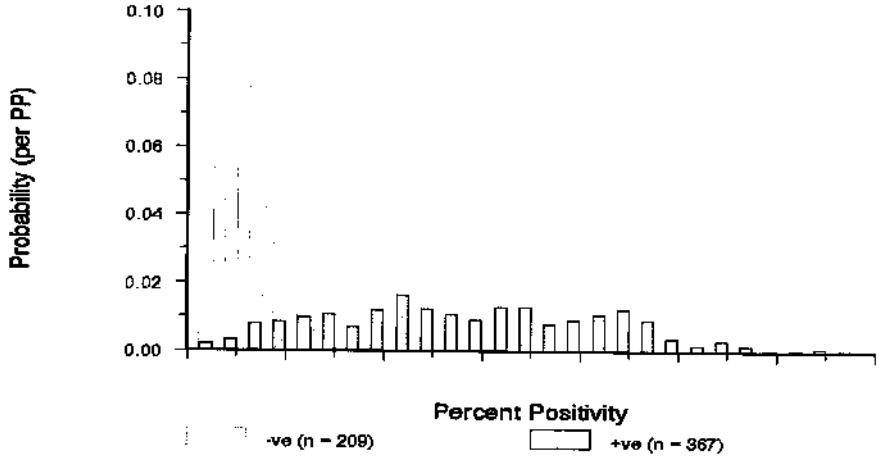

FIG. 3. Distributions of percentage positivity (PP) in Ab-ELISA for sera from trypanosome-infected (n = 367) and uninfected (n = 209) cattle expressed as probability density.

3.2.2. Sensitivity and specificity

The relationships between cut-off and sensitivity and specificity of the Ab-ELISA for both sera and bloodspots are shown in Figs 5 and 6.

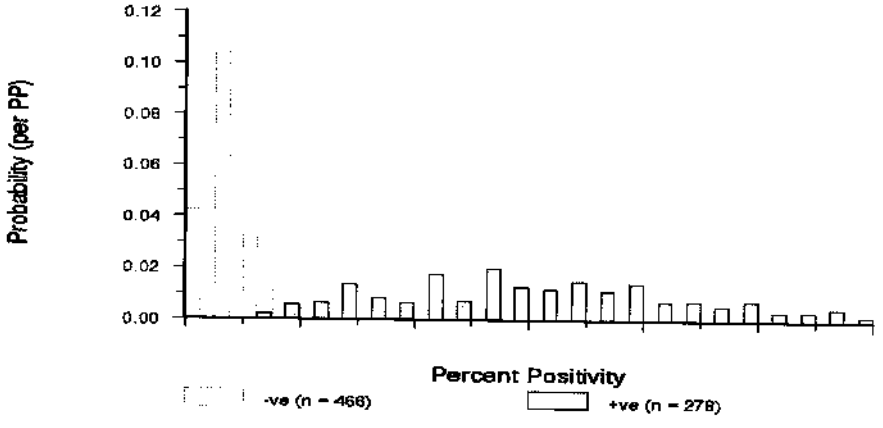

FIG. 4. Distributions of percentage positivity (PP) in Ab-ELISA for bloodspots from trypanosome-infected (n = 278) and uninfected (n = 466) cattle expressed as probability density.

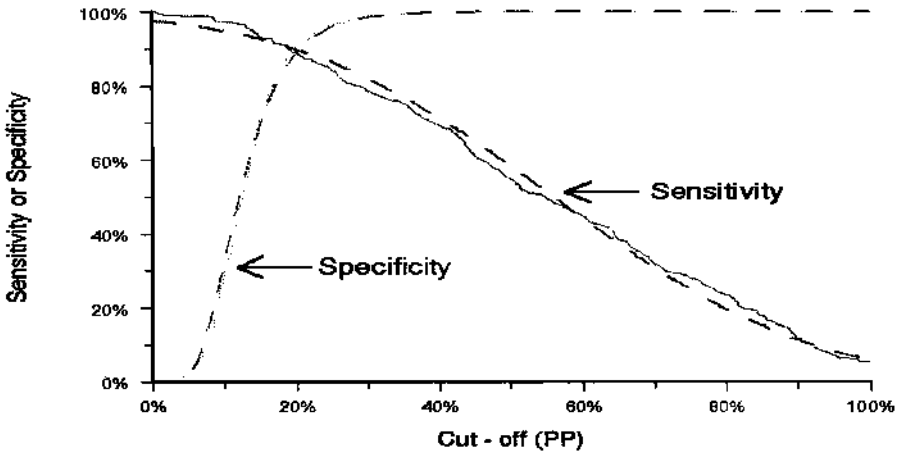

FIG. 5. Relationships between cut-off and sensitivity and specificity of the trypanosomosis Ab-ELISA for sera. Solid lines: sensitivity and specificity calculated using raw data. Dashed lines: sensitivity and specificity calculated using cumulative normal distribution for positive samples, cumulative log-normal distribution for negative samples.

For sera, high sensitivity and specificity were also obtained over a range of cut-off values of approximately 20.0% to 25.5% positivity (Fig 5). At a cut-off of 20.0% positivity, sensitivity and specificity were 88.8% and 90.9% respectively. At a cut-off of 25.6% positivity, sensitivity and specificity were 83.7% and 96.7% respectively. At an intermediate cut-off value of 23.0% positivity, sensitivity and specificity were 86.1% and 95.2%, respectively.

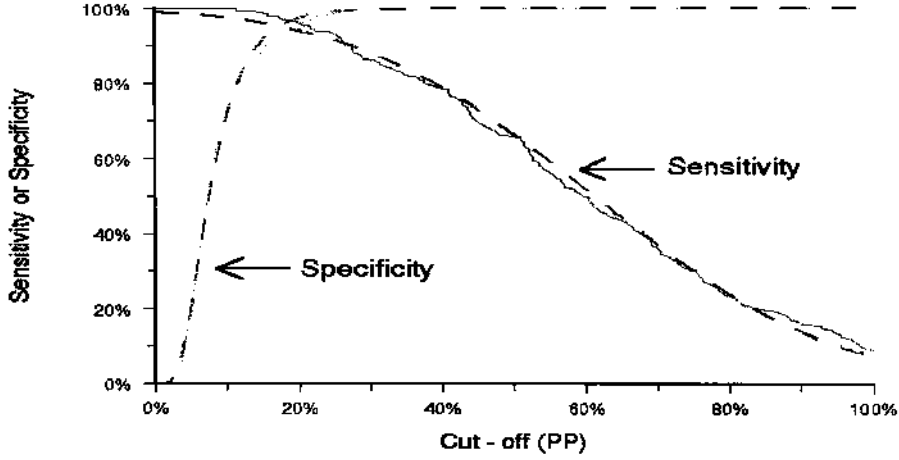

FIG. 6. Relationships between cut-off and sensitivity and specificity of the trypanosomosis Ab-ELISA for bloodspots. Solid lines: sensitivity and specificity calculated using raw data. Dashed lines: sensitivity and specificity calculated using cumulative normal distribution for positive samples, cumulative log-normal distribution for negative samples.

For bloodspots, high sensitivity and specificity were obtained over a wider range of cut-off values of approximately 16.5% - 26% positivity (Fig 6). At a cut-off of 16.5% positivity, sensitivity and specificity were 97.8% and 92.5% respectively. At a cut-off of 26% positivity, sensitivity and specificity were 91.4% and 98.9% respectively. At an intermediate cut-off value of 18.8% positivity sensitivity and specificity were 96.8% and 95.7% respectively.

3.2.3. Concordance of results for sera and bloodspots

Results for paired serum and bloodspots, based on a cut-off value of 23.0% positivity for sera and 18.8% positivity for bloodspots are shown in Table VI. Concordance was observed in 394 of 455 samples, while the expected concordance based on chance was 228, these data resulting in a *kappa* value of 0.731.

TABLE VI. CONCORDANCE OF TRYPANOSOMOSIS AB-ELISA RESULTS FOR PAIRED SERUM AND BLOODSPOT SAMPLES FROM TRYPANOSOME-INFECTED (n = 266) AND UNINFECTED (n = 189) CATTLE, BASED ON A CUT-OFF VALUE OF 23.0% POSITIVITY FOR SERA AND 18.8 % POSITIVITY FOR BLOODSPOTS

	Serum results		
Bloodspot results	Positive	Negative	Total
Positive	223	51	274
Negative	10	171	181
Total	233	222	455

4. DISCUSSION

The sensitivity and specificity of the FAO/IAEA trypanosome antigen detection ELISA's 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 [2] was used as a reference method for parasitological diagnosis. These investigations showed that although the Ag-ELISA's had good diagnostic specificity for trypanosome non-infections, diagnostic sensitivity and hence overall diagnostic efficiency were unsatisfactory. Furthermore, the ability of the tests to differentiate trypanosome species (i.e. trypanosome species specificity) was poor.

The sensitivity of the phase-contrast buffy-coat technique for tsetse-transmitted *T. congolense* infections in cattle was reasonably high (67.0%) when results for all samples collected from day 1 following tsetse challenge were included in the analysis. However, 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%). 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. It should be noted, however, that all the results were obtained with experimentally-infected cattle in excellent animal handling facilities that were in close proximity to a well-equipped laboratory. Consequently, the sensitivity estimates for the phase-contrast buffy-coat technique may not reflect the situation under field conditions.

In contrast to the phase-contrast buffy-coat technique, the sensitivities of the *T. congolense* and *T. vivax* Ag-ELISA's for infections with homologous parasite species were very low at the recommended cut-off value of 10% positivity (Tables II - IV). For *T. congolense*-infected cattle, the sensitivity of the *T. congolense* Ag-ELISA was a lowly 4.0% for all samples collected post-tsetse challenge, whereas the sensitivity of the phase-contrast buffy-coat technique was 67.0% for the same period. For *T. vivax*-infected cattle, the sensitivity of the *T. vivax* Ag-ELISA was 14.5% over the same experimental period, whereas the sensitivity of the phase-contrast buffy-coat technique was 59.5%.

When results for samples taken during the first 14 days following tsetse challenge were excluded (i.e. the prepatent period), the advantage of the phase-contrast buffy-coat technique over the Ag-ELISA's was even greater. The sensitivity of the *T. congolense* Ag-ELISA remained low at 5.7%, whereas the sensitivity of the phase-contrast buffy-coat technique for *T. congolense*-infected cattle

was 96.0%. The corresponding figures for *T. vivax*-infected cattle were 19.6% (*T. vivax* Ag-ELISA) and 76.3% (phase-contrast buffy-coat technique).

Lowering the cut-off percentage positivity improved the sensitivity estimates (Figs. 1 and 2), but at the expense of specificity. The optimal diagnostic performance for the *T. congolense* ELISA occurred at a cut-off of approximately 1% positivity, at which both sensitivity and specificity were approximately 60%. This result differs markedly from that obtained by Masake and Nantulya [8], in which a sensitivity of 82.5% was observed when 691 samples from seven *T. congolense*-infected steers were tested. However, in that study sensitivity and specificity were not determined contemporaneously using a double-blind design as was the case here.

For the *T. vivax* ELISA there was no value for the cut-off at which both sensitivity and specificity were above 50%. Poor sensitivity of the Ag-ELISA for *T. vivax* was reported previously for experimentally infected small ruminants, and for both naturally and experimentally infected cattle [19, 20].

Since no *Trypanosoma brucei*-infected cattle were investigated, it was not possible to assess the sensitivity of the *Trypanosoma brucei* Ag-ELISA in these studies.

In addition to poor sensitivity, the results obtained here suggest that the species specificity of the three Ag-ELISA's for salivarian trypanosome infections is also poor, at least for *T. congolense* and *T. vivax* infections. This contrasts markedly with the original description of the Ag-ELISA method [5] in which cross reactivity was considered not to occur.

In contrast with the Ag-ELISA, when large numbers of field sera from cattle of known infection status, both positive and negative, using the Ab-ELISA for bovine trypanosomosis, estimates of sensitivity and specificity were high. This assay worked well using either conventional serum samples or eluates of dried bloodspots on filter paper.

Use of blood spots simplifies the tasks of collection and management of samples, and greatly reduces the expense involved, particularly because of the reduced requirement for centrifugation and cold-chain facilities in remote areas. In addition, for the collection of bloodspots the requirement for handling animals is no more than for the standard phase-contrast buffy-coat technique, when heparinised capillaries are filled directly from a marginal ear vein. Common practice of field survey teams is to collect two such capillaries from each animal. The second capillary tube is a backup in case the first breaks during the microhaematocrit centrifugation, although generally it is not required and may be used for preparing a bloodspot. In contrast, to obtain sera, collection of an additional sample by jugular or caudal venipuncture adds considerably to the workload of the field team, and may reduce the number of animals that may be examined on each occasion of sampling.

Discrimination between infected and non-infected cattle was slightly better using dried bloodspots on filter papers than using sera. This was the case whether all available data for sera (n = 576) and bloodspots (n = 744) were analysed (Fig. 5), or whether the analysis was restricted to paired samples (n = 455; data not shown). The reasons for this are not clear but it is possible that non-specific factors present in sera interfere with the detection of specific antibody and /or contribute to background reactions of negatives. These factors may be present in bloodspot eluates in lower concentrations than in sera diluted for testing in the ELISA.

Seropositivity does not necessarily indicate current infections in individual animals and should not be used as basis for chemotherapy. Nevertheless, for the purposes of targeting tsetse and trypanosomosis control seroprevalence may provide useful information. Detection of antibody titres which are likely to persist for some time following elimination of infection either by self-cure or as a result of chemotherapy may give a better indication of the magnitude of the disease problem in an area than parasitological methods.

More information is required regarding persistence of Ab after chemotherapy, although studies in West Africa suggest that a period of four to six months may be typical [21]. Also, results from cattle less than six months old should be interpreted with caution in view of the possibility of maternal antibody transfer.

No attempt was made to differentiate between antibodies against the different species of pathogenic African trypanosomes. An approach to species differentiation has been described [22], but has not seen widespread use. For the practical purposes of tsetse/trypanosomosis control, differentiation between the various pathogenic salivarian trypanosomes may not be of great importance, although differentiation of the ubiquitous, non-pathogenic stercorarian trypanosome of

cattle, *T. theileri*, is clearly desirable. No cross-reactions were observed in earlier studies using Ab-ELISA for trypanosomosis on *T. theileri* infected cattle [10]. The large population of cattle from a tsetse-free area studied in the present work could reasonably be expected to be infected with *T. theileri*. The high specificity of the assay based on samples from this population provides further evidence that such cross-reactivity does not occur.

In summary, the diagnostic sensitivities of the current FAO/IAEA Ag-ELISA's for *T. congolense* and *T. vivax* are inadequate for these tests to be useful in sero-epidemiological investigations of bovine trypanosomosis. In contrast, the studies described here have shown that under optimised conditions, good diagnostic sensitivity is possible using the phase-contrast buffy-coat technique. Moreover, data obtained using an antibody-detection ELISA for this disease suggest that excellent sensitivity and specificity may be obtained using this technique. Although the detection of antibodies is not appropriate for individual animal diagnosis, Ab-ELISA has been adopted in a number of southern-African (RTTCP) countries, notably Zambia and Zimbabwe, as a tool for characterising areas in terms of trypanosomosis risk and for prioritisation of control activities.

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CONTROL OF *TRYPANOSOMA EVANSI* IN BUFFALO IN INDONESIA: IDENTIFICATION OF INFECTED ANIMALS BY AG- AND AB-ELISA TESTS AND TREATMENT WITH DIMINAZENE ACETURATE

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Abstract

CONTROL OF *TRYPANOSOMA EVANSI* IN BUFFALO IN INDONESIA: IDENTIFICATION OF INFECTED ANIMALS BY AG- AND AB-ELISA TESTS AND TREATMENT WITH DIMINAZENE ACETURATE.

In order to control effectively infections with *Trypanosoma evansi* it is necessary to identify all infected individuals, including not only animals with patent infections but also those with non-patent infections. In order to provide a reliable means of diagnosing infected individuals, a combination of Ab-ELISA, Ag-ELISA and parasitological tests were used to identify for treatment groups of buffalo from Central Java, Indonesia in an area in which trypanosomosis caused by *T. evansi* is endemic. The animals were divided into four groups, Group A comprised animals positive by both Ab- and Ag-ELISA and/or parasitological examination. Group B consisted of animals positive by Ag-ELISA and Group C by Ab-ELISA only. Group D was a control group of animals that were negative by both serological and parasitological tests. All of Group A were treated with diminazene aceturate and the prevalence and incidence of infection in the four groups determined over approximately 12 months. The prevalence of infection declined in Group A declined from 100% to <25% in four months and remained at this low level until the end of the study. In the other groups, the serological prevalence increased up to 50%, although in Group D, the initially uninfected group, the increase was lower. Although treatment was effective in reducing the prevalence of infection, the cumulative incidence in the four groups was found to be similar.

1. INTRODUCTION

Trypanosomosis ("surra") caused by *Trypanosoma evansi* occurs as an endemic disease causing production losses in Indonesia, India, Malaysia, The Philippines, Vietnam, Thailand and China [1, 2, 3, 4]. In Indonesia it has been ranked for many years as a livestock disease of economic and strategic importance and cases of trypanosomosis are reported throughout the islands of the Indonesian archipelago [5]. Serious epidemic outbreaks of disease occur from time to time [6] but infection is seen usually as a chronic disease that causes abortion, decrease in growth rates and can adversely affect work output [7, 8, 9, 10, 11, 12]. Indeed, in Indonesia, and in other areas, production parameters such as weight loss or decreased milk yield are probably of greater importance than mortality, but this needs to be tested. If effective control of *T. evansi* is to be achieved, it is essential that reliable means are available for diagnosing and treating infections. Presently, control of trypanosomosis is still reliant on treatment of infected animals identified by parasitological tests or, more commonly, by clinical signs, in response to serious disease epidemics or to infections occurring in well managed farms. The absence of a wildlife reservoir and the non-cyclical mode of transmission should favor the use of control based solely on the use of chemotherapy, nevertheless, *T. evansi* persists as an endemic disease in many countries [1, 2, 12]. This suggests that the method of diagnosis is not sufficiently reliable to identify infected animals that harbor non-patent infections and thereby remain as a source of infection to susceptible livestock. In the following study, a combination of parasitological and serological tests were used to identify infected animals and to form a basis for trypanocidal drug treatment to determine how effective this approach was in controlling infection.

2. MATERIALS AND METHODS

2.1. Location of control site

Five villages in Pemalang District, Central Java, namely Surajaya, Kejambon, Penggarit, Peguyangan and Banjarmulya were selected for the study. The prospective longitudinal survey commenced in September 1996 and finished in December 1997.

and the conjugate, diluted in PBST–0.5%, was added to each well. The plate was placed on the incubator/shaker for 30 minutes and then washed again. Freshly prepared TMB was added to each well and the plate was incubated for 10 minutes at 37°C; the reaction was stopped by the addition to each well of 50µl 2M sulphuric acid. The OD's were read with a 450 nm filter using a Titertek Multiskan ELISA plate reader. The specificity and sensitivity of this test was 75% and 71% respectively [13].

2.4. Trypanocidal drug treatment

The animals comprising Group A were treated with diminazene aceturate commencing in November. All buffalo were treated at a dose rate of 7 mg/kg body weight. Follow-up visits were made over the next 12 months in December, March, June, October and December. At each visit, sera were collected and parasitological examination carried out. Any animal from Group A that showed evidence of re-infection, or persistent infection, when compared to its previous status, was treated again with diminazene aceturate at the same dose rate.

3. RESULTS

An animal was scored infected if it was positive to either: (1) direct visualization of parasites, or (2) serology (i.e., parallel interpretation). Serological positivity was defined serially (at 20%PP) with the Ab-ELISA conducted first, followed by the Ag-ELISA. The period of observation extended over 16 months, commencing in September, at the end of the dry season, and through the wet season in December to March. The prevalence of infection in the different groups of buffalo is shown in Figs 1, 2, 3 and 4.

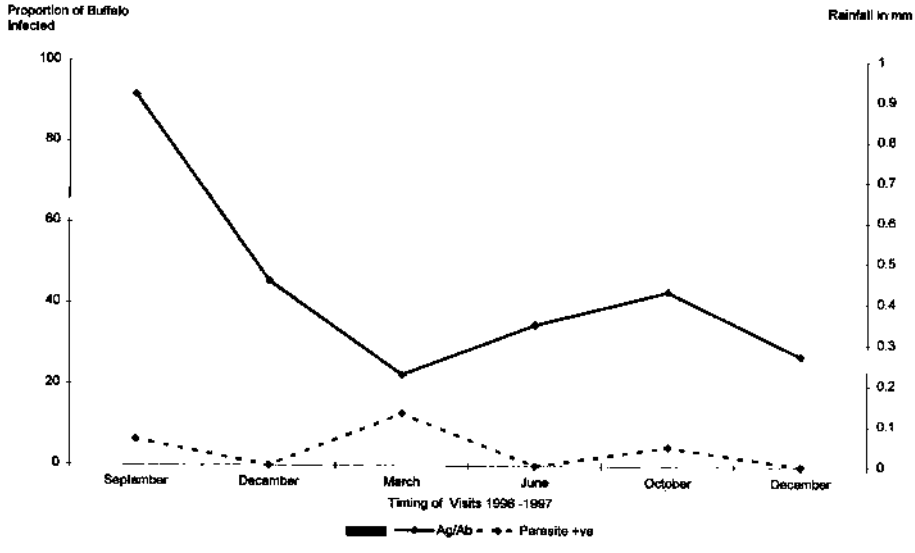

FIG. 1. Point prevalence of infections with Trypanosoma evansi *in village buffalo in Group A, originally positive by Ab- and Ag-ELISA or by parasitological tests, and treated with diminazene aceturate.*

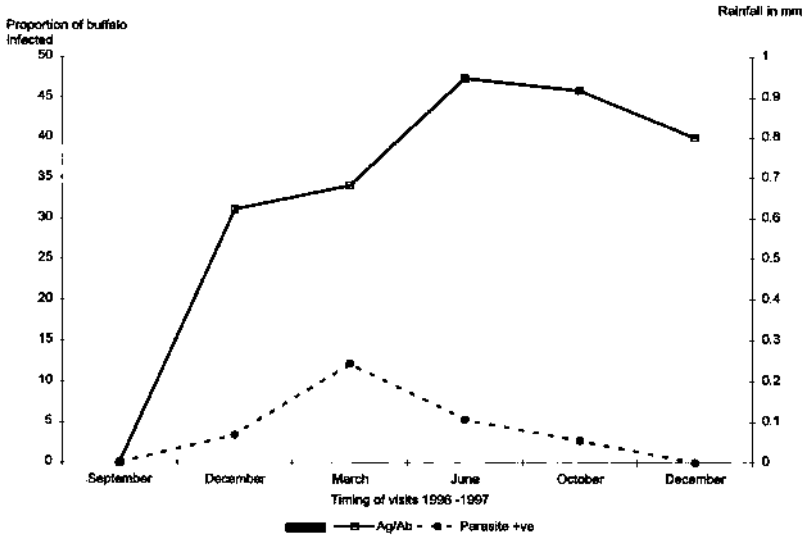

FIG. 2. Point prevalence of infections with Trypanosoma evansi *in village buffalo in Group B, originally positive by Ag-ELISA only.*

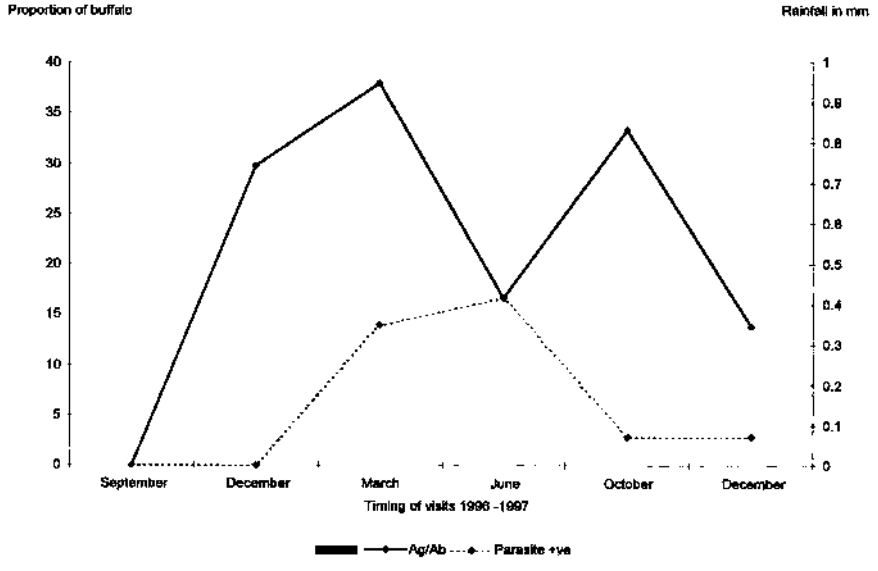

FIG. 3. Point prevalence of infections with Trypanosoma evansi *in village buffalo in Group C originally positive by Ab-ELISA only.*

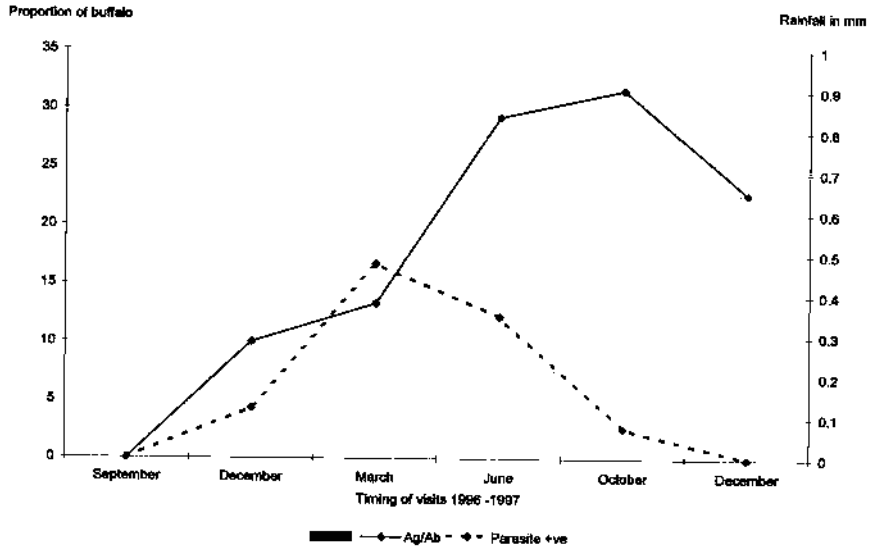

FIG. 4. Point prevalence of infections with Trypanosoma evansi *in village buffalo in Group D originally negative by both Ab- and Ag-ELISA and parasitological tests.*

3.1. Parasitological findings

The proportion of parasite positive animals in Group D increased to nearly 5% by December, peaking at over 10% in March, thereafter falling, so that by the following December there was no evidence of infection parasitologically. The proportion of parasite positive infected animals in Groups B and C increased to 10% by March and, in the case of Group C, there was further increase in June. Thereafter, the proportion of infections in both groups decreased through to the following December. In the diminazene-treated buffalo from Group A, no parasite positive animals were detected in December, three months after treatment, but new infections were detected four months later in March, and again, seven months later, in October. No patent infections were detected at the end of the study in December.

3.2. Serological findings

Buffalo in Group D, that were considered uninfected at the start of the observation period, became serologically positive by September and the proportion infected increased progressively thereafter to reach approximately 30% by October the following year. Thereafter, the proportion declined to 20% by the end of the study (Fig. 1). The major increase in infection occurred after the end of the rains in March. In Groups B and C, the serological prevalence of infection increased markedly during December and March to over 30%. In Group B, in which buffalo were positive by Ag-ELISA at the start of the observation period, this rise continued until over 50% of the animals were serologically positive. This high level was maintained until the following December. In Group C, after the initial rise, the proportion of infected animals decreased in June, and by the end of the study period, only 20% of the buffalo were positive by Ab- and Ag-ELISA. In the buffalo in Group A that had been treated with trypanocidal drug, the proportion of serologically positive animals decreased from over 95% in September to <25% by March. This was followed by a slight increase to >30% by October, but had decreased to <25% at the conclusion of the experiment.

Although there was a difference in prevalence rates between the treated and the untreated groups, there was no evidence that the cumulative incidence of infection was reduced by treatment (see Table I). The cumulative incidence varied between 0.68 and 0.77 and there was no statistically significant difference by χ^2 test between the values for the various groups ($p = 0.83$).

TABLE I. CUMULATIVE INCIDENCE OF INFECTIONS WITH *TRYPANOSOMA EVANSI* IN FOUR GROUPS OF BUFFALO FROM CENTRAL JAVA, INDONESIA

Buffalo	Period of observation (weeks)	Numbers positive	Point estimate of true cumulative incidence
Group A	64	24	0.77
Group B	64	32	0.75
Group C	64	33	0.68
Group D	64	40	0.76

Group A: Initially infected; treated with trypanocidal drug diminazene aceturate
Group B: Initially uninfected; Ag-ELISA positive only
Group C: Initially uninfected; Ab-ELISA positive only
Group D: Initially uninfected; negative by both parasitological and serological tests

4. DISCUSSION

Since there are few drugs available for the treatment of *T. evansi* and it is essential that reliable and effective strategies are employed in their application and that due attention is paid to their cost-effectiveness [14]. For control of *T. evansi* it is usual to treat those animals that are showing patent infection or are considered to be infected on the basis of the unreliable clinical signs [12]. Where treatment has been applied in response to major disease outbreaks even these criteria may be shelved in a situation where widespread use of drugs is indicated to protect a susceptible population. The effectiveness of the strategy employed is rarely called into question since there is often no surveillance following control so it is not possible to assess the overall impact or sustainability. Where the treatment is directed at proven infected or clinically affected animals, it fails to address the underlying problem of carrier animals. The low sensitivity of parasitological techniques and the unreliability of clinical signs ensure that this approach cannot decrease the number of cases as effectively as would treating all infected individuals. If wildlife reservoirs are of no importance, and if small ruminants and dogs present little or no problem, then it should be possible to effect control based solely on the use of chemotherapy [1, 2, 12]. In applying this hypothesis to the control of *T. evansi* in Central Java it was assumed that there are no wild animal reservoirs of infection since the area is intensively cultivated and that sheep, goats and dogs were unlikely to contribute significantly to the epidemiology of disease. Finally, it was assumed that conventional control fails to address the underlying problem of carrier animals because treatment of animals with overt evidence of infection or disease will not diminish the actual number of cases as effectively as treatment of all infected individuals. It is beneficial to the individual but of little relevance to the larger number of animals with non-patent infections whose presence contributes more significantly to the overall morbidity and provides a constant source of infection. Parasitological tests alone may fail to detect 50–70% of animals with active infections [14] hence the use of sensitive serological tests to assist in the diagnosis of infection with *T. evansi* has been advocated by a number of authors [15, 16]. By combining both parasitological and serological tests the predictive value of the diagnostic strategy would be increased and a greater proportion of infected animals would be targeted for treatment [17].

Treatment with trypanocidal drug reduced the proportion of buffalo infected with *T. evansi*, whereas in contrast, in the untreated groups, there was a rise in the proportion of infected animals. In Groups B and C, the increase in serologically positive animals (i.e., positive by both Ab- and Ag-ELISA) was greater than in Group D, reaching 30% within three months of the start of the study. In Group D the rise was slower, not reaching such a level until 10 months later. This was probably due to the fact that animals in Group D comprised animals that did not harbor infection. In contrast, in Groups B and C, although buffalo were not positive by both ELISA tests, the fact that they did already show the presence of antigen or antibody and it is likely that a proportion of the animals indeed infected. The incidence of infection was found to be similar in all four groups, so that although the treatment had reduced the prevalence of infection in Group A, they were still being exposed to new infection. There was a considerable reservoir of infection to enable this to happen – the overall prevalence of infection in the animals included in the study was 15% by parasitological test and over 40% by serological tests. Since the treatment protocol targeted only about 25% of the total buffalo population in the villages, if infections were similar in the remaining animals a considerable

proportion of un-treated, infected buffalo were likely to serve as reservoir hosts, harboring infections. This finding supports the conclusion that in order to control *T. evansi* it will be necessary to treat all infected animals within the population.

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THE USE OF PCR IN THE DIAGNOSIS AND EPIDEMIOLOGY OF ANIMAL TRYPANOSOMOSIS

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Abstract

THE USE OF PCR IN THE DIAGNOSIS AND EPIDEMIOLOGY OF ANIMAL TRYPANOSOMOSIS.

The pathogenic trypanosomes of cattle in Africa are identified by the traditional parasitological techniques as *Trypanosoma brucei, T. evansi, T. equiperdum, T. congolense, T. simiae* and *T. vivax*. The species *T. brucei, T. evansi* and *T. equiperdum* on the one hand and *T. congolense* and *T. simiae* on the other hand are not distinguishable by their morphology in a thin film. These trypanosomes are distinguished by their characteristic pathogenicity and epidemiology. New tools developed by molecular biologists now make it possible to characterise these parasites both in the vectors and the hosts. The polymerase chain reaction (PCR) makes possible the separation of *T. congolense* from *T. simiae* and the characterisation of five different 'taxa' within the species *T. congolense*. Epidemiological studies undertaken in Burkina Faso, combining the characterisation of the parasites in cattle and tsetse flies, the identification of the origin of the blood meals in the vectors and the precise location of collection, give a more complete image of the transmission of these parasites. These studies are essential for the design of more effective methods of control.

1. INTRODUCTION

The trypanosomoses are a major constraint to the development of livestock keeping in many areas. In sub-Saharan Africa for example, in addition to the enormous direct losses, more than 20 million American dollars are spent per annum on trypanocidal drugs, accounting for 44% of the total expenditure in veterinary drugs [1]. The animal trypanosomoses, transmitted by vectors or sexually in the case of dourine, have a cosmopolitan distribution. In the vertebrate hosts, microscopic examination makes it possible to recognize the major groups (sub-genera) of trypanosomes thanks to their morphology. One can thus easily distinguish *T. brucei* s.l. (*Trypanozoon* sub-genus) from *T. vivax* (*Duttonella* sub-genus) and from *T. congolense* (*Nannomonas* sub-genus). In tsetse flies in Africa, morphology is of no help in identifying the trypanosomes. The different trypanosomes take similar forms during their cycle in the vector. Classically, only the precise localization of the parasites in the insect allows an identification. The three sub-genera quoted above present different cycles in the tsetse fly. If the parasites are discovered only in the proboscis of the insect, the trypanosomes are known to belong to the *Duttonella* sub-genus; if they are discovered in the midgut and the proboscis, they are known to belong to the *Nannomonas* sub-genus; and if in addition they are discovered in salivary glands, they are known to belong to the *Trypanozoon* sub-genus. When the midgut alone is infected, one cannot draw a definitive conclusion and an immature infection is reported [2]. The use of molecular biological tools, and in particular the Polymerase Chain Reaction (PCR), introduced an exceptional sensitivity and especially the possibility of characterization at the specific or infraspecific level, which had been impossible previously. This paper reports the results achieved recently using the molecular tools to clarify the epidemiology of the animal trypanosomoses in West Africa.

2. MATERIALS AND METHODS

2.1. Experimental infections

General tsetse flies were given their initial bloodmeal using infected rabbits or mice. The infecting meal was a single one and lasted approximately 10 minutes. Subsequently, the engorged flies were separated from the non engorged ones, which were eliminated. The selected tsetse flies were fed 5 days out of 7 on a healthy rabbit. When the cycle of the trypanosome was completed (14 days on average for *T. congolense*, 10 days for *T. vivax*), the tsetse flies were killed and dissected according to a classical protocol.

2.2. Dissection of the tsetse flies

The tsetse flies from the Cirad-IRD insectarium in Montpellier or the ones captured in the field in Africa (studies undertaken at CIRDES, Bobo-Dioulasso), were dissected by separating the various organs. The instruments of dissection were cleaned between each organ and each tsetse fly (bleach followed by distilled water) to avoid contaminations. Following observation using a phase contrast microscope to locate and index the infections, the organs were suspended separately in Eppendorf tubes containing 50µl sterile distilled water, and frozen at –20° C until laboratory analysis by PCR.

2.3. Blood samples from cattle

Blood samples were taken from the jugular vein. The most frequently used parasitological technique is the haematocrit centrifugation technique [3] which provides the value of the packed red cell volume (PCV) and allows at the same time the examination of the buffy coat under the microscope. PCR was applied directly to the buffy coat suspended in distilled water. The concentration of the trypanosomes in the buffy coat increases the sensitivity of detection and eliminates most of the red blood cells, thus limiting the risks of inhibition of Taq polymerase. In order to eliminate any residual inhibiting effects, the suspension was purified by using a chelating resine.

2.4. PCR

Application of PCR on the mouthparts and salivary glands of the insect did not require preliminary purification of the samples. The organs were simply suspended in sterile distilled water, a fraction of which was used for PCR. The midgut suspensions of tsetse flies were like the bovine blood samples, treated with a purification kit containing a resine. The primers used for amplification are indicated in Table I.

TABLE I. PRIMERS USED FOR PCR AND THE SIZE OF THE FINAL PRODUCT

Species/taxa	Sequences	Size of products (bp)	References
T. congolense "savannah"	ILO344:5'CGAGCGAGAACGGGCAC 3' ILO345:5'GGGACAAACAAATCCCGC3'	320	[4]
T. congolense "Kilifi"	TCK1:5'GTGCCCAAATTTGAAGTGAT3' TCK2:5'ACTCAAAATCGTGCACCTCG3'	294	[5]
T. congolense "forest"	TCF1:5'GGACACGCCAGAAGGTACTT3' TCF2:5'GTTCTCGCACCAAATCCAAC3'	350	[5]
T. congolense "Tsavo"	ILO892:5'CGAGCATGCAGGATGGCCG3' ILO893:5'GTCCTGCCACCGAGTATGC3'	400	[6]
T. simiae	TSM1:5'CGGTCAAAAACGCATT3' TSM2:5'AGTCGCCCGGAGTCGAT3'	437	[5]
T. brucei	TBR1:5'GAATATTAAACAATGCGCAG3' TBR2:5'CCATTTATTAGCTTTGTTGC3'	177	[7]
T. vivax West Africa	TVW1:5'CTGAGTGCTCCATGTGCCAC3' TVW2:5'CCACCAGAACACCAACCTGA3'	150	[5]

2.5. Geographical information system

A geographical information system (GIS) was set up for the agropastoral zone of Sideradougou, Burkina Faso [8]. It combined investigations in the field with high resolution satellite data (SPOT images). The GIS allowed the combination of various layers of georeferenced information relating to entomology (3600 tsetse flies captured, 1 trap placed every 100 m over 120 km of hydrographic network), parasitology (diagnostic microscopy and PCR), the cattle (distribution, census, age structure, sentinel animals), the natural environment (70 parameters characterizing mainly vegetation, topography, soil use and its dynamics) and pastoralist practices (type of producers, sedentarisation, points of watering). The integration of all these layers of information permitted a classification of the biotopes favorable to tsetse flies.

3. RESULTS

3.1. Vectorial capacity of the tsetse flies

Four species or subspecies of tsetse flies were infected in experiments using clones of *T. congolense* identified by PCR [9]. The Dinderesso/80/CRTA/3 clone belonged to the type 'West African riverine forest' and the clone E 325 to the type 'savannah'. By studying the infection rate of the midgut, the labrum and the hypopharynx of *Glossina morsitans morsitans*, *G. m. submorsitans*, *G. palpalis gambiensis* and *G. tachinoides*, the Intrinsic Vectorial Capacity (IVC) [10] of each one of these species could be compared for each clone.

The results show that *G. m. submorsitans* is an excellent vector of *T. congolense* 'savannah type' (IVC = 0.809) followed by *G. m. morsitans* (IVC = 0.411), *G. tachinoides* (IVC = 0.204) and *G. p. gambiensis* (IVC = 0.082) in descending order. For *T. congolense* 'forest type', the IVC calculated are considerably lower: 0.125 for *G. m. s.*, 0.047 for *G. t.*, 0.040 for *G. p. g.* and 0.027 for *G. m. m.*

3.2. Comparison between detection of parasites by microscopy and PCR

(1) In a study of 139 *G. longipalpis* captured in the field in Côte d'Ivoire [11], 50 tsetse flies were found to be infected and were subjected to the PCR. The results are summarized in Table II.

TABLE II. DETECTION OF PARASITES IN TSETSE FLIES FROM CÔTE D'IVOIRE BY MICROSCOPY AND CHARACTERISATION BY PCR

		PCR			
Parasite	Microscopy	*Trypanozoon*	*Nannomonas*	*Duttonella*	Negative
Trypanozoon	1	0	1	0	0
Nannomonas	20	0	16	1	3
Duttonella	17	0	3	3	11
Immature	12	0	3	0	9

PCR = polymerase chain reaction

(2) In a study of 298 infected tsetse flies (177 *G. tachinoides* and 121 *G. p. gambiensis*) captured in the agropastoral area of Sideradougou [12], the following observations were made (Table III).

TABLE III. DETECTION OF PARASITES IN TSETSE FLIES FROM SIDERADOUGOU BY MICROSCOPY AND CHARACTERISATION BY PCR

		PCR (in %)			
Parasite	Microscopy	*Trypanozoon*	*Nannomonas*	*Duttonella*	Negative
Nannomonas	33	3	54.5*	18.2	27.3
Duttonella	107	0	3	86	11
Immature	158	4	14.5	0	84.2

* with 21.2% mixed infections; PCR = polymerase chain reaction.

(3) In the game reserve of Nazinga in the east of Burkina Faso, 166 infected tsetse flies (110 *G. m. submorsitans* and 56 *G. tachinoides)* were studied. The results are summarized in Table IV [13].

TABLE IV. DETECTION OF PARASITES IN TSETSE FLIES FROM NAZINGA BY MICROSCOPY AND CHARACTERISATION BY PCR

		PCR (in %)			
Parasite	Microscopy	*Trypanozoon*	*Nannomonas*	*Duttonella*	Negative
Nannomonas	40	0	62.5*	17.5	20
Duttonella	54	0	18.5	13	68.5
Immature	72	2.8	31.9	4.2	66.7

* with 7.5% mixed infections; PCR = polymerase chain reaction

We also noticed during this latter study that the PCR gave 9.7% of positive results for proboscides negative in microscopy, 14.8% of positive results for microscopically negative midguts and 1 positive infection of salivary glands for the 166 microscopically negative ones.

3.3. Comparison of the PCR results from cattle and tsetse fly samples

During an epidemiological investigation carried out in the pastoral zone of Yalé in the east of Burkina Faso *G. tachinoides* was recognized as the principal vector of cattle trypanosomosis [14]. The taxonomic composition of the midgut infections was characterized by PCR for 50 infected tsetse flies. The results are compared with the analysis by PCR of 44 blood samples collected from cattle infected in the same zone (Table V). No infections with *T. brucei* were observed during this study.

TABLE V. COMPARISON OF PCR RESULTS FROM TSETSE FLIES AND BOVINE BLOOD SAMPLES COLLECTED IN THE AGROPASTORAL ZONE OF YALÉ

	PCR (in %)	
	G. tachinoides (n = 50)	Bovine samples (n = 44)
T. congolense 'savannah'	46	52**
T. congolense 'forest'	8	
T. simiae	24	
T. congolense 'savannah' + 'forest'	8	
T. vivax	6	16
T. congolense 'savannah' + *T. simiae*	4	
T. simiae + *T. vivax*	4	
T. congolense unidentified*		9
T. vivax unidentified *		23

* = not caracterized with the available primers; ** = in association with an unidentified *T. vivax* on one occasion; PCR = polymerase chain reaction.

3.4. GIS

Analysis by GIS in the area of Sideradougou revealed the major discriminating factors influencing the distribution and the densities of *G. tachinoides* and *G. palpalis gambiensis* along the principal hydrographic network [8]. The maps of current distribution were compared with those obtained fifteen years earlier. The impact of the human activity resulted in either an increase, reduction or disappearance of these riverine tsetse flies. Some factors, in particular edaphic, can limit the agricultural expansion near the forest galleries, and support the maintenance or the increase in the populations of vectors (preserved biotopes). This observation was particularly interesting, since it goes against the generally held opinion, that tsetse flies rarely venture in zones, which are under high human population pressure. The high resolution satellite images allowed the classification of natural and human spaces, directed primarily towards the landscapes of the valleys. Certain units of landscapes appeared definitely more favorable to the tsetse flies. These results need to be validated in comparable bioclimatic zones. The factor 'tsetse fly density' is a major element for the evaluation of the risk of parasite transmission. However, it seems that the vectors do not present the same danger everywhere and the molecular biology analyses showed that the tsetse flies do not harbour always the same trypanosomes (pathogenic/non pathogenic species) depending on their geographical position along the hydrographic network. The transmission of trypanosomes to cattle depends primarily on the interface between vector and host. In particular, the interface is influenced by livestock husbandry systems and animal movements.

4. DISCUSSION

Traditional parasitology recognizes 6 different species of pathogenic trypanosomes for man or cattle in Africa. Microscopic examination allows the morphological differentiation of *T. brucei*, *T. congolense* and *T. vivax*. Nosological and epidemiological factors further allow the separation of *T. brucei brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. evansi* and *T. equiperdum* within the group *'brucei'*, and *T. congolense* and *T. simiae* within the group *'congolense'*. These distinctions can only

be made in samples from the vertebrate host. The tools provided by molecular biology now make it possible to consider a precise characterization of the parasites both in the vector and the vertebrate host. PCR distinguishes within the group *'congolense'* 6 different entities: *T. congolense* 'savannah form', *T. congolense* 'West African riverine forest', *T. congolense* 'Kilifi form', *T. congolense* 'Tsavo form', *T. simiae* and finally *T. godfreyi* (not considered in the present study). The taxonomic place of these forms is not yet well defined, in a group of parasites where the importance of sexual reproduction relative to clonal division in the field is not known. However, biological observations can be made to compare some of these forms. For example the intrinsic vectorial capacity of *G. morsitans submorsitans* is 0.809 for *T. congolense* 'savannah form' whereas it is 0.125 for *T. congolense* 'forest form'. In the same way, studies are in progress concerning the differences in pathogenicity of the different forms (Z. Bengaly, personal communication). The possibility of characterizing the parasites by PCR enabled us to compare, in different epidemiological situations, the results of traditional parasitology with those of the PCR performed on infected flies. In the case of the study on *G. longipalpis* in Côte d'Ivoire, 34% of infections with *T. vivax* (s.g. *Duttonella*) had been identified in the field. PCR confirmed only 6% of them, for which it is necessary to add 2% identified as *Nannomonas* in the field; 22% correspond to trypanosomes having the morphology of *T. vivax* but not recognized by the available primers; 6% finally are identified as *Nannomonas*. In the field 40% of the infections had been attributed to the *Nannomonas* sub-genus; PCR recognized 46% of them. Finally, the infection of a salivary gland by *Trypanozoon* observed in the field appeared to be an infection with *Nannomonas*, thus confirming the possibility of contamination of the various organs during the traditional dissection method.

In general, traditional parasitology seems to overestimate the infection rate with *Duttonella* and to underestimate the infection rate with *Nannomonas*, compared to the PCR method. Furthermore, the results showed that 46% of the infections were not identified by the PCR primers available, including 22% of trypanosomes that one could qualify as '*T. vivax*-like'.

Similar studies were carried out in the pastoral zone of Sideradougou where domestic cattle are most abundant, and in the wildlife reserve of Nazinga. In Sideradougou, 86% of the infections with *Duttonella* were confirmed by PCR, thus showing the importance of infections with *T. vivax* in domestic cattle. In Nazinga, only 13% of the infections with *Duttonella* were confirmed by PCR; 68.5% were negative and corresponded to *T. vivax*-like trypanosomes in the wild fauna. The studies highlighted the importance of mixed infections, explaining the totals higher than 100% in Tables II and III. In the same way the high sensitivity and specificity of the PCR were confirmed.

In the pastoral zone of Yalé, the different trypanosomes identified by PCR could be compared between cattle and tsetse flies. The majority of trypanosomes identifiable by PCR were found in the principal vector, *G. tachinoides*. *T. congolense* 'savannah form' with 46% and *T. simiae* with 24% were the most abundant parasites detected. In cattle, only *T. congolense* 'savannah form' (52%) and *T. vivax* (16%) were identified. The absence of *T. simiae* and *T. congolense* 'forest form' in cattle is noteworthy.

The development of a GIS for the area of Sideradougou allowed us a global vision of the disease by including not only the traditional interaction 'parasite-vector-host', but also the assessment of environmental and sociological factors, thus, providing a better understanding of the complex epidemiology of the disease.

5. CONCLUSION

The application of molecular tools showed that *T. congolense*, and undoubtedly the other 'classical species' of pathogenic trypanosomes, actually consisted of complexes of species. The use of these tools in the field in already known epidemiological situations allowed a better understanding of the complexity of the pathogenic system 'parasite-vector-host'. A new epidemiological interpretation of the trypanosomoses is thus emerging. The integration of these new data with all the environmental and socio-economic parameters in a geographical information system will lead to a better definition of both the epidemiologically dangerous zones, and of the priority zones where the available funds should be focused to effectively control the disease.

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TRYPANOSOMOSIS DUE TO *TRYPANOSOMA VIVAX* IN RUMINANTS IN LATIN AMERICA: A REVIEW

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Abstract

TRYPANOSOMOSIS DUE TO *TRYPANOSOMA VIVAX* IN RUMINANTS IN LATIN AMERICA: A REVIEW.

The history and the present situation of *T. vivax* infections in Latin America are reviewed. Clinical signs, diagnostic aspects, therapy and control of the disease are briefly discussed. In view of the recent emergence of bovine trypanosomosis in areas where it previously never existed, it is advisable to invest in improving the diagnosis and control of the disease in Latin America.

1. INTRODUCTION

Trypanosomosis is a disease present in 36 African countries affecting humans and animals alike. The disease hampers animal production in an area of roughly 10 million km^2 of the African continent. Various trypanosome species cause disease in domesticated ruminants, namely *Trypanosoma vivax*, *T. congolense* and to a lesser extent *T. brucei* and *T. evansi*. Similarly, horses can be affected by these trypanosomes in particular by *T. evansi*, the causative organism of surra and by *T. equiperdum*, the causative organism of dourine. Another trypanosome species, *T. simiae*, can cause fulminating disease in pigs. The most important vector of trypanosomes affecting ruminants in Africa is the tsetse fly, *Glossina* spp., in which the parasite undergoes a cyclical development. Consequently, to prevent the spread of the disease from one animal to another, one can either control or eradicate the tsetse flies. Control has been effective in some places using insecticides, traps and targets, but ineffective in other places due to civil unrest, shortage of funds or logistical difficulties. Eradication has mostly failed due to the geographical extent of the problem, reinvasion of cleared areas and the large number of tsetse species (23) capable of transmitting the disease. Unfortunately, no vaccine is available, but animals can be successfully cured from the disease by using (a limited number of) trypanocidal drugs. Mass treatment of susceptible hosts using curative or prophylactic drugs will also prevent the spread of disease. Trypanosomes can also be transmitted mechanically through the mouthparts of haematophagous biting flies, which is the mode of transmission for *T. evansi* and the only possibility of disease transmission in Latin America, since tsetse flies do not occur on that continent.

2. HISTORY IN LATIN AMERICA

Curasson [1] has suggested that in 1830 the disease was introduced into Latin America by a shipment of Zebu cattle imported from Senegal. Whatever the cause of introduction, *T. vivax* was first described in French Guyana in 1919, when Leger and Vienne described an epizootic in dairy cattle with a mortality of more than 52% [2]. They named the parasite *T. guyanense*, but subsequently, it was found to be identical with *T. vivax* [3]. The parasite was soon detected in many other countries in Latin America. Plata [4] identified *T. vivax* on morphological and biometrical grounds in blood samples collected from cattle in Columbia, where the disease was reported to be widespread along the Atlantic coast, more commonly occurring in the dry season of the year and in lowland areas of the country [5]. The disease was also reported from Venezuela [6], Panama [7], Surinam [8] and from two islands in the lesser Antilles, namely Guadeloupe [9] and Martinique [10]. In Colombia, Virviescas [11] estimated that the situation was rather acute and that in the previous two years at least 12,000 head of cattle had died of trypanosomosis in three Departments of the Costa Atlántica. It was predicted that the disease would spread to other areas of Colombia as indeed it did during the

following years [12]. In 1972, Shaw and Lainson [13] described *T. vivax* isolated from water buffalo in Belém, Pará State, Brazil. The same authors mentioned that local Animal Health Department records did mention the finding of *T. vivax* in Brazilian cattle back in 1946. Romero *et al.* [14] described a single case in a young steer in Paraguay. In a serological survey using the indirect fluorescent antibody test, antibodies against *T. vivax* were detected in serum samples originating from El Salvador, Costa Rica, Colombia, Ecuador, Peru, Brazil and Paraguay [15]. However, in some cases the number of serum samples collected was rather small, for example only 15 samples came from Paraguay (6 were positive). Moreover, the presence of antibodies is of epidemiological importance and indicates previous exposure, but not necessarily a current infection. Nevertheless, the serological survey showed that the possible distribution of *T. vivax* in South America extended from at least 12° N to the Tropic of Capricorn.

3. PRESENT SITUATION IN LATIN AMERICA

Trypanosoma vivax is presently considered endemic in the tropical region of Colombia [16]. Recently outbreaks of trypanosomosis due to *T. vivax* have been reported from the Pantanal region in Brazil [17], from all lowland provinces of Santa Cruz Department and from all provinces of Beni Department in Bolivia [18; S. Angus, personal communication]. Infection rates in selected cattle ranged from 34% (10 out of 29 animals infected) in the Pantanal in 1995 to 86% in Laguna Concepción, Bolivia in 1996. The authors suggest that the spread of the disease from the north (as detected by Shaw and Lainson in 1972) to the South of Brazil has been due to an increase in cattle movement facilitated by extensive road construction linking Cuiabá with Belém [18]. Furthermore, an increase in cattle trading between Brazil and Bolivia in the years 1995 and 1996, due to depressed prices for Brazilian cattle, facilitated the spread of the disease to Bolivia [18].

4. CLINICAL SIGNS

The disease caused by *T. vivax* in adult cattle in South America is accompanied by fever, parasitaemia, suppression of milk yields, abortion and occasional deaths [19]. Anaemia, lethargy and loss of condition lead to the characteristic "fly-struck" appearance. Camus and Martrenchar [20] described a rapid weight loss of 10-17 kg in one month as compared to the controls during an experimental infection of Zebu cattle in French Guyana. Moreover, Otte *et al.* [21] showed that in an endemic situation the disease can become subclinical, but still produce measurable production losses. Perinatal infection has been reported in Venezuela [22] and under experimental conditions [23], but field observations from Africa indicate that the transplacental route is an abnormal method of transmission [24] and, therefore, is unlikely to play an important role in the epidemiology of the disease. Animals that recover from the disease can become reservoirs, showing low and undetectable parasite levels in the blood [25]. Thus, aparasitaemic phases can be part of a *T. vivax* infection in animals and the parasites have been shown to hide in extravascular locations such as the choroid plexus and the aqueous humor of the eye [26]. However, latent infections can be reactiviated due to stress. Consequently, the disease can be quickly spread by a combination of movement of cattle along trade routes and the presence of sufficient numbers of vectors. *Trypanosoma vivax* has not only been detected to be widespread in cattle, but also affects water buffalo [13], sheep [27; 28] capybara [29] and wild deer in South America [30]. Most *T. vivax* stocks are restricted to ruminant hosts and do not infect dog, rabbit, guinea pig or smaller laboratory animals, thus severely hampering experimental investigations [8; 31]. Leeflang *et al.* [32] described a *T. vivax* stock isolated from a Zebu cow in Nigeria that could be maintained in mice. The clinical signs and pathology of *T. vivax* infections in different susceptible hosts has been reviewed previously [33]. The parasite has been reported to infect horses and cause a mild, chronic disease with emaciation, anaemia and oedema of the genitalia and legs [34]. It should be pointed out that horses in South America are frequently infected with *T. evansi*, a distantly related, but different trypanosome. This parasite causes the disease known locally as Mal de Cadeiras, but will not be discussed here.

Fujita *et al.* [35] detected *T. theileri* in 40% of cattle blood samples from Paraguay. Although frequently encountered in cattle all over the world and a potential cause for cross reactions and false positives in serological tests, *T. theileri* is generally considered a non pathogenic parasite [36].

5. TRANSMISSION AND EPIDEMIOLOGY

It appears that *T. vivax* in South America has become well established in the absence of its original vector, the tsetse fly. Although it has been suggested that the parasite could be transmitted cyclically in South America in an unknown vector other than *Glossina* [37], no evidence for this hypothesis has been provided as yet. On the other hand, the parasite is transmitted in Latin America by the bite of bloodsucking flies such as tabanids in which it does not undergo a cyclical development. Mechanical transmission of the parasite from one bovine to another has been successfully achieved with the tabanids *Cryptotylus unicolor* [38] and *Tabanus importunus* [39] in French Guyana and with *Tabanus nebulosus* in Colombia [40]. *Stomoxys calcitrans* might also be a potential vector in South America. It has been pointed out that the level of parasitaemia of the infected donor is probably an important factor influencing the success rate of mechanical transmission [41]. In addition to direct evidence, there is also indirect evidence of mechanical transmission from epidemiological studies in Colombia, which demonstrated a significant temporal relationship between the feeding activity of tabanids and *T. vivax* incidence, and which showed that *T. vivax* infections were associated with low-lying swampy areas, where tabanids breed [21]. It is possible that the South American trypanosome stock (s) may have lost the ability to be transmitted by tsetse [42; 43]. Deer, such as the cariacou (*Odocoïleus gymnotis*) can serve as a wild reservoir of the parasite [29; 30] in Venezuela, but there is no conclusive proof that deer act as a reservoir from which cattle can become infected. Poor nutrition, intercurrent diseases and stress factors are considered as predisposing factors for infection in cattle [44].

6. DIAGNOSIS

Diagnosis of the disease is possible by parasitological, serological or molecular techniques. The parasitological techniques can range from the microscopic examination of a fresh drop of blood to that of Giemsa-stained thin or thick blood smears. In addition, concentration methods such as the micro-haematocrit centrifuge technique [45], or the dark-ground/phase-contrast technique [46] have the advantage of a higher sensitivity, thus detecting lower levels of parasites in the blood. Once detected the parasite is characterised by fast movements in wet smears and by its morphological characteristics in stained smears, such as a rounded posterior end, a free flagellum, a large and terminal kinetoplast, an inconspicuous undulating membrane and a body length of 15-30µ, with short and long forms [47]. Measurements of various South-American *T. vivax* strains showed that they were morphologically closer to the smaller West African forms than to the larger strains from East-Africa [13; 48]. The serological techniques, such as the immune fluorescent antibody test [49] or the enzyme-linked immunosorbent assay [50] have the advantage that a considerably larger number of samples can be examined in a short time period. However, the disadvantage is that cross reactions with other parasites can occur producing false positives. Moreover, these techniques usually detect antibodies, which indicate a past infection, but not necessarily a current infection. An enzyme-linked immunosorbent assay detecting trypanosomal antigens has been developed [51], but produced disappointing results with an isolate of *T. vivax* from South America [52]. However, the serological techniques are very useful for epidemiological investigations to assess the occurrence and distribution of the disease in a country. Other more sophisticated detection techniques have recently become available and have been applied to the study of South American *T. vivax*, such as DNA probes, isoenzyme analysis [53] and polymerase chain reaction (PCR). The DNA hybridisation technique using species-specific DNA probes has improved the accuracy of trypanosome identification [54], but has a lower sensitivity than the PCR method. Isoenzyme electrophoresis is an effective method of parasite characterization and can be useful as part of epidemiological studies of the disease. However, no enzymic division was detected between stocks from Africa and a South American *T. vivax* stock [55]. The PCR method has been evaluated for a South American *T. vivax* stock [56] and gave an excellent result in comparison

with other diagnostic techniques [57]. Under experimental conditions the technique proved consistently more sensitive than parasitological techniques in detecting parasite DNA in dried blood samples [58]. However, the primers used for the PCR do not amplify the DNA of all *Duttonella* stocks circulating in a particular region [59]. Furthermore, the molecular techniques are not suitable yet for wide scale application under field conditions.

7. TREATMENT

Trypanosomosis in cattle can be treated with a number of drugs. The most commonly used is diamidine diminazene aceturate (Berenil®, Ganaseg®), which must be administered intramuscularly at 3.5 mg/kg. As some stocks in Colombia [43] and French Guyana [60] were found to be resistant at the recommended dose, it might be advisable to routinely double the dosage [61]. Another drug is isometamidium chloride (available as Trypamidium® in South America), which has therapeutic as well as prophylactic effects. It must be administered by deep intramuscular injection at 0.5 or 1.0 mg/kg body weight (for the 2% aqueous solution), but can cause strong reactions at the injection site. Under experimental conditions the drug was found to protect cattle against a tsetse transmitted *T. vivax* infection during a period of one to two months depending on the sensitivity of the trypanosome population [62]. To prevent underdosage and, consequently, the induction of resistant parasite strains, it is advisable to weigh animals before treatment. Two other trypanocidal drugs are occasionally available, firstly phenanthridines homidium (Ethidium®, Novidium®) for treating *T. vivax* infections through deep intramuscular injection at 1 mg/kg (careful intravenous injection is the preferred route in horses) and secondly quinoline-pyrimidine quinapyramine (Antrycide®, Trypacide®) used for treating *T. evansi* in horses at 5 mg/kg by subcutaneous injection, but it can produce cross-resistance to the other three trypanocidal drugs when used in cattle [63].

8. CONTROL

Control of the disease must be based on a combination of restriction of movement of (infected) animals, treatment of infected animals, epidemiological monitoring of the distribution and severity of the disease and on vector avoidance and/or control. Wells pointed out that a priority in Latin America is to determine the economic impact of trypanosomosis relative to other diseases and groups of diseases [64]. If the economics are such as to justify it, then further epidemiological studies should be carried out to determine optimal control or eradication strategies. The need for an economic assessment of bovine trypanosomosis is the same today, although it will be through epidemiological studies that economic answers will be provided. To date there have been very few studies to estimate the impact of this disease among livestock in Latin America. In Colombia, trypanosomosis of domestic animals has been ranked third in economic importance of the parasitic diseases, after ticks and tick-borne diseases and distomatosis [65]. Betancourt and Wells [66] estimated losses in an outbreak among dairy cattle in Colombia as averaging US$ 56.5 per animal, at 1976 prices. Otte *et al.* [21] suggested that, on a regional scale, even the inapparent losses of subclinical infestations could be considerable. The financial impact of *Trypanosoma vivax* on the Brazilian Pantanal and Bolivian lowlands was recently assessed [67]. It was estimated that the cost of the 1995 outbreak on seven farms in the Poconé region of the Pantanal was equivalent to approximately US$ 15 per breeding cow (4% of the total value of the animal). If the outbreak had gone untreated the estimated losses would have been nearly US$ 64 per brood cow (or 17% of the total value of the animal). In view of the recent introduction of bovine trypanosomosis to the Pantanal and lowland Bolivia, and the economic losses associated with the disease, it is advisable to invest in improving the diagnosis and control of the disease in Latin America.

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EVALUATION OF ENZYME LINKED IMMUNOSORBENT ASSAYS FOR THE DIAGNOSIS OF BOVINE TRYPANOSOMOSIS IN BOLIVIA

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Abstract

EVALUATION OF ENZYME LINKED IMMUNOSORBENT ASSAYS FOR THE DIAGNOSIS OF BOVINE TRYPANOSOMOSIS IN BOLIVIA.

Four ELISA systems were evaluated as potential diagnostic tools for bovine trypanosomosis in Bolivia. The assays identified antibody positive animals in three of the four areas sampled with the highest rates in animals in the two areas where *Trypanososma vivax* infection had been detected previously by parasitological methods. Further evaluation of these assays is required to establish their ability to differentiate between infections with *T. evansi* and *T. vivax*.

1. INTRODUCTION

Bovine trypanosomosis due to *T. vivax* infection was first described in Bolivia following an outbreak of severe disease in 1995 [1] and was considered to have been introduced via animals imported from the neighbouring Pantanal region of Brazil [2]. Subsequently, *T. vivax* infection was identified from animals in several other areas of Bolivia associated with severe wasting disease and up to 40% mortality. Initial diagnosis was based principally on the detection of trypanosomes in stained blood smears, which has a much lower detection rate compared with other indirect, diagnostic methods such as antibody detection assays. Four antibody-capture ELISA kits developed by IAEA were evaluated as potential tools that could be used by Bolivian laboratories to improve their diagnostic capability for *T. vivax* infection to obtain new information on the epidemiology of *T. vivax* infection. Such information would be an important prerequisite for the development of appropriate control regimes. This paper describes the results of an initial evaluation of four ELISA kits using serum samples from cattle in Bolivia.

2. MATERIALS AND METHODS

LIDIVET staff collected serum samples during routine health screening from 79 cattle located in four areas of the Santa Cruz Department of Bolivia – Cabezas, Concepción, Guaryos and San Ignacio. *T. vivax* infection had been detected previously in cattle in Guaryos and Concepción but the status of the other two areas was unknown although San Ignacio was on the main importation route from Brazil.

Each serum sample was tested at CTVM using four ELISA kits supplied by the Animal Production and Health Section, IAEA, Vienna. Each kit used a different antigen system – *T. congolense* native (TcAGn), *T. congolense* denatured (TcAGd), *T. vivax* native (TcAGn) or *T. vivax* denatured (TvAGd). Assays protocols were all carried out according to the instructions supplied with each kit. The result from each serum sample was expressed as the percentage positive value (PP) of the positive control serum supplied with each kit. Positive: negative discrimination was then based on PP cut-off points as recommended by IAEA.

3. RESULTS

Overall PP values ranging from 4 to 115 were obtained with the 79 serum samples with the two *T. vivax* antigens showing widest range of values (Fig. 1). Broadly similar distribution patterns were seen for each antigen system consisting of a compact cluster of values at the lower end of the range with a much wider spread of values covering the remainder of the range. This pattern was seen most clearly with the results from the two denatured antigen systems. The recommended cut-off values for each antigen system also corresponded to the differentiation point between the two distributions, although this was less marked with the native *T. vivax* antigen than with the other antigen systems.

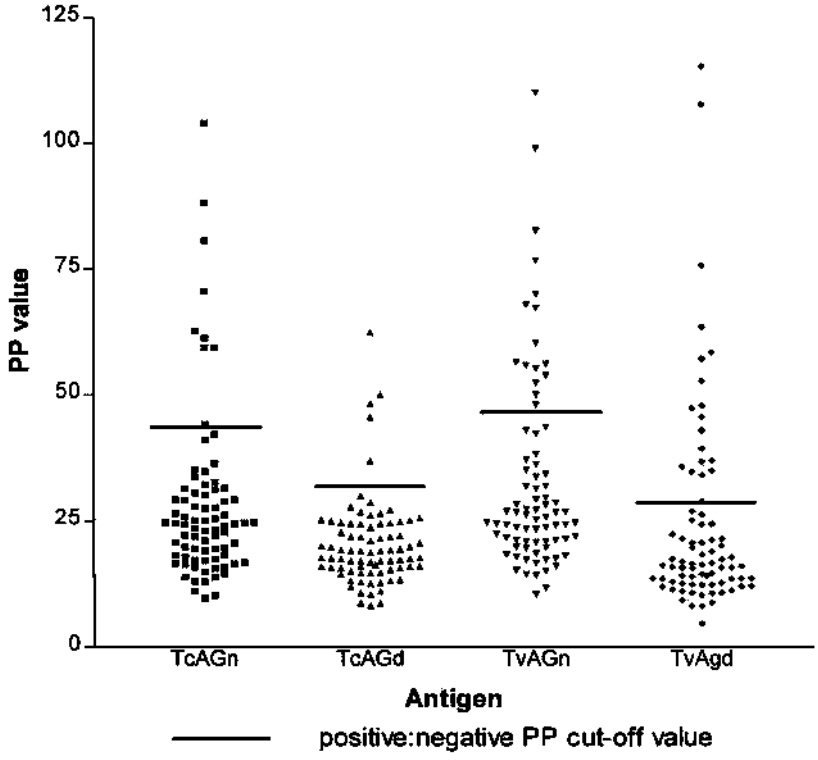

FIG. 1. Distribution of percent positvity (PP) values of serum samples from animals in the Santa Cruz Department.

Antibody positive animals were detected with at least one of the antigen systems in three of the four areas (Fig. 2). Animals from the Concepción and Guarayos areas had the highest proportion of antibody positives to all four antigen systems with Guarayos having the highest proportion of positive animals to 3 of the four antigens. Furthermore, in Concepción and Guarayos the proportion of animals positive to both *T. vivax* antigens was higher than proportion positive to either of the *T. congolense* antigens. No differences were seen between the antibody-positive rates to the two *T. vivax* antigens in Concepción and Guarayos. However, the antibody-positive rate to the native *T. congolense* antigen was higher than that to the denatured *T. congolense* antigen in these two areas.

4. DISCUSSION

Examination of the distribution pattern of PP values from each assay confirmed that the recommended cut-off values obtained with animals in Africa would be suitable for use with cattle from Bolivia as in most cases the cut-off value acted as a separator between two populations. Furthermore, values below the cut-off had a more restricted range than those above the cut-off value. Uninfected animals might be expected to show a more restricted range of values than infected animals, which could be at different stages in the course of infection.

Antibody-positive animals were detected by all four ELISA systems in two of the four sampling areas based on the recommended cut-off values for each assay. None of the animals in the San Ignacio area were antibody positive by any of the assays and only one animal positive by TcAGd in the Cabezas area. Prior to this study, *T. vivax* infected animals had been reported from both the Concepción and Guarayos areas with animals in Guarayos area being most severely affected by *T. vivax* infection. Therefore, the results from the ELISA testing appear to confirm the parasitological

results in that the two *T. vivax* ELISA detected the highest proportion of antibody positive animals in these two areas.

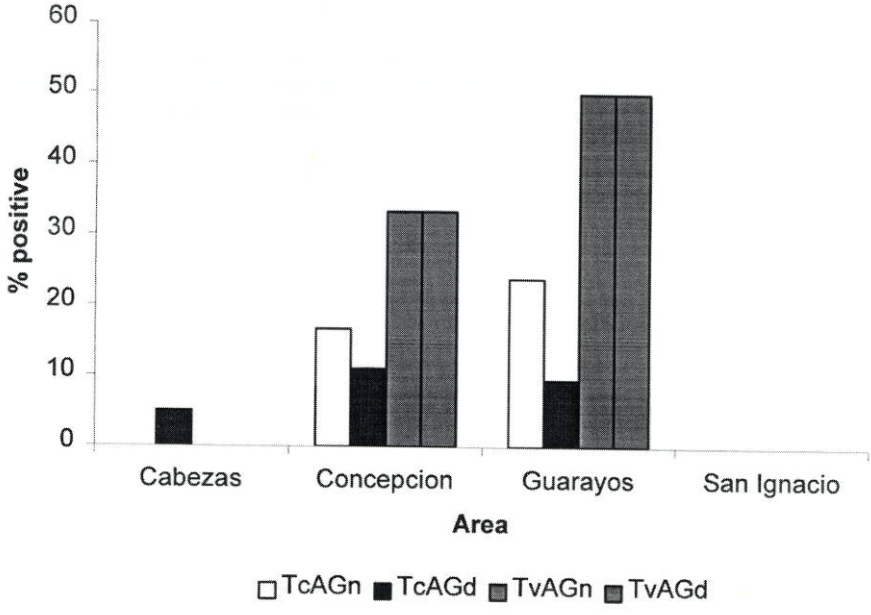

FIG. 2. Antibody positive animals in the Santa Cruz Department.

However, animals positive to the two *T. congolense* ELISAs were also found in these areas that, in the absence of *T. congolense* in S. America, is likely to be due to cross-reactivity with either *T. vivax* or *T. evansi* [3].

Differences were seen between the antibody-positive rates obtained with the two *T. congolense* assay systems. The lower positive rates obtained with the denatured antigen indicates that the denaturing process might reduce cross-reactivity problems encountered with native antigen preparations but does not eliminate them. No difference was seen between the results obtained with the two *T. vivax* antigens which suggests that cross-reactivity between *T. vivax* antigens and *T. evansi* antibodies is not as extensive as between *T. congolense* and other trypanosome species. If so, then the *T. congolense* antigens could be used for the detection of *T. evansi* infections in S. America. No cross-reactivity is considered to occur between Salivarian and Stercorarian trypanosome species and the results obtained in this study are, therefore, likely to be an indication of infection with *T. vivax* and/or *T. evansi*.

Although this study was carried out with a limited number of samples the results suggest that the ELISA systems developed for African bovine trypanosomosis could be used with little if any modification for studies on New World bovine trypanosomosis. However, further studies are needed on the diagnostic specificity of the assays in order to evaluate their ability to distinguish between *T. vivax* and *T. evansi* infections.

ACKNOWLEDGEMENTS

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FIELD VALIDATION OF ANTIBODY ELISA'S FOR DIAGNOSIS OF BOVINE TRYPANOSOMOSIS AND MONITORING THE EFFICACY OF A TSETSE AND TRYPANOSOMOSIS CONTROL CAMPAIGN IN THE AGROPASTORAL ZONE OF YALE, BURKINA FASO

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Abstract

FIELD VALIDATION OF ANTIBODY ELISA'S FOR DIAGNOSIS OF BOVINE TRYPANOSOMOSIS AND MONITORING THE EFFICACY OF A TSETSE AND TRYPANOSOMOSIS CONTROL CAMPAIGN IN THE AGROPASTORAL ZONE OF YALE, BURKINA FASO.

Four trypanosomal antibody-detection-ELISA's systems based on the use of either native or denatured crude lysate antigens prepared from *in-vitro* propagated *T. congolense* bloodstream forms and *in-vitro* propagated *T.* vivax epimastigote forms, have been validated for their diagnostic performance and their utility for monitoring the efficacy of a tsetse and trypanosomosis control program. For that purpose, 600 samples of which 34 were parasite-positive, collected at different intervals in an area where tsetse eradication was implemented, were analysed. In addition, 200 sera from non-infected cattle in tsetse-free-area were also analysed. Except the native *T. vivax* antigen which showed high background and unreliable data, the three other ELISA assays were found acceptable for trypanosomal antibody detection. The cut-off values determined by doubling the mean of the known negative population were slightly lower than those obtained by visual analysis. The sensitivity ranged from 91% to 100% while the specificity was between 95% and 98% depending on the assay system used. For monitoring the efficacy of the tsetse control program, the other three antigens were found suitable but only denatured *T. vivax* antigens showed a significant decrease of the seroprevalence of *T. vivax* infection between the beginning and the end of the control program. The native and denatured *T. congolense* assay systems suggested that *T. congolense* infections might occur at a chronic stage and were being controlled by the cattle.

1. INTRODUCTION

Trypanosomosis is probably one of the most important diseases of cattle in Burkina Faso. It is estimated that 90 000 km^2, 32% of the landmass is tsetse fly -infested and the prevalence of trypanosomosis in cattle ranges from 0 to 13% [1].

The control of the disease relies on chemotherapy, chemoprophylaxis and tsetse control using deltametrin pour-on technology combined with insecticide impregnated traps and screens along the main rivers. Tsetse control gave successful results in terms of a decrease of the tsetse fly population and improvement of cattle productivity in the agropastoral zone of Yalé [2]. In order to monitor the efficacy of this tsetse control program, blood samples were collected from cattle and examined for parasites by buffy-coat/dark ground technique [3] and by stained-thin smear method. Since parasitological detection methods have a low sensitivity and may lead to under-reporting of disease prevalence [4], trypanosomal antibody detection could be used to reveal the occurrence of undetected levels of parasiteamia in cattle. The diagnostic performance of indirect antibody-detection enzyme-linked immunosorbent assays (ELISA) is reported. In addition, the tests were applied for assessing the impact of the tsetse control programme on the prevalence of trypanosomosis in cattle in Burkina Faso.

2. MATERIALS AND METHODS

2.1. Tsetse and trypanosomosis control strategy

An integrated campaign against two tsetse fly species (*Glossina tachinoides* and *G. morsitans submorsitans*) was carried out from 1994 to 1996 in the agropastoral zone of Yalé, Burkina Faso (Fig. 1). This area covers approximately 400 km^2 and is located at the border with the Republic ofGhana. The control strategy consisted of a bimonthly systematic topical treatment of all cattle in the agropastoral zone with a 1% pour-on formulation of deltamethrin, in combination with the use of insecticide-impregnated targets during 6 months of the dry season each year.

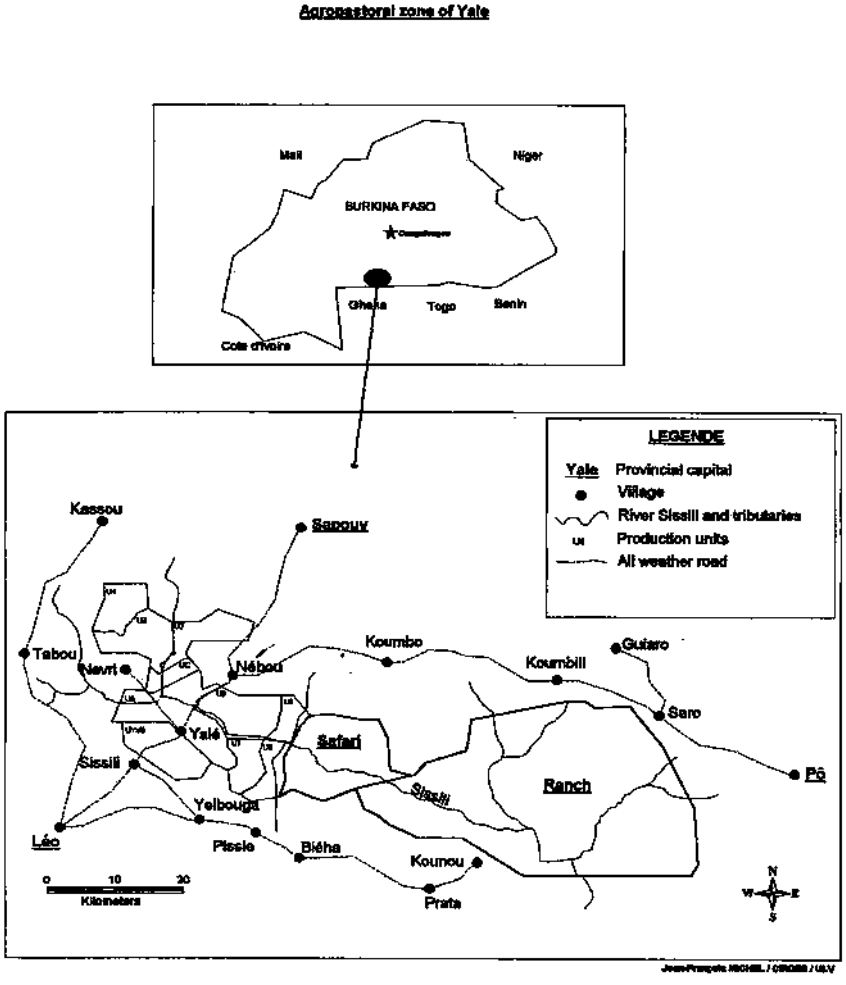

FIG. 1. Map of Burkina Faso, showing the study area of the agropastoral zone of Yalé.

2.2. Assessment of the impact of the tsetse and trypanosomosis control programme

In addition to the entomological investigations [2], an epidemiological survey was carried out to assess the impact of the tsetse control programme on the prevalence of trypanosome infections in cattle.

A randomly selected population of 250 cattle was eartagged and screened four times at four-monthly intervals for the presence of trypanosomes by buffy-coat/darkground technique (BCT) and by stained- thin smear (STS) method. Blood samples were also collected from cattle by jugular venipuncture using plain vacutainer tubes (Becton-Dickinson, USA) without anticoagulant. Sera were separated from clotted blood by conventional centrifugation and stored at -20°C for future use. A total of 600 serum samples (160 samples collected during each of the first three visits and 120 samples during the fourth visit) were analysed to assess seroprevalence. During the initial sampling, all cattle were treated with diminazene aceturate at a dose of 7 mg/kg body weight. Thereafter, only animals testing positive for trypanosomes by BCT or animals with a packed red cell volume (PCV) percent below 25 were treated with the trypanocidal drug. The packed red cell volume was another parameter that was monitored during the observation period.

2.3. Antibody-detection ELISA's

The immunoassay system is based on an indirect ELISA [5] and was developed and standardised by the FAO/IAEA Agriculture and Biotechnology Laboratory in Seibersdorf, Austria, for the detection of bovine serum antibodies directed at either *Trypanosoma congolense* or *T. vivax*.

The antigens were prepared as crude lysates following ultrasonic disruption of *in vitro* propagated *T. congolense* bloodstream forms of strain CP81 and *in vitro* propagated *T. vivax* epimastigote forms of strain IL3905. Native and denatured preparations were prepared from each type of antigen and applied onto Immulon 1 polystyrene 96-well microplates (Dynex, USA).

The precoated plates were supplied with all other reagents necessary to conduct the assays (buffer tablets, anti-species conjugate, blocking detergent agent, substrate, chromogen and stopping solution) together with standardised bench protocols.

The percent positivity (PP) of data expression and quality assurance was used [6]. The positive reference sera (C++, C+) and the negative one (C-) were also obtained from IAEA. Four replicates of internal quality controls (IQC) at the four levels (C++, C+, C- and the conjugate control CC) were included in every ELISA plate. For acceptance of results from individual microtitre plates, the median OD of the four C++ replicates, and the PPs of at least three of the four replicates at all four levels of IQC had to fall within predetermined values (upper control and lower control limits).

2.4. Establishment of the diagnostic threshold PP values

Two threshold (cut-off) values were retained for each of the ELISA systems. The first one was calculated by doubling the mean PP values of the disease-free group of cattle. The second one was obtained by visual inspection [7] based on the frequency distribution of the percent positive values of the negative (n=200) and infected (n=34) cattle population.

Known negative serum samples were available from non-infected cattle in the tsetse-free area around Dori located in the north of Burkina Faso. The positive samples were from the BCT/STS-positive cattle obtained during the survey in the pastoral zone of Yalé. Of the 34 BCT/STS-positive cattle, 27 were infected with *T. congolense*, 7 with *T. vivax* and one with a mixed infection of *T. vivax* and *T. congolense*.

2.5. Statistical analyses

Statistical analysis was used to compare seroprevalence values of trypanosomosis from one visit to the next using confidence intervals [8]. The concordance between the parasitological and the serological technique was assessed using kappa statistics [9].

3. RESULTS

3.1. Analyses of IQC responses for acceptance of microtitre plates

The IQC responses (OD and PP values) were consistent (coefficient of variation below 10%) and fell within the predetermined target ranges in 16 out of the 20 microtitre plates tested with *T. congolense*-antigen native (TcAgn) or denatured (TcAgd) systems (Figs 2 and 3). With the *T. vivax* antigen systems, only the denatured one (TvAgd) gave acceptable results in 13 out of the 20 microtitre plates (Fig. 4) and was used for the analysis of samples tested.

3.2. Diagnostic threshold PP, sensitivity and specificity values

The threshold values resulting from doubling the mean of PP data for sera from the negative cattle population were 26 PP (mean = 12.75 PP, SD= 8.1 PP) and 29 PP (mean = 14.36 PP, SD= 10 PP) for the TcAgn and TcAgd, respectively. The value was estimated at 19 PP (mean = 9.18 PP, SD= 5.4 PP) for the TvAgd system.

The frequency distribution of Ab-ELISA responses of sera from negative (n=200) and positive (n=34) cattle are shown in Figs 5, 6 and 7. Based on visual analysis, the cut-off points were estimated at 30% for the TcAgn and TcAgd and at 25% for the TvAgd system.

The diagnostic sensitivity and specificity values were calculated using the different thresholds determined previously (Table I).

TABLE I. DIAGNOSTIC SENSITIVITY AND SPECIFICITY FOR THREE DIFFERENT ELISA'S

ELISA system	Cut-off (%)		Sensitivity (%)	Specificity (%)
TcAgn	Arithmetic	26	100	97.5
	Estimated	30	100	99.5
TcAgd	Arithmetic	29	100	97.0
	Estimated	30	100	97.5
TvAgd	Arithmetic	19	94.1	95.0
	Estimated	25	91.1	97.5

TcAgn = ELISA plates coated with native *T. congolense* antigen; TcAgd = ELISA plates coated with denatured *T. congolense* antigen; TvAgd = ELISA plates coated with denatured *T. vivax* antigen.

3.3. Concordance of the results obtained by BCT/STS and Ab-ELISA

The kappa statistic values were 0.002 for TcAgn and TcAgd, and 0.005 for TvAgd, while a value of 0.4 is required to consider the existence of an accordance.

3.4. Estimates of trypanosomosis prevalence in the agropastoral zone of Yalé

The kinetics of the seroprevalence of trypanosomosis as determined by the TcAgn or TcAgd systems, using the arithmetic thresholds, did not show a decrease during the progress of the control programme and neither a significant difference between the initial and the last visit (Table II).

TABLE II. PREVALENCE OF BOVINE TRYPANOSOMOSIS OBTAINED BY DIFFERENT ELISA's AND THE BUFFY-COAT TECHNIQUE IN THE PASTORAL ZONE OF YALÉ

Date	BCT (%)	TcAgn (%)	TcAgd (%)	TvAgd (%)	PCV (%)
23-02-1994	13.1	99.3	100	100	28.62
	(8.5-19.6)*	(99-99.9)	(97.1-99.9)	(97.1-99.9)	
22-06-1994	6.25	95.6	95	92.5	30.35
	(3.2-11.5)	(90.8-98)	(90-97.6)	(86.9-95.8)	
27-10-1994	2.5	98.1	98.1	88,75	32.70
	(0.8-6.6)	(94.1-99.5)	(94.1-99.5)	(82.5-93)	
23-03-1995	0	97.5	97.5	80.8	34.33
		(92.3-99.3)	(92.3-99.3)	(72.4-87.2)	

* confidence interval; for explanation of TcAgn, TcAgd and TvAgd see Table I.

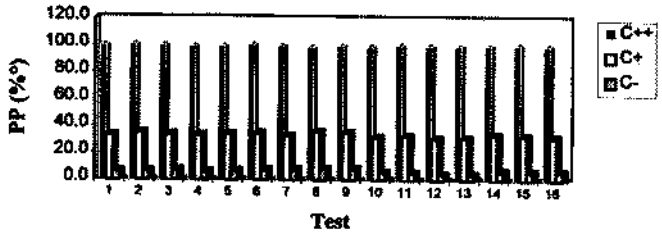

FIG. 2. Internal quality control percentages for 16 tests using ELISA plates pre-coated with T. congolense *native antigen.*

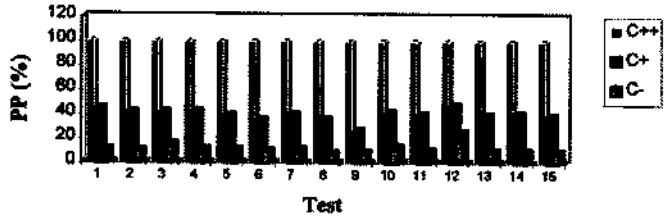

FIG. 3. Internal quality control percentages for 15 tests using ELISA plates pre-coated with T. congolense *denatured antigen.*

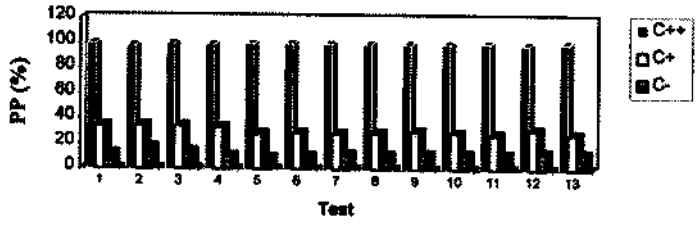

FIG. 4. Internal quality control percentages for 13 tests using ELISA plates pre-coated with T. vivax *denatured antigen.*

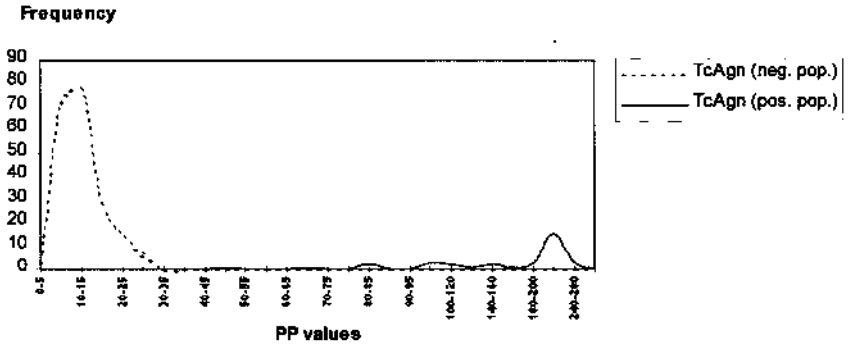

FIG. 5. Frequency distribution of percentage positivity (PP) values of a negative (n=200) and positive (n=34) population using ELISA plates pre-coated with T. congolense *native antigen (Tc Agn).*

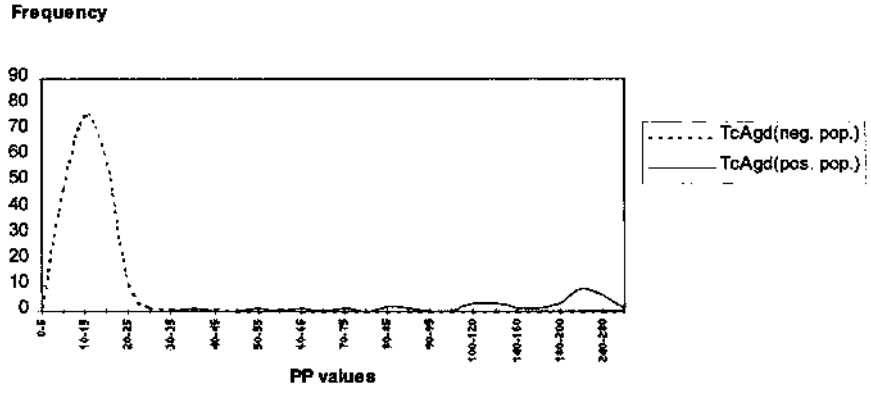

FIG. 6. Frequency distribution of percentage positivity (PP) values of a negative (n=200) and positive (n=34) population using ELISA plates pre-coated with T. congolense *denatured antigen (TcAgd).*

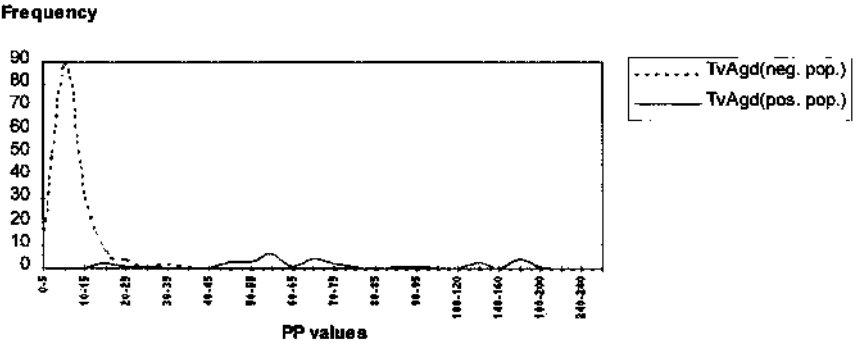

FIG. 7. Frequency distribution of percentage positivity (PP) values of a negative (n=200) and positive (n=34) population using ELISA plates pre-coated with T. vivax *denatured antigen (TvAgd).*

In contrast, a constant decrease was observed and the seroprevalence was significantly lower ($p< 0.05$) at the final visit as compared to the initial visit with the TvAgd system. By using estimated thresholds for the ELISA systems the previous observations were not altered, except for the TvAgd system showing a more important decrease.

The prevalence of trypanosomosis as determined by the parasitological methods showed a decrease from 13% to 0% between the initial and the last visits. Simultaneously, an increase of the average PCV values of the cattle was observed.

4. DISCUSSION

The objective of the present study was to assess the diagnostic performance of native and denatured antigens prepared from *in vitro* cultured trypanosomes and used in an ELISA for the detection of trypanosomal antibodies in cattle and for monitoring the efficacy of a tsetse and trypanosomosis control programme.

Except for the *T. vivax* native antigen system in which high background levels were recorded, the control reference sera worked well in the three other ELISA systems. The discrimination between infected and uninfected cattle was acceptable according to the frequency distribution of the PP values. A few infected cattle showed PP values similar to those of the negative population using the TvAgd system, but this could be explained by the absence of antibodies until 8 to 21 days after primary infection [10, 11], if parasite detection is used as a "gold standard".

Excellent specificity and sensitivity values for each system were obtained when the arithmetic cut-off point as well as when the estimated one was used. Analysis of a larger number of samples from the known positive cattle population, might have influenced the sensitivity value. However, in the present study attention was focused on specificity, since a high specificity is required to minimise the number of false positive results, which is of importance when implementing a tsetse and trypanosomosis control programme.

The lack of a significant decrease in seroprevalence using the *T. congolense* antigen systems suggests that infections due to this species were probably chronic in nature and were being controlled by the animals. Moreover, false positive results might have occurred as a result of antibodies persisting from 4 to 10 months after treatment [12, 13].

It is possible that non-specific factors (associated with other parasites such as *T. theileri, Babesia* or *Anaplasma*) present in sera from an endemic area interfered with the detection of specific antibodies and/or contributed to background reactions of negative samples.

The *T. vivax* denatured antigen system seemed to work better, although a much lower seroprevalence value was expected at the final visit as compared to the initial one. The decrease as observed in the seroprevalence suggests that no new *T. vivax* infections occurred in the area under control.

However, more information is required regarding the persistence of antibody titres in the field since most studies were carried out under controlled conditions. Nevertheless, the detection of antibody titres using pre-coated plates as part of monitoring a tsetse control programme may give a better indication of the magnitude of the disease problem in a given area than detection of parasites using conventional techniques.

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EVALUATION OF FOUR INDIRECT ELISA SYSTEMS FOR THE DETECTION OF TRYPANOSOMAL ANTIBODIES IN BOVINE SERUM

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Abstract

EVALUATION OF FOUR INDIRECT ELISA SYSTEMS FOR THE DETECTION OF TRYPANOSOMAL ANTIBODIES IN BOVINE SERUM.

Four indirect-ELISA systems developed by the Joint FAO/IAEA Division for the detection of trypanosomal antibodies in bovine serum were evaluated in the field. Internal quality control data obtained were good showing that pre-coating plates with antigen increase the robustness of the assay and contribute to its standardisation. ELISA systems derived from *Trypanosoma vivax* antigen lysates gave a better performance than ELISA systems using *T. congolense* antigens. Sensitivity and specificity corresponding to the highest accuracy were 86-87% and 83-85% respectively. When comparing the two ELISA systems utilising *T. vivax* antigens, there was no significant difference between native and denatured antigens and diagnostic threshold was higher for denatured antigens.

1. INTRODUCTION

Trypanosomosis is a major constraint to livestock production in sub-Sahara Africa. For a long time, detection of parasite in blood samples was the only diagnostic method available. Several parasitological techniques were developed. Although their specificity was good, the sensitivity remained relatively low. This situation necessitated the development of alternative diagnostic methods such as ELISAs which can be used for epidemiological investigation of trypanosomosis [1, 2].

The results are presented of an evaluation of four indirect-ELISA systems recently developed by the Joint FAO/IAEA Division for the detection of trypanosomal antibodies in bovine serum samples.

2. MATERIALS AND METHODS

2.1. ELISA systems evaluated

The four ELISA systems evaluated were produced at the Animal Production Unit of the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria. They are based on a standard indirect enzyme-linked immunosorbent assay (ELISA) technique using antigens derived from *T. congolense* and *T. vivax* lysates. For each trypanosome species, Immulon I polystyrene 96-well microplates were pre-coated with crude trypanosomal antigen, either native (AGn) or denatured (AGd). Thus the four ELISA systems are described as TcAGd, TcAGn, TvAGd and TvAGn.

Precoated plates were stored dry and dark at room temperature. Control sera and anti-species conjugate were hand carried from Seibersdorf to the laboratory in Africa and kept at –20°C until use. Substrate and chromogen were stored at +4°C.

2.2. Serum samples chosen for test evaluation

Serum samples used for evaluation of the four ELISA systems came from the LANAVET bovine serum bank. They were chosen on the basis of the parasitological status with regard to *T. brucei*, *T. congolense*, *T. vivax*, *T. theileri* and microfilaria. The status was defined following the testing of blood samples using the buffy coat technique, the micro haematocrit centrifugation technique and Giemsa-stained thin smears [3]. Samples found negative for the above parasites or positive to only one parasite were selected for evaluation.

2.3. Evaluation protocol

The assay procedure for the evaluation of each ELISA system was designed and written by the Animal Production Unit of the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria. All tests were ran within the time period of one week by the same operator. Protocols were

strictly followed. Optical density readings were retrieved using EDI 2.2 software. MedCalc 5.0 software was used for statistical analysis.

2.4. Evaluation of diagnostic proficiency of assays

The Receiver operating characteristic (ROC) analysis method [4, 5] was used to evaluate and to compare the diagnostic proficiency of the ELISA systems evaluated.

3. RESULTS

3.1. Parasitological status of samples used for tests evaluation

A total number of 912 serum samples were selected for evaluation of the tests. They had been collected in the field between year 1994 and 1997 as part of a study to assess the prevalence of bovine trypanosomosis in northern Cameroon. The area is known as endemic for *T. brucei*, *T. congolense* and *T. vivax* [6]. Few samples were found positive to only *T. brucei* or *T. congolense*. A large number of samples were negative or positive to only *T. vivax*, *T. theileri* or microfilaria. The samples positive for *T. congolense* showed the lowest average packed red cell volume (PCV) percentage, followed by *T. vivax* samples and the samples containing microfilaria (Table I). PCV were normal for the group without parasites (negatives).

TABLE I. NUMBER AND STATUS OF SAMPLES USED FOR THE EVALUATION OF ELISA TESTS

Parasitological status of cows from which serum samples originated	Number of samples tested	Packed red cell volume (Mean ± SD)
Trypanosoma brucei	37	27,892 ± 5,496
Trypanosoma congolense	53	21,132± 4,433
Trypanosoma vivax	239	24,979 ± 5,260
Trypanosoma theileri	157	28,497 ± 5,095
Microfilaria	178	25,612 ± 3,880
Negatives	248	30,286 ± 4,991
Total	912	

SD = standard deviation

3.2. ELISA results

3.2.1. Assay performance

For each ELISA system, 23 antigen pre-coated plates were necessary to analyse all the 912 selected field samples. In addition, one extra plate was used for samples that needed retesting. ELISA systems using antigens derived from *T. congolense* gave the best performance. Of the 48 plates tested, 38 were within control limits as defined by quality assurance criteria. For the *T. vivax* systems, only 19 plates of 48 tested were within the recommended range. A total of 39 plates showed internal quality control values close to, above or below the defined limits. Nevertheless, the results of the assays were accepted. The mean ± 1 standard deviation calculated for each internal quality control is presented in Table II.

TABLE II. INTERNAL QUALITY CONTROL LIMITS AND MEANS AS CALCULATED FOR EACH ELISA SYSTEM

Indirect ELISA systems	C++ Optical densities		C+ Percent positivity (%)		C- Percent positivity (%)		Cc Percent positivity (%)	
	QA	IQC	QA	IQC	QA	IQC	QA	IQC
TcAGd	0.936 – 1.406	1.196 ±0.240	38 – 49	43.396 ±2.159	9 – 14	10.833 ±1.554	1 – 4	3.135 ± 0.675
TcAGn	0.974 – 1.323	1.268 ±0.161	32 – 39	35.115 ±1.886	5 – 11	8.219 ±1.370	1 – 3	1.990 ± 0.657
TvAGd	0.983 – 1.507	1.048 ±0.130	43 – 53	51.427 ±7.443	10 – 16	13.313 ±1.611	1 – 5	3.000 ±1.046
TvAGn	0.808 – 1.299	1.118 ±0.258	40 – 62	43.656 ±4.986	11 – 19	13.479 ±2.462	-4 – 10	2.490 ±1.399

C++ = Strong positive control serum; C+ = Moderate positive control serum; C- = Negative control serum; Cc = Conjugate control.
QA = Quality assurance information; IQC = Internal quality control data.
IQC data are represented by the mean ± 1 standard deviation.

3.2.2. Diagnostic proficiency of assays

Frequency distribution of percent positivity values of samples tested with each ELISA system are presented in Figs 1 to 4. PP values obtained for samples without parasites (negative group) and those containing *T. congolense* or *T. vivax* parasites (diseased group) were used for evaluation of diagnostic proficiency of assays. ROC analysis of PP values showed that the four ELISA systems can be use to discriminate *T. congolense* and *T. vivax* cases from negative samples. Cut-off point corresponding to minimal false negative and false positive for each ELISA system is presented in Table III. Sensitivity was higher than specificity for all ELISA systems and their values were better for ELISA systems derived from *T. vivax* antigens. Area under ROC curve which is an expression of the test value was larger for ELISA systems using *T. vivax* antigens, indicating a better performance for these systems. Comparison of ROC curves showed no significant difference between native and denatured antigens when they are prepared from *T. vivax* lysates (Fig. 5).

3.2.3. ELISA results for samples containing parasites other than T. congolense *or* T. vivax

Many samples containing *T. brucei*, *T. theileri* or microfilaria showed PP values above cut-off points calculated for different ELISA systems tested. The proportion of these samples was high, between 37% and 58% (Table IV).

TABLE III. ROC ANALYSIS OF PP VALUES OBTAINED FOR SERUM SAMPLES PARASITOLOGICALLY NEGATIVE OR INFECTED WITH *T. CONGOLENSE* OR *T. VIVAX*.

(ROC) analysis	TcAGd	TcAGn	TvAGd	TvAGn
Sample size of positive group (*T.c.*, *T.v.*)	292	292	292	292
Sample size of negative group	248	248	248	248
Disease prevalence (%)	54,1 %	54,1	54,1	54,1
Area under the ROC curve	0,730	0,812	0,891	0,882
Standard error	0,021	0,018	0,014	0,015
Diagnostic threshold (≥)	19 %	24 %	30 %	23 %
Sensitivity	79,5 %	77,4 %	85,6 %	87,0 %
Specificity	57,7 %	71,0 %	84,7 %	82,7 %
Comparison of ROC curves				
- Difference between areas	0,082		0,009	
- Standard error	0,017		0,009	
- Significance level (P)	0,000		0,300	

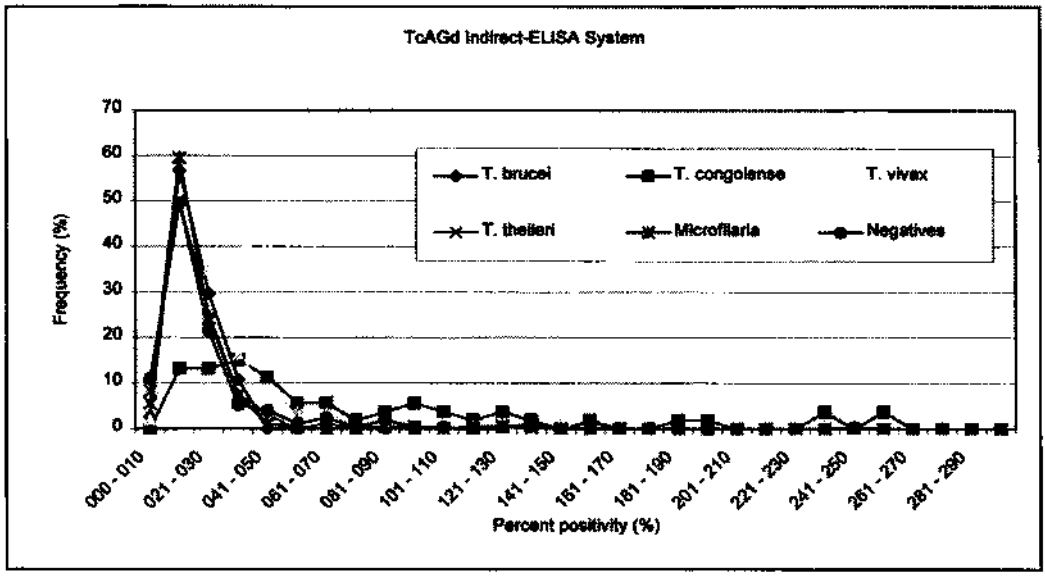

FIG. 1. Frequency distribution of PP values for TcAGd indirect-ELISA system.

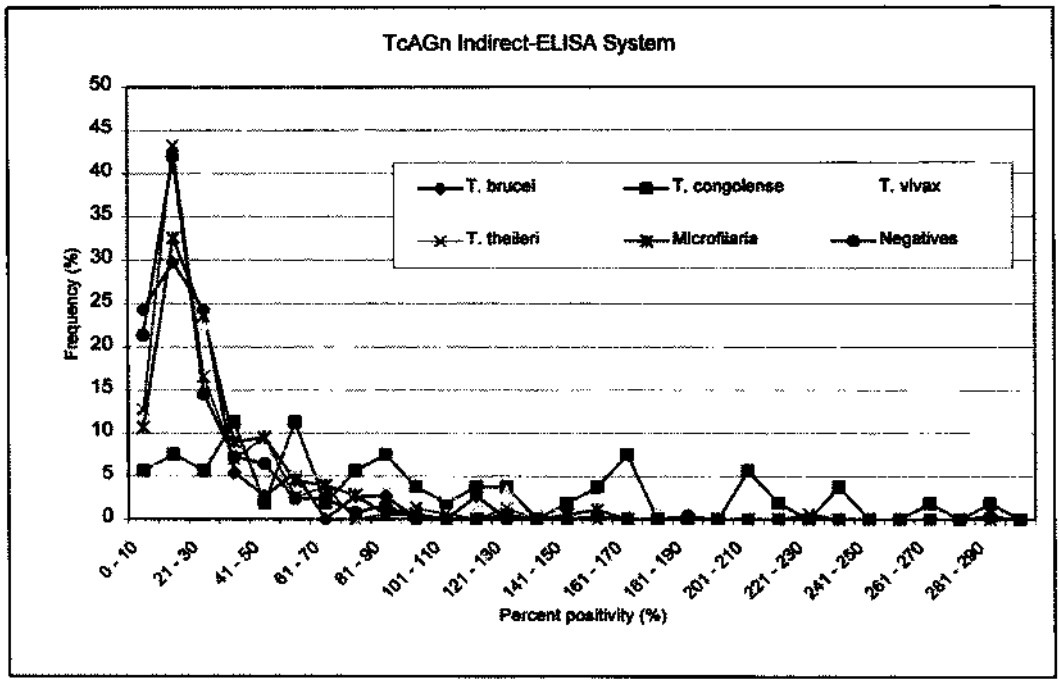

FIG. 2. Frequency distribution of PP values for TcAGn indirect-ELISA system.

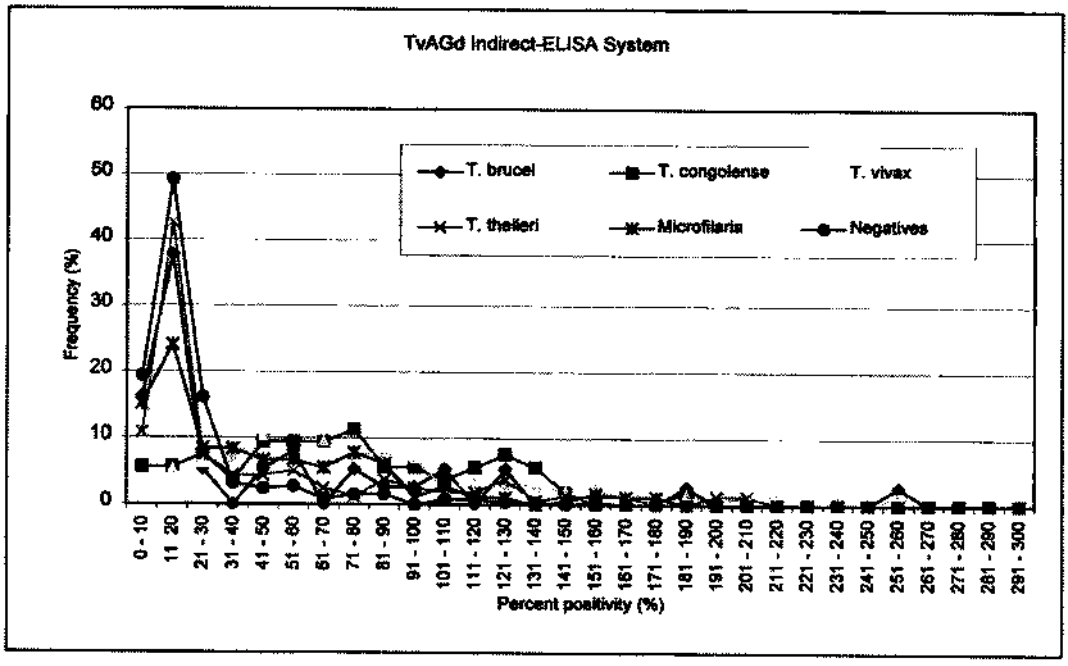

FIG. 3. Frequency distribution of PP values for TvAGd indirect-ELISA system.

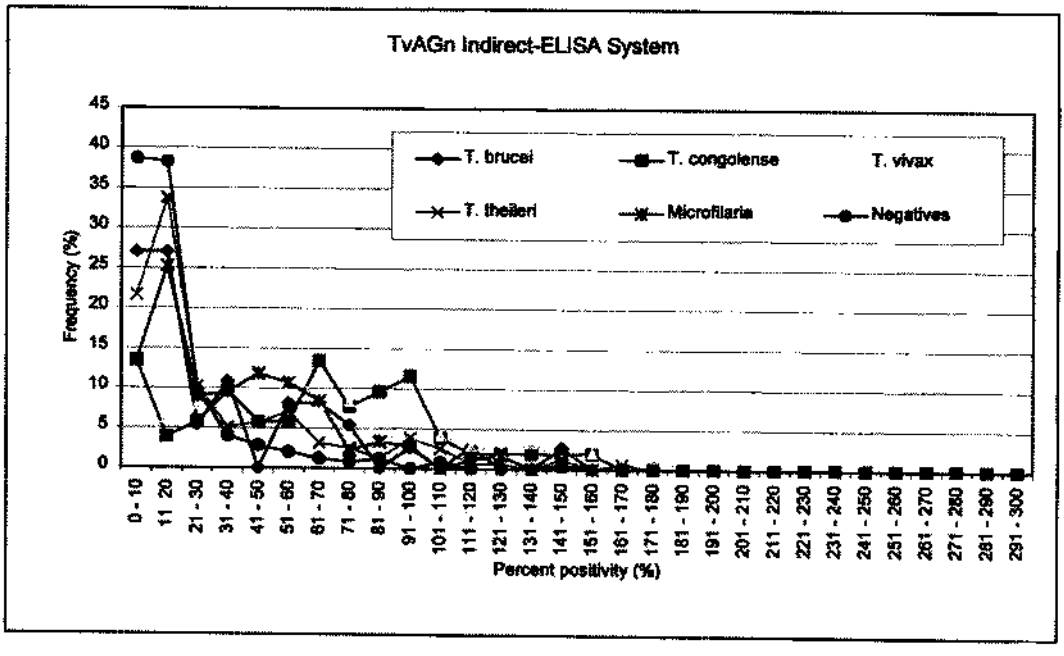

FIG. 4. Frequency distribution of PP values for TvAGn indirect-ELISA system.

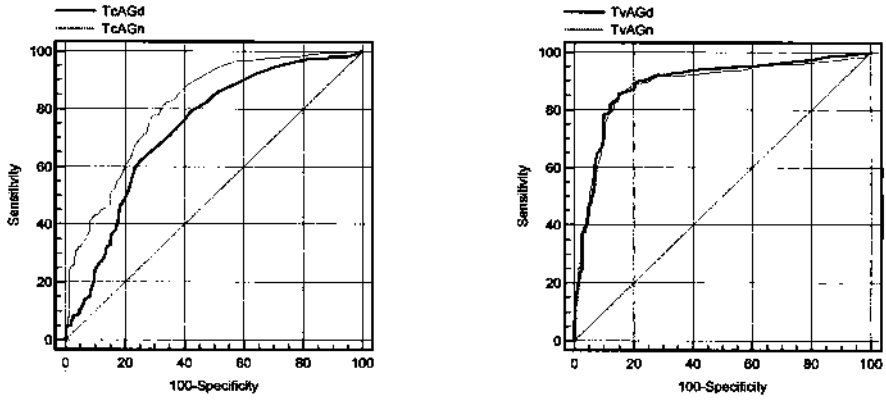

FIG. 5. Comparison of ROC curves generated using PP values obtained for serum samples without parasites (negative group) or containing T. congolense *or* T. vivax *(positive group).*

TABLE IV. PERCENTAGE OF SAMPLES CONTAINING PARASITES OTHER THAN *T. CONGOLENSE* OR *T.VIVAX* FOUND POSITIVE USING DIFFERENT ANTIBODY ELISA SYSTEMS.

	Samples tested	TcAGd (%)	TcAGn (%)	TvAGd (%)	TvAGn (%)
Diagnostic threshold		≥ 19	≥ 24	≥ 30	≥ 23
T. brucei	37	48.65	40.54	40.54	45.95
T. theileri	157	52.23	37.58	38.85	40.76
Microfilaria	178	48.88	51.69	52.25	58.43

4. DISCUSSION

4.1. Assay performance

Although the results of internal quality controls for some plates were close to, above or below the defined limits, all the 48 ELISA plates tested could be accepted. The control limits are preliminary and pre-defined limits which had been determined under single laboratory conditions at the Animal Production Unit of the FAO/IAEA Agriculture and Biotechnology Laboratory in Seibersdorf, Austria. As a result of the field evaluation, a readjustment of quality assurance criteria might be required.

Precoating plates by the producer of the assay has solve the problem of the need to maintain a cold chain for the shipment of antigen to the end user. It also minimises risk of errors during precoating step by the users. As a result precoating has contribute to the standardisation and the robustness of the assay. This confirms results of a study by Rebeski *et al.* [1] who found that precoating plates with denatured trypanosome antigens following storage at +37°C for at least 7 weeks and shipment to laboratories in Africa under uncontrolled conditions has improved the stability of the test.

4.2. Serum samples chosen for test evaluation

The study area where originates serum samples used for evaluation is known to be endemic for animal trypanosomosis. Because there was no trypanosome free area elsewhere in the country, negative population used for the study was chosen on the basis of the parasitological status of blood samples. It has been established that none of the parasitological diagnostic methods used to analyse

blood samples operates efficiently at levels of less than 10^2 trypanosomes par ml [3]. Therefore a parasitologically negative tested sample does not exclude the presence of trypanosome in endemic area. It is highly probable that samples considered negative for the evaluation study contains trypanosome antibodies.

4.3. Diagnostic proficiency of assays

ELISA systems derived from *T. vivax* showed the highest accuracy with a diagnostic threshold of 30% for denatured antigens and 23% for native antigens. There was no significant difference between areas under ROC curves of TvAGd and TvAGn ELISA systems, suggesting that any of these two ELISA systems can be use for field studies.

The sensitivity and specificity of 86-87% and 83-85% respectively obtained with ELISA systems derived from *T. vivax* are good enough if we consider that negative population used for validation of the assay originates from trypanosome endemic area. Hopkins *et al.* [2] obtained a sensitivity and a specificity of 86.1 and 95.2% respectively for a similar assay using known negative samples from tsetse free area.

Diagnostic proficiency of the assay would probably be increased by testing few samples collected from known negative animals in the study area. Another solution consists in theselection of two cut-offs rather than one [7]. One cut-off would include 99% of the values from infected animals and the other 99% of the values from uninfected animals. The values between these percentiles would be considered as suspicious.

Under the particular field conditions in Cameroon, the use of *T. vivax* based ELISA systems rather than *T. congolense* based ELISA systems are recommended for reliable estimates of the disease prevalence based on antibody detection.

4.4. ELISA results for samples containing parasites other than *T. congolense* or *T. vivax*

Luckins [8] found no evidence of cross-reactivity between trypanosome antigens and serum samples from cattle infected with *T. theileri*. High seroprevalence observed in our study for samples parasitologically positive to only *T. theileri* or microfilaria probably reflects the presence of trypanosome antibodies rather than a cross-reactivity. This assumption is supported by the fact that samples were collected in tsetse endemic area.

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APPLICATION OF AN ANTIBODY-DETECTION ELISA FOR THE DIAGNOSIS OF BOVINE TRYPANOSOMOSIS IN CÔTE D'IVOIRE

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Abstract

APPLICATION OF AN ANTIBODY-DETECTION ELISA FOR THE DIAGNOSIS OF BOVINE TRYPANOSOMOSIS IN CÔTE D'IVOIRE.

An antibody-detection ELISA was used in a tsetse control area in the central region of Côte d'Ivoire for the serodiagnosis of bovine trypanosomosis, in combination with parasitological techniques. Of 1553 samples examined by buffy coat technique (BCT), 24 (1.54%) were positive for pathogenic trypanosomes: 7 *Trypanosoma congolense*, 4 *Trypanosoma vivax*, and 13 *Trypanosoma brucei*. The low prevalence could be due to the frequent use of trypanocidal drugs in these herds and the use of tsetse traps.

Using an ELISA to detect *T. congolense* antibodies, 231 (48%) of 480 samples examined, showed high percent positivity values. All 7 animals parasitologically positive for *T. congolense* showed a high percent positivity. Using an ELISA to detect *T.vivax* antibodies, 136 samples (28.33%) showed a high percent positivity.

1. INTRODUCTION

The three pathogenic species of bovine trypanosomosis (*T. brucei*, *T. congolense* and *T. vivax*) are important causes for livestock disease in Côte d'Ivoire. The country is situated between 4° and 10° latitude North and the entire surface area is infested with tsetse flies.

The national tsetse control service based in Bouaké and Korhogo controls the northern and central regions. Furthermore, breeders often use chemotherapy and chemoprophylaxis, while some of them also use tsetse traps. However, frequent use of trypanocidal drugs is expensive and can be toxic for the animals. A rapid and accurate diagnosis would be the best way for restricting treatingdiseased animals only.

The purpose of the study was to validate an ELISA for detecting antibodies against *T. congolense* and *T. vivax* in field samples using precoated plates as supplied by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

2. MATERIALS AND METHODS

2.1. Survey area

Samples were collected from a tsetse control area in the central region of Côte d'Ivoire near the city of Toumodi. The study area is in a rain savannah region, situated at the limit of a forest region. The climate is dominated by two rainy seasons (April to July and October to November) with an annual rainfall varying between 1000 - 1600 mm. The temperature varies between 20°C and 32°C. Two major rivers are present in the District, the N'zï in the East and the Bandama in the West.

Approximately 50 000 cattle are present in the area, consisting of trypanotolerant animals like N'Dama and Baoulé; and Zebu and crossbred animals. They are mainly raised for meat but also for milk.

The principal vectors of trypanosomes in the area are considered to be *Glossina longipalpis*, *G. palpalis*, *G. morsitans*, *G. medicorum* and other insects such as Tabanids.

2.2. Animals

Twenty herds totalling about 3000 animals were selected. The animals were kept under traditional management conditions, in settled herds. A common grazing system was practised, while some herds received additional cotton seed. Veterinary cares was provided by the local veterinary services consisting of chemotherapy and chemoprophylaxis against trypanosomosis, ticks control and

helminth control. The animals were treated in December, 1997 and January, 1998. Furthermore, some breeders used tsetse traps. Two visits were carried out in the survey area.

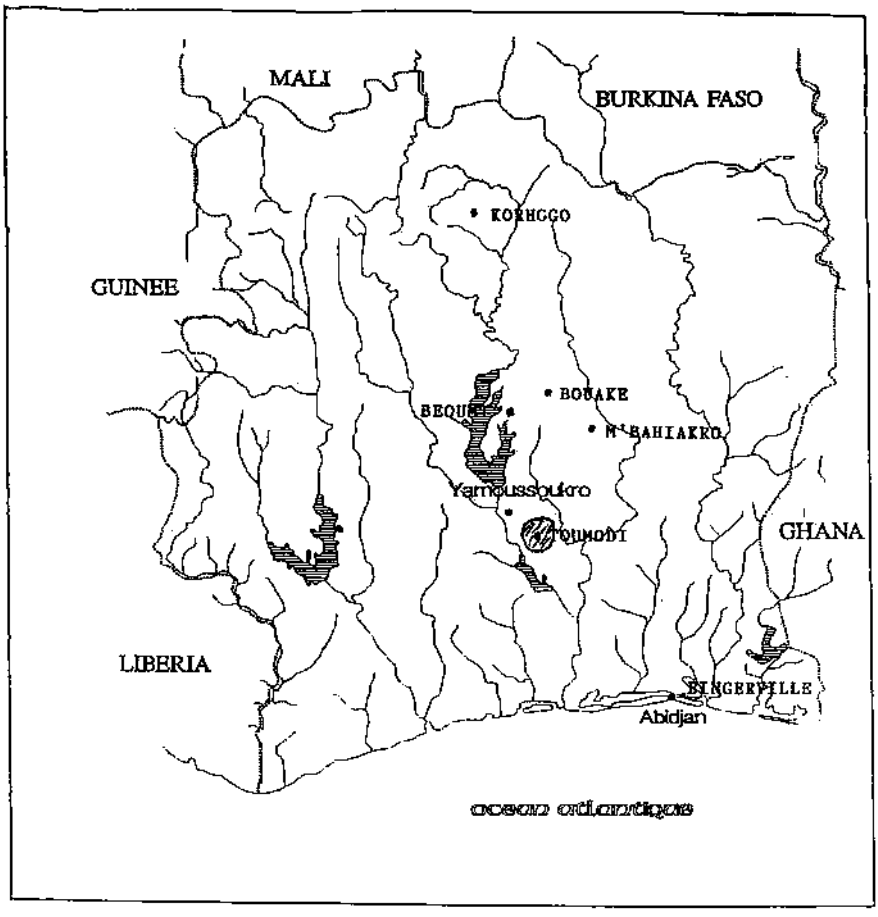

FIG 1. Map of Côte d'Ivoire showing the study area

2.3. Sampling methods

A total of 1553 male and female animals between one and ten years old were sampled. Samples were collected during the dry (February, 1998) and wet (April, 1998) season.

Peripheral blood was collected from the ear vein (two capillary tubes per animal), centrifuged and examined in the field using buffy coat technique (BCT) to determine the presence of trypanosomes and the packed red cell volume (PCV) levels [1].

Jugular blood was collected in sterile vacutainer tubes and serum samples were collected after centrifugation, and were stored in 1 ml aliquots at –20°C until analysis.

During the second visit, feacal samples were collected from the rectum of 216 animals.

3. RESULTS

3.1. Parasitological results

Microscopical examination of 1553 samples using the BCT showed 24 animals (1.54%) positive for pathogenic trypanosomes (Table I). Of the 767 blood smears examined, 7 (0.91%) were positive (Table II). All three species pathogenic for cattle were present: *Trypanosoma brucei*, *T. congolense* and *T. vivax*.

TABLE I. RESULTS OBTAINED USING THE BUFFY COAT TECHNIQUE

Date of visit	No. of samples	No. of positives	Pathogenic trypanosomes			Other parasites	
			T. brucei	*T. congolense*	*T. vivax*	*T. theileri*	microfilaria
February, 1998	767	15	11	3	1	0	88
April, 1998	786	9	2	4	3	2	83
Total	1553	24	13	7	4	2	171
%	100%	1.54	0.83	0.45	0.26	0.13	11

TABLE II. RESULTS FOLLOWING THE EXAMINATION OF BLOOD SMEARS

Date of visit	No. of samples	No. of positives	*T. brucei*	*T. congolense*	*T. vivax*	Babesia
February, 1998	767	7	5	1	1	52
%		0.91	0.65	0.13	0.13	6.78

Faecal sample examination using the McMaster technique showed that Strongyles were the predominant helminth species present (Table III).

TABLE III. COPROLOGICAL RESULTS

Helminth species	No. of cattle positive	%	Range of egg count (e.p.g.)
Strongyles	63	29.16	100-1400
Strongyloides	6	2.77	100-400
Coccidia	13	6	100-1600
Ascarides	1	0.46	
Trichuris	1	0.46	
Cestodes	7	3.24	

e.p.g. = eggs per gram faeces

3.2. Serological results

As a validation of the indirect ELISA to detect *T. congolense* and *T. vivax* antibodies a total of 480 sera were collected in the field and tested for both species using Immulon 1 plates, precoated with *T. congolense* denatured antigen and *T. vivax* denatured antigen (Table IV).

TABLE IV. RESULTS OBTAINED USING THE ANTIBODY-DETECTION ELISA

	T. congolense antibody ELISA		*T. vivax* antibody ELISA		PCV
	OD average	PP average	OD average	PP average	
Samples positive for *T. congolense* using BCT					
1	1.376	85	1.782	146	30
2	1.698	105	0.246	20	31
3	2.471	153	1.697	141	34
4	1.684	104	0.767	64	42
5	0.849	53	0.259	21	31
6	1.185	73	1.382	114	32
7	1.304	81	0.700	58	44
Samples positive for *T. vivax* using BCT					
1	1.131	70	2.221	184	26
2	0.785	49	0.439	36	24
3	0.319	20	0.415	34	42
4	1.288	80	0.338	28	42
Samples positive for *T. brucei* using BCT					
1	2.191	136	0.594	79	37
2	1.070	66	0.338	28	28
3	0.525	32	0.299	25	32
4	1.555	96	1.282	106	30
5	2.134	132	0.968	80	34
6	0.365	23	1.933	160	28
7	0.668	41	0.217	18	37
8	1.747	108	2.115	175	25
9	0.347	21	0.195	16	44
10	0.428	26	0.594	49	43
11	0.324	20	0.162	13	46
12	0.606	38	1.460	121	31

OD: optical density; PP: percent positivity; BCT: Buffy Coat technique; PCV: packed red cell volume.

3.2.1. T. congolense *antibody-detection ELISA*

All seven animals positive for *T. congolense* by BCT showed high OD values (0.849 to 2.471) and high PP values (53 to 153).

Of the four animals positive for *T. vivax* by BCT, 3 had high OD values (0.785 to 1.288) and high PP values (49 to 80).

Six of the twelve animals positive for *T. brucei* by BCT showed high OD values (0.606 to 2.191) and high PP values (41 to 136).

Of the 456 animals that were negative for trypanosomes using the BCT, 215 (47%) showed high OD values and high PP values using *T. congolense* antibody ELISA.

In total 231 (48%) of the 480 samples examined, showed high OD and PP values using the *T. congolense* antibody ELISA.

3.2.2. T. vivax *antibody-detection ELISA*

One of four animals positive for *T. vivax* by B.C.T showed high OD values (2.221) and high PP values (184), the three others showed OD values between 0.338 and 0.439 and PP values between 28 and 36.

Five animals positive for *T. congolense* by BCT showed high OD values (0.700 to 1.782) and high PP values (58 to 146).

Five of twelve animals positive for *T. brucei* by BCT showed high OD values (0.968 to 2.115) and high PP values (80 to 175).

From the 456 animals that were negative for trypanosomes using BCT, 125 (27.41%) showed high OD and PP values with the *T. vivax* antibody ELISA.

In total, from the 480 sera examined, 136 (28.33%) showed high OD and PP values with the *T. vivax* antibody ELISA.

4. DISCUSSION

Results obtained using the BCT showed a trypanosome prevalence rate of 1.54%. The low prevalence could be due to the frequent use of trypanocidal drugs in these herds. Moreover, some breeders also used insecticide impregnated traps and treated animals against ticks.

Using the *T. congolense* antibody ELISA, 231 (48%) of the 480 samples examined, showed high percent positivity. All seven animals positive for *T. congolense* using BCT showed high percent positivity values, while three of the four animals positive for *T. vivax* showed high percent positivity and six of 12 animals positive for *T. brucei* also showed high percent positivity values. In total, 16 of the 23 animals parasitologically positive for trypanosomes showed high positivity percent values (70%).

Using the *T. vivax* antibody ELISA, 136 (28.33%) of the 480 samples examined, showed high percent positivity values. One of four animals positive for *T. vivax* by BCT showed high percent positivity, five of the animals positive for *T. congolense* and seven of the 12 positive for *T. brucei* showed high percent positivity. In total, 13 of the 23 animals parasitologically positive for trpanosomes showed a high percent positivity with the *T. vivax* antibody ELISA (56%).

Using the antibody ELISA especially the *T. congolense* antibody ELISA, more animals were detected positive than with parasitological techniques. However, since some parasitologically positive animals showed low percent positivity values in the antibody ELISA, it will be necessary to combine serological and parasitological (BCT) techniques to complete the epidemiological pivcture.

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VALIDATION OF AN IMPROVED ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DIAGNOSIS OF TRYPANOSOMAL ANTIBODIES IN GHANAIAN CATTLE

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Abstract

VALIDATION OF AN IMPROVED ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DIAGNOSIS OF TRYPANOSOMAL ANTIBODIES IN GHANAIAN CATTLE.

The validation of an enzyme-linked immunosorbent assay (Ab-ELISA) for the detection of antibodies to pathogenic trypanosomes in cattle is described. Two hundred known negative sera obtained from the tsetse-free zone of Dori (Burkina Faso) were analyzed using microtitre plates pre-coated with crude antigen lysates of *Trypanosoma congolense* and *T. vivax*. A pre-test optimization was carried out and a percent positivity (PP) of 50% was chosen (specificity: >82%) for assaying field sera. A total of 440 serum samples collected from cattle in areas of known and unknown disease prevalence were assayed. For all animals the packed red cell volume (PCV) was determined and the buffy coat technique (BCT) and blood smears were examined to detect trypanosomes at the species level. A comparison of the BCT and Ab-ELISA results showed there was a much higher prevalence of antibodies to both species than the parasite prevalence as shown by the BCT (10 fold). The rate of agreement between BCT-positive and Ab-ELISA- positive samples for both species was low (<10%). No conclusion could be drawn from this finding because of the low number of known BCT positive cases that were identified. There was a better, albeit highly variable, agreement between BCT- negative and Ab-ELISA- negative samples (30-70%).

Proposals for further improvement of the Ab-ELISA and prospects for the use of the assay in the monitoring of trypanosomosis control in Ghana are discussed.

1. INTRODUCTION

Pathogenic trypanosome species prevalent in cattle in Ghana are *Trypanosoma* (Nannomonas) *congolense*, *T.* (Dutonella) *vivax* and to a lesser extent *T.* (Trypanozoon) *brucei* and its subspecies [1]. More than 65% of all trypanosome infections in ruminants is caused by *T. vivax*, while *T. brucei* is responsible for less than 10% of the infections except in places where trypanocidal drugs have been used extensively over a prolonged period [2]. However *T. brucei* has been frequently encountered in dogs and pigs in all ecological zones of Ghana [1, 2]. Field diagnosis by veterinarians in rural areas is often based on history and clinical signs. Unfortunately, no pathognomic clinical signs differentiate trypanosomosis from other haemoparasitic infections.

The standard parasitological tests (wet blood films and stained blood smears) are quite insensitive and can not always be relied upon especially in chronic cases [3]. Even the improved parasitological methods like the haematocrit centrifuge technique [4] or its variant, the buffy coat technique (BCT), do not detect all infected animals [5].

As part of efforts to improve the diagnosis of trypanosomosis using serological tests, the enzyme-linked immunosorbent assay (ELISA) for the detection of circulating antigens (antigen ELISA) of different trypanosome species was introduced by the FAO/IAEA Joint Division in 1987. Following a period of validation the test was temporarily shelved due to its poor sensitivity. In the meantime an ELISA for the detection of circulating antibodies specific for *T. congolense* and *T. vivax* was introduced. The results are presented of the validation of the ELISA system for the detection of circulating trypanosomal antibodies to pathogenic trypanosomes in naturally infected cattle using microtitre plates pre-coated with crude antigen lysates of *T. congolense* and *T. vivax*. Moreover, the test was used to monitor ongoing tsetse and trypanosomosis control programmes in Ghana.

2. MATERIALS AND METHODS

2.1. Sampling

Single visit surveys were carried out of herds in the Saboba /Chereponi, Savelugu/Nanton and Tolon/Kumbungu districts of the Guinea Savannah Zone of the Northern Region of Ghana (Fig.1). Sample sizes were chosen at 95% confidence level within an accuracy of 5% and a presumed prevalence of 20% [6]. Because of the large numbers of animals presented in a herd by the owners,

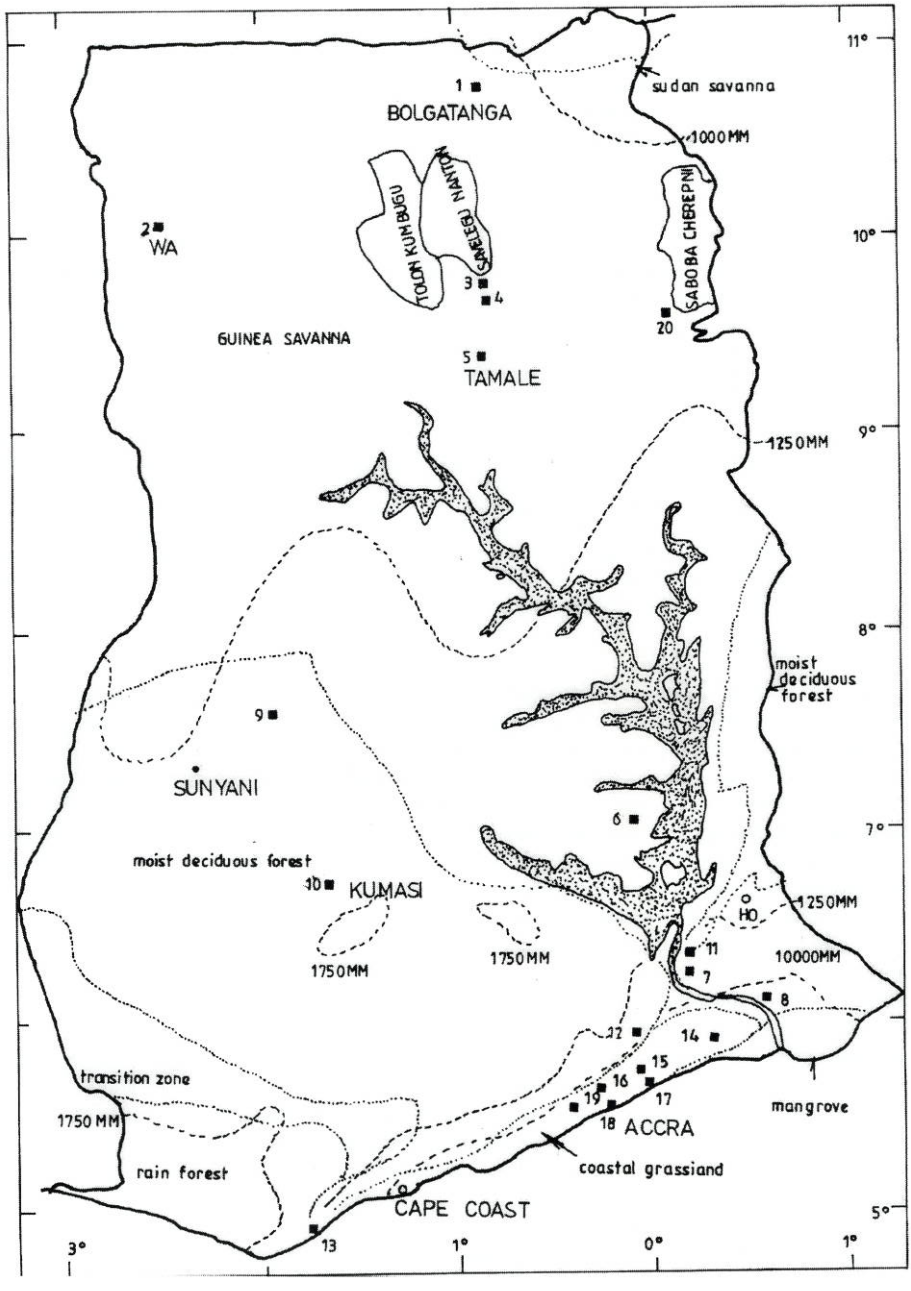

FIG. 1. Map of Ghana to show sampling sites indicated by numbered black squares the approximate boundaries of the natural vegetation as dotted line, the main rainfall isohyets as dashed lines, and other cities indicated with open circles, lake Volta is shown dashed.

systematic sampling was adopted. Therefore, invariably sample sizes slightly higher than the calculated size were selected. This had the advantage of ensuring that there were more than enough samples for testing. It is important to note that there was a marked preponderance of samples taken from adult cattle (>90%). Serum samples from the three districts of various trypanosomosis prevalence status were used.

Few reports of clinical disease had been reported from Saboba/Chereponi, where surveys had never been carried out previously. The district was classified as presumably a low challenge area. The Savelugu/Nanton and Tolon/Kumbungu districts were classified as a medium and high challenge area, respectively.

Samples from a known tsetse/trypanosomosis-free area were obtained from Dori district, Northeast Burkina Faso (courtesy Dr. Bengaly, CIRDES). All locations were geographically referenced.

2.2. Tsetse species and densities

Tsetse surveys were carried out in 1997 and again at the time of sampling in all three districts [1]. Tsetse challenge in these districts could be classified as low, medium and high in the Saboba/Chereponi, Savelugu/Nanton and Tolon/Kumbungu districts, respectively. While *Glossina palpalis gambiensis* and *G. tachinoides* were the tsetse species present in the fist two districts, *G. m. submorsitans* could be found in the Tolon/Kumbungu district in addition to the *G. palpalis* species depending on the season and the amount of movement of livestock into the adjacent Mole Game park lying to the west of the district. High pressure on land for crop cultivation and the constant removal of tree cover for fuel wood appeared to restrict tsetse distribution to the gallery forests along the major river systems.

2.3. Herd history

More than 80% of cattle in the 10 herds sampled in the three districts were of the West African shorthorn (WAS) or Sanga breed (Zebu x WAS). Stock bulls were usually Zebu or Sanga. Herds were sedentary and owned by either individuals or households. The cattle were herded by either hired Fulani herdsmen or the children of the owners. They were kraaled at night near the owners' dwellings or just outside the village. Hardly any feed supplementation was provided during the dry season. Moving westwards towards *G. morsitans* infested areas, herd size decreased and herd composition shifted in favor of the WAS.

2.4. Parasitology

Blood for parasitological examination was collected from the ear vein early in the morning into two haematocrit capillary tubes. Capillaries were centrifuged at 7000 revs/min for 5 min and microscopically examined (or put on ice if examination was delayed). The BCT method was used to detect trypanosomes. Wet smears were made from positive samples for species identification. Thick and thin smears were prepared whenever it was not possible to identify the parasite species.

2.5. Serology

Blood was collected from the jugular vein into plain vacutainer tubes (10 ml). Serum was separated by centrifugation (5 min at 3000 revs/min), decanted into storage vials and stored at-20°C until required for analysis.

Each serum sample was tested for the presence of trypanosomal antibodies using two types of pre-coated plates according to the bench protocol [7]. All reagents and biologicals were supplied by the Joint FAO/IAEA Division.

2.6. Indirect antibody-detection ELISA

Two sets of ELISA plates coated with either *T. congolense* or *T. vivax* antigens were provided by the FAO/IAEA Agriculture and Biotechnology Laboratory at Seibersdorf, Austria. The antigen-coated plates were incubated an 37°C for one hour under continuous shaking after which they were washed 4 times with washing buffer.

Test serum (1:100 for *T. congolense* and 1: 400 for *T. vivax*) was added and incubated at 37°C for 1 hour under continuous shaking. Plates were again washed 4 times with washing buffer.

The conjugate was added at 1: 14000 and plates were incubated at 37°C for 1 hour under continuous shaking after which period they were washed 4 times. Substrate/chromogen was added and incubated for 15 minutes at 37°C under continuous shaking of plates.

A stopping solution (ortho-phosphoric acid) was added, the plates were shaken briefly and read immediately using a 450nm filter. ELISA plates were also visually inspected to compare OD values.

3. RESULTS

Analysis of 200 negative sera obtained from Dori, a tsetse-free zone in the North Eastern Province of Burkina Faso, showed that at 0% and 30% percentage positivity (PP), all sera tested positive. Therefore, the PP was adjusted to 50% at which level a specificity of 82% and 88% was obtained for *T. congolense* (Fig. 2) and *T. vivax*, respectively. The frequency distribution for *T. vivax* antibodies was similar to the one for *T. congolense*.

A total of 400 samples were collected from animals originating from areas with a known and unknown disease prevalence. The parasitological and serological findings have been summarized in Tables I, II and III. A frequency distribution of PCV values as a parameter of animal health in the various study areas is shown in Figure 3.

TABLE I. COMPARISON OF BCT AND AB-ELISA RESULTS FOR *T. CONGOLENSE* POSITIVE SAMPLES PER DISTRICT

District	Locality *	No. of samples	BCT+ve	Ab-ELISA +ve	BCT+ve Ab-ELISA +ve	BCT+ve Ab-ELISA -ve	BCT-ve Ab-ELISA +ve	BCT-ve Ab-ELISA -ve
Savelugu/ Nanton	Savemed	100	4 (4%)	25 (25%)	2 (2%)	2 (2%)	23	73 (73%)
Saboba/ Chereponi	Sabobaunk	100	0	4 (4%)	0	0	4	96 (96%)
Tolon/ Kunbungu	Tolonknhi	240	10 (4%)	102 (43%)	6 (3%)	4 (2%)	102	134 (56%)
Dori	Dori	200	0	36 (18%)	0	0	36	164 (82%)
TOTAL		640	14 (2%)	167 (26%)	8 (1%)	6 (1%)	157	467 (72%)

* Savemed: known medium prevalence of trypanosomosis; Sabobaunk: unknown (few reports of trypanosomosis); Tolonknhi: known high prevalence of trypanosomosis; Dori: known negative population from Burkina Faso; BCT = buffy coat technique; ELISA = enzyme linked immunosorbent assay.

TABLE II. COMPARISON OF BCT AND AB-ELISA RESULTS FOR *T. VIVAX* POSITIVE SAMPLES PER DISTRICT

District	Locality*	No. of samples	BCT +ve	ELISA +ve	BCT+ve/ELISA+ve	BCT+ve /ELISA -ve	BCT-ve /ELISA +ve	BCT-ve ELISA -ve
Savelugu/ Nanton	Savemed	100	5 (5%)	55 (55%)	4 (4%)	1 (1%)	51	44 (44%)
Saboba/ Chereponi	Sabobaunk	100	0 (0%)	30 (30%)	0 (0%)	0	30	70 (70%)
Tolon/ Kunbungu	Tolonknhi	240	14 (6%)	126 (53%)	11 (5%)	3 (1%)	115	71 (30%)
Dori	Dori	200	0	23 (12%)	0 (0%)	0	23	177 (89%)
Total		640	19 (3%)	234 (37%)	15 (2%)	4 (1%)	219	362 (57%)

* Savemed: known medium prevalence of trypanosomosis; Sabobaunk: unknown (few reports of trypanosomosis); Tolonknhi: known high prevalence of trypanosomosis; Dori: known negative population from Burkina Faso; BCT = buffy coat technique; ELISA = enzyme linked immunosorbent assay.

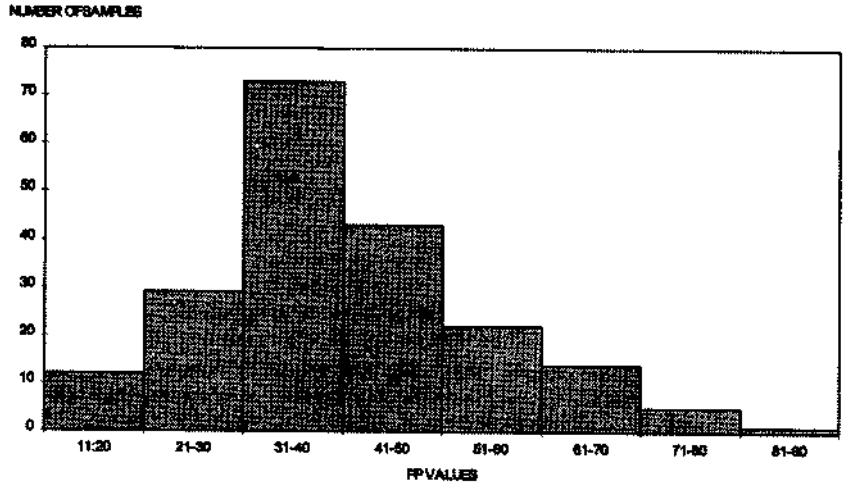

FIG. 2. Frequency distribution of percent positivity (PP) values of sera from a disease free area (Dori, Burkina Faso).

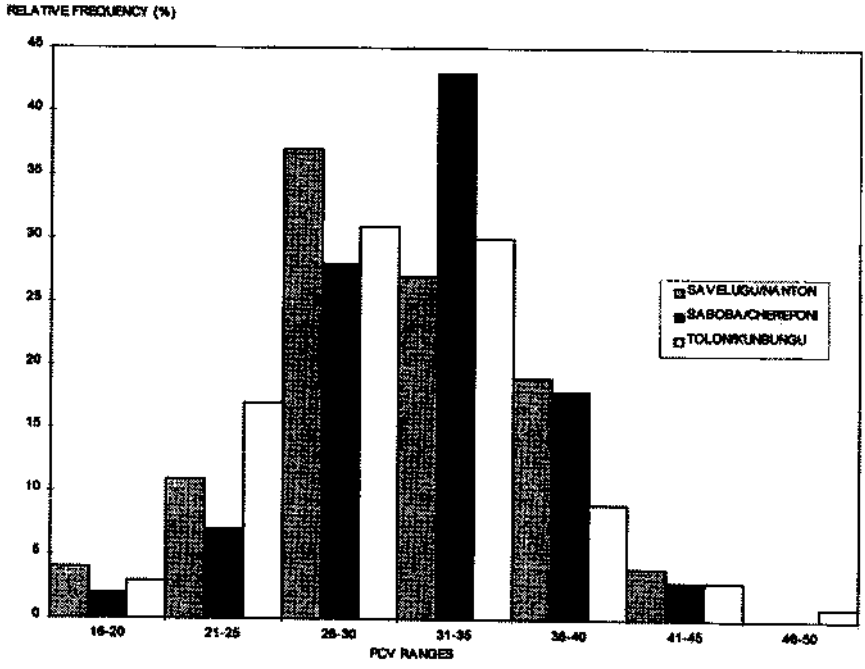

FIG. 3. Frequency distribution of packed red cell volume (PCV) values of sera from three areas with different disease prevalence rates.

TABLE III. BCT AND ELISA POSITIVE RESULTS FOR MIXED INFECTIONS ACCORDING TO DISTRICT

District	Locality*	No. of samples	BCT	Ab-ELISA
Savelugu/Nanton	Savemed	100	2 (2%)	13 (13%)
Saboba/Chereponi	Sabobaunk	100	0 (0%)	2 (2%)
Tolon/Kunbungu	Tolonknhi	240	7 (3%)	65 (27%)
Total		440	9 (2%)	80 (18%)

* Savemed: known medium prevalence of trypanosomosis; Sabobaunk: unknown (few reports of trypanosomosis); Tolonknhi: known high prevalence of trypanosomosis; BCT = buffy coat technique; ELISA = enzyme linked immunosorbent assay.

4. DISCUSSION

The successful control of animal trypanosomosis will require simple, accurate and easily applicable diagnostic tools. Diagnosis could be either used for the identification of active infections (parasitological examination or antigen detection) or exposure to infection (antibody detection and/or parasitological examination). Field validation of any diagnostic test is an indispensable if the test is to be relied upon for epidemiological investigations or the diagnosis of individual animals. Validation requires that testing known negative and positive field samples to determine the specificity and sensitivity of the test. However, in the absence of a tsetse-free zone in Ghana, trypanosome-negative samples were obtained from a tsetse-free zone in Burkina Faso. It was assumed that the serum composition of Zebu cattle (from Dori, Burkina Faso) and the main breeds found in Ghana, West African Shorthorn (WAS) and their crosses (Sanga), did not differ which are. Similarly, it was assumed that the cattle in Burkina Faso had not been exposed to *T. vivax*, which can be mechanically transmitted in tsetse free zones.

Even at a PP of 50% a specificity of 82% was obtained for the assay of the negative sera; this could be due to cross-reactivity to antibodies directed against other haemoprotozoa.

According to the results the prevalence of antibodies against *T. congolense* and *T. vivax* was much higher in all study areas than the parasite prevalence as determined by the BCT: antibodies were detected about 10 times more often in the 440 test samples than trypanosomes. This finding is in agreement with previous reports indicating that the disease in adult cattle in Ghana is usually chronic in nature with low parasitaemias (more than 90% of the serum samples originated from adult animals).

The high disease prevalence of Tolon was confirmed by both the BCT and Ab-ELISA. In Sabobaunk, an area with unknown, but probably low disease prevalence (low tsetse challenge, few reports of clinical disease and no previous disease surveys) a prevalence was detected of antibodies directed against *T. vivax* of 30% as compared to 0% infection rate as determined by BCT. However, the assay for *T. congolense* antibodies showed a low prevalence of 4% in the same area. The predominant cattle breed in this area is the WAS. Although there are few reports of clinical trypanosomosis in the area, it is possible that this is due to the trypanotolerance of the breed, i.e. its superior ability to control parasite levels of *T. vivax*. Moreover, the frequency distribution of PCV values (Fig. 2) suggests that the majority of animals in the study areas had a PCV within the normal range for trypanotolerant breeds. According to the farmers animals had not been treated with trypanocidal drugs during the past 12 months before sampling.

The percentage of samples that were simultaneously positive by BCT and by ELISA was low (0-5%). The poor agreement between the two tests can be due to the fact that firstly the total number of BCT positive cases was low (23 out of 440 samples or 7.5% of the total). Secondly, the presence of antibodies does not necessarily imply active infection; hence antibody prevalence could far exceed the prevalence of trypanosomes as determined parasitologically.

A comparison of the results of antibody assays for single and mixed infections suggests that cross-reactivity did occur between the two trypanosome species tested. Such cross-reactivity might limit the use of the Ab-ELISA, but from a practical point of view it is of no consequence as treatment in the field is often effected regardless which species of pathogenic trypanosome is detected.

In conclusion, the Ab-ELISA demonstrated a high capacity to detect trypanosomal antibodies. During the optimization stage of the test a higher PP value (70%), could have improved the specificity, but would undoubtedly have compromised sensitivity. The precise sensitivity of the test

could not be ascertained due to the limited number of confirmed positive samples available. Consequently, it is necessary to assess the sensitivity using a sufficiently large number of positive cases from the field. Comparison with the distribution curve of the negative population would show the "doubtful zone" and allow a more precise determination of the margin of error.

Despite the gaps in understanding of the performance of the test, it can be an effective diagnostic tool for the determination of antibody status of herds before, during and after tsetse and trypanosomosis control programmes.

The provision of plates pre-coated with trypanosomal antigen is an improvement in the robustness of the test. Furthermore, it offers the opportunity for harmonization in the interpretation of results. There is however a need to modify the assay to a "penside" test, preferably in the form of dipsticks. This would be particularly useful for the diagnosis of individual animals in the field where results are required instantly leading to immediate treatment.

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As representatives for the participants of the Co-ordinated Research Programme we wish to express our appreciation and gratitude through the Joint FAO/IAEA Division to the Government of the Netherlands for providing the funds for the programme. During the final Research Co-ordination Meeting held at ILRI, Ethiopia, from 6-10 September 1999, it was decided to urge the Government of the Netherlands to reconsider the decision to terminate the funding for the programme at such a critical stage when encouraging results are beginning to show.

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VALIDATION OF INDIRECT ELISA SYSTEMS FOR THE SERODIAGNOSIS OF BOVINE TRYPANOSOMOSIS IN ENDEMIC AREAS OF KENYA

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Abstract

VALIDATION OF INDIRECT ELISA SYSTEMS FOR THE SERODIAGNOSIS OF BOVINE TRYPANOSOMOSIS IN ENDEMIC AREAS OF KENYA.

The present study was aimed at validating the performance of four indirect ELISA systems developed for the detection of anti-trypanosomal antibodies in bovine serum. The assay systems employ the use of either native or denatured crude lysate antigens prepared from *Trypanosoma congolense (Tc)* and *Trypanosoma vivax (Tv)*. Assay systems were designated as TcAGd, TcAGn, TvAGd or TvAGn depending on the trypanosome species from which the antigen was prepared (*Tc* or *Tv*) and whether the antigen was denatured (AGd) or native (AGn). The microtitre plates used were precoated with the above antigen preparations at the International Atomic Energy Agency laboratories in Vienna, Austria and shipped to Kenya. Diagnostic sensitivities and specificities were assessed using both known infected and uninfected bovine sera, respectively. All the positive samples were collected from cattle kept in trypanosomosis endemic areas of Galana and Ukunda in Coast province and Mfangano Island in Nyanza province of Kenya. Known negative sera were obtained from animals kept in a non-trypanosomosis endemic area in Muguga, near Nairobi, Kenya. Assay sensitivity ranged from 86% to 97%, while specificity was between 82% and 100% depending on the assay system used. Systems employing denatured antigens had slightly higher, diagnostic sensitivity and specificity. The study has demonstrated that antigen pre-coated plates are useful in circumventing the problem of antigen instability. However, further studies need to be undertaken using a larger sample size to determine if there are any significant differences between plates pre-coated with native and denatured antigens. The present version of indirect ELISA is a useful epidemiological tool and can be incorporated in mapping out the extent of disease.

1. INTRODUCTION

African animal trypanosomosis caused by *Trypanosoma brucei, T. congolense* and *T. vivax* remains one of the major constraints to health and productivity of cattle and other domestic animals in the tsetse endemic areas of sub-Saharan Africa. Owing to its varied clinical manifestations, diagnosis of trypanosomosis cannot be based on clinical signs alone [1]. Reliable diagnostic methods are a prelude to understanding the epidemiology of the disease and an important consideration when assessing the success of trypanosomosis control programmes. Conventional parasitological methods have a limited sensitivity and may lead to under-estimation of the prevalenceof the disease [2]. More sensitive diagnostic tests, including those for the detection of *Trypanosoma* specific antibodies [3] and [4, 5] have therefore been developed. One of the problems that has characterised users of the IAEA ELISA kits for bovine trypanosomosis has been that of maintaining the stability of the antigen during shipment to counterpart laboratories. This study utilised antigen pre-coated plates as a way to try and circumvent the problem. This paper reports on the validation of four indirect ELISA systems using antigen pre-coated polystyrene microplates. The usefulness of the current version of indirect ELISA as an epidemiological tool for studies in bovine trypanosomosis is discussed.

2. MATERIALS AND METHODS

2.1. Sample collection and description

Cattle populations kept in two different trypanosomosis endemic areas of coastal Kenya, namely; Galana, and Ukunda and on Mfangano island in Nyanza Province were used in this study. Trypanosomosis control programmes were on-going in Galana and Ukunda, but not on Mfangano. All the animals were monitored once a week for trypanosomosis using the buffy coat technique (BCT) and packed red cell volume (PCV). Blood for serum preparation was collected once a month and all field sera classified as either positives or negatives on the basis of whether or not parasites could be demonstrated in the blood by microscopy. For the purpose of validating the current version of indirect ELISA, a pool of 500 known positive sera obtained from any of the above locations was used. It is therefore worth noting that even though the samples were collected sequentially over a period of time,

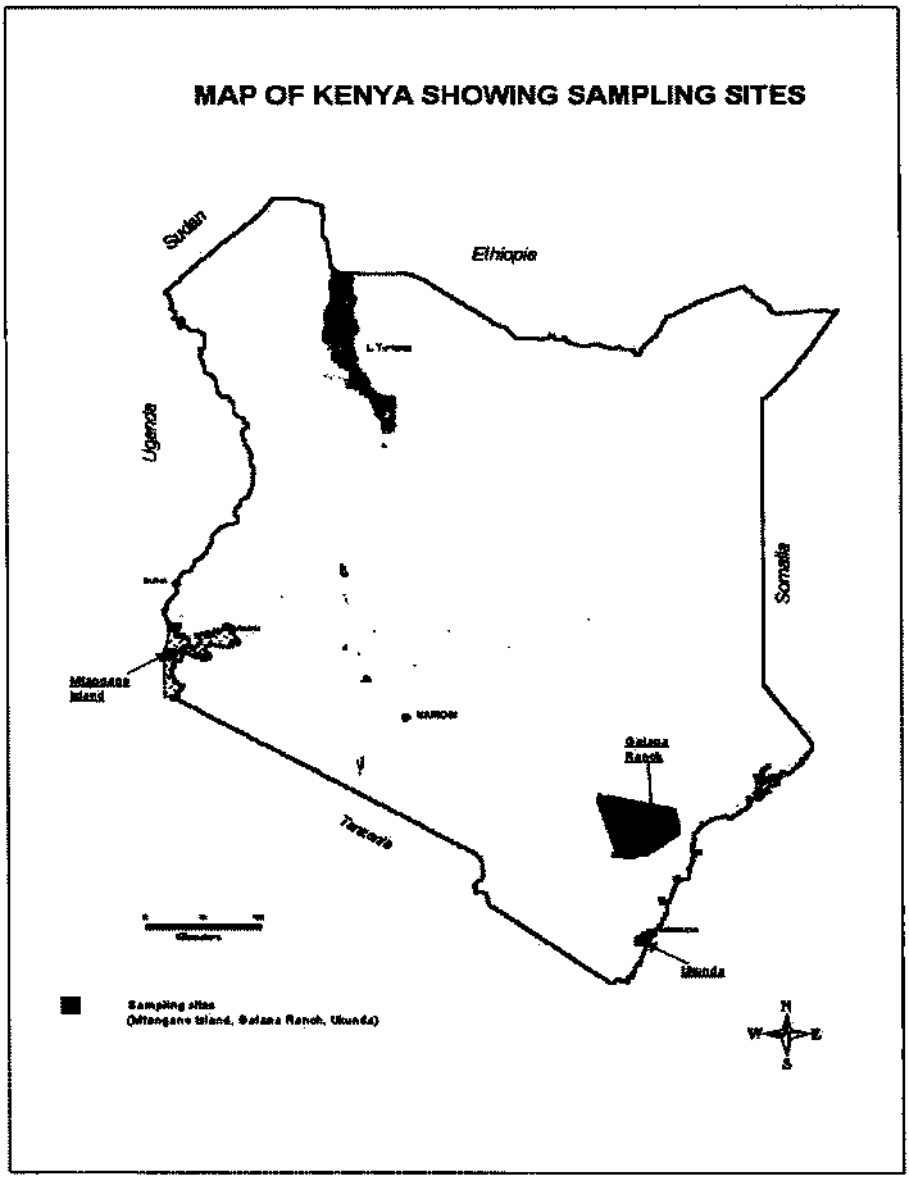

FIG.1. Map of Kenya showing the study areas.

the diagnostic sensitivities reported in this paper were calculated from BCT positive samples only. However, for each of the assay systems a set of samples from the disease endemic, but BCT negative were included in the analysis. Known negative serum samples used to determine the cut-off percent positivity (PP) value were obtained from cattle belonging to the Kenya Agricultural Research Institute (KARI) and the Kenya Trypanosomiasis Research Institute (KETRI) dairy herd. Both the KETRI and

KARI animals are kept in a non-trypanosomosis endemic area in Muguga, near Nairobi. It was against this background that the animals were classified as a 'known negative' population.

2.2. Validation of *Trypanosoma congolense* and *Trypanosoma vivax* indirect ELISA's

Serum samples collected from cattle of known infection status in trypanosomosis endemicareas (described above) and known uninfected cattle were tested using the indirect TcAGd, TcAGn, TvAGd and TvAGn ELISA systems to determine the diagnostic sensitivity (D-SN) and diagnostic specificity (D-SP) of the test. The reagents used for the assay were supplied by the International Atomic Energy Agency (IAEA) and included internal quality control reference sera of C++, C+ and C-, conjugate and antigen pre-coated polystyrene microplates.

2.3. ELISA protocol

The indirect *T. congolense* and *T. vivax* ELISAs were used to test both the negative and positive sera following the bench protocol version iTAB 1.0, provided by FAO/IAEA [6]. Briefly, antigen-precoated Immulon® 1 microplates were reconstituted by adding ice-cold PBS (100 µl/well) and incubated at 37°C for 1 hour while shaking. The microplates were then washed four times with PBS containing Tween 20 (PBS-T, pH 7.4). Test and control sera diluted 1/400 (TvAGd and TvAGn), 1/100 (TcAGd) and 1/200 (TcAGn) in sample diluent buffer (PBS-T with 5% w/v skimmed milk) were then dispensed in duplicate (100 µl/well) and incubated at 37°C for 1 hour with rotation. Anti-bovine IgG conjugate diluted 1/20,000 was added and incubated for one hour. After four cycles of washing, hydrogen peroxide and TMB (substrate/chromogen) were mixed at a ratio of 1:1 and dispensed (100 µl/well) then incubated at 37°C for 15 minutes after which 1M orthophosphoric acid was added (100 µl/well) to stop the reaction. The absorption was measured at 450 nm wavelength using a multichannel spectrophotometer (Multiskan® Plus MKII, Labsystems, Finland).

2.4. Data expression

The optical density (OD) values were expressed as percent positivity (PP) values. PP values for internal quality control (IQC) samples were calculated as part of the quality assurance and used as a basis of accepting the test sera results. The PP values for each plate were calculated based on the OD value of the respective C++ as follows:

$$\frac{\textit{Replicate OD values of each control}}{\textit{Median OD value of C++ control}} \times 100 = \textit{PP values for quality control}$$

$$\frac{\textit{Replicate OD value of test serum}}{\textit{Median OD value of C++ control}} \times 100 = \textit{PP values for acceptance of test sera}$$

2.5. Determination of cut-off PP values

A number of methods are in existence for the determination of seropositive/seronegative threshold (cut-off) values. However, for this study, the cut-off selection was done according to the visual inspection method described by Jacobson [7]. Briefly, based on the visual inspection of frequency distributions of test results from known infected and uninfected animals, the cut-off was placed at the intersection of the two distributions. The cut-off was also determined by a modified receiver-operator characteristic (ROC) analysis [8]. In both cases, a PP value of 30% was taken as the cut-off value. Any value from the negative population above 30% was considered as false positive, while those from the positive population falling below this value were false negatives.

2.6. Determination of diagnostic sensitivity and specificity

The diagnostic sensitivity (D-SN) and diagnostic specificity (D-SP) were calculated as shown in Table I and as described by Jacobson [7].

TABLE I. CALCULATION OF DIAGNOSTIC SENSITIVITY (D-SN) AND DIAGNOSTIC SPECIFICITY (D-SP)

Test	Animals of known infection status		Total
	Infected	Uninfected	
AB-ELISA +VE	True positive (TP)	False positive (FP)	TP+FP
AB-ELISA -VE	False negative (FN)	True negative (TN)	FN+TN
Calculation	D-SN = TP/TP+FN	D-SP = TN/FP+TN	

Ab-ELISA = antibody-detection enzyme linked immunosorbent assay

3. RESULTS

3.1. Diagnostic sensitivity (D-SN) of the test systems

The diagnostic sensitivities and specificities achieved by the four assay systems are shown in Tables II and III.

TABLE II. SENSITIVITY OF THE FOUR ASSAY SYSTEMS CALCULATED FROM BCT POSITIVE SAMPLES

Assay System	Total no. tested by Ab-ELISA (BCT -ve from endemic areas)	BCT positive (a)	Ab-ELISA positive (b)	Diagnostic Sensitivity (%) (b/a x 100)
TcAGd	920 (420)	500	476	95
TcAGn	880 (380)	500	455	91
TvAGd	920 (420)	500	485	97
TvAGn	720 (220)	500	430	86

BCT = buffy coat technique; -ve = negative; The four tests (TcAGd, TcAGn, TvAGd, TvAGn) used as antigen either *T. congolense* (Tc) or *T. vivax* (Tv) in a denatured (AGd) or native (AGn) state.

TABLE III. SPECIFICITY OF THE ASSAY SYSTEMS AS DETERMINED BY SERA COLLECTED IN A NON TRYPANOSOMOSIS ENDEMIC AREA

Assay system	No. of known -ve samples tested	-ve by ELISA (TN)	+ve by ELISA (FP)	Diagnostic specificity (%) (TN/TN+FP) ×100
TcAGd	80	76	4	95
TcAGn	120	98	22	82
TvAGd	80	80	0	100
TvAGn	160	151	9	94

-ve = negative; +ve = positive; The four tests (TcAGd, TcAGn, TvAGd, TvAGn) used as antigen either *T. congolense* (Tc) or *T. vivax* (Tv) in a denatured (AGd) or native (AGn) state; TN is True negative; FP is False positive.

4. DISCUSSION

The four indirect ELISA systems validated during this study have been shown to have a diagnostic sensitivity of between 86% and 97% and a specificity of 82% to 100%. The highest diagnostic sensitivity (97%) and specificity (100%) were achieved using TvAGd. The reason for the relatively high sensitivity and specificity obtained using assay systems employing denatured antigen preparation is not known. However, past experience (Ouma *et al.*, unpublished data; Rebeski, personal communication) has shown that use of native antigen for coating ELISA plates results in a general decrease in the optical density (OD) signals. It is possible that by treating the antigen prior to coating of micro-plates, certain factors that either deteriorate or cause deactivation of the antigen are stabilised. In the present study, the OD values for both the native and denatured antigen-coated

microplates showed very little variation over time. The ODs of the quality control sera (C++, C+ and C-) also remained within the expected lower and upper control limits throughout the study. Given that antigen instability has been one of the major setbacks that has characterised IAEA test kits before, it appears the pre-coating of ELISA plates offers a solution to this problem.

One of the major aims of this study was to incorporate the indirect ELISA in monitoring trypanosomosis control programmes in Kenya. A major obstacle to achieving this objective is the fact that antibodies are known to persist in circulation up to 10 months after successful therapy or self-cure [9], hence introducing the phenomenon of false positives in a herd. Interpretation of results gets even more complicated when dealing with serum samples from animals kept in tsetse infested (disease endemic) areas. In such areas it is difficult to discriminate between past and current infection. In the present study, some of the animals from disease endemic areas that were BCT negative were positive by Ab-ELISA. This phenomenon implies that the antibody detection assays cannot be used as a basis to institute treatment. However, Ab-ELISA is considered an important epidemiological tool for monitoring sero-prevalence of the disease and not for diagnosis of individual animals [10]. Combining serological (Ab- ELISA) with standard parasitological tests (BCT) produces more reliable results than using Ab-ELISA alone.

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EVALUATION OF AN ANTIBODY-DETECTION ELISA USING PRE-COATED PLATES IN THE DIAGNOSIS OF *TRYPANOSOMA CONGOLENSE* AND *T. VIVAX* INFECTIONS IN CATTLE IN MALI

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Abstract

EVALUATION OF AN ANTIBODY-DETECTION ELISA USING PRE-COATED PLATES IN THE DIAGNOSIS OF *TRYPANOSOMA CONGOLENSE* AND *T. VIVAX* INFECTIONS IN CATTLE IN MALI.

Under the Coordinated Research Programme " Use of Immunoassay for improved diagnosis of trypanosomosis and monitoring trypanosomosis control programmes", a new ELISA test has been validated. The test was designed for trypanosome specific antibody detection. The purpose of the work was to study the main parameters of the test (sensitivity and specificity) using reference sera provided by IAEA and field sera collected in Mali. The field sera were collected in different ecological zones and included sera from trypanosomosis endemic areas as well as sera from tsetse free areas. The test was performed according to the protocol proposed by IAEA. The results have shown a poor stability of the reference sera for both *T. congolense* and *T. vivax*. By using de-iointized water, we got better OD's for *T.vivax* C++ reference serum, while the *T.congolense* C++ had to be replaced by a locally produced reference serum. Under the above conditions, the *T. congolense* system gave quite encouraging results (sensitivity: 100%; specificity: 87.5-95%). This system was able to differentiate quite well animals from tsetse infected areas from those originating from tsetse free areas and could therefore be used in epidemiological surveys.

The *T. vivax* system showed a good sensitivity (100%), but a poor specificity (37.5-57.5%). The *T. vivax* system could be improved by establishing a cut-off point based on local negative populations.

1. INTRODUCTION

In Africa, animal trypanosomosis is a major constraint to rural development, especially to livestock production. The distribution of the disease and its economic importance are not yet fully determined in many parts of sub-Saharan Africa. This is partly due to the low sensitivity of currently used parasitological techniques [1]. Problems with parasitological diagnosis affect not only studies on disease prevalence, but also the effective application of trypanocidal drugs since infected animals with no patent parasitaemia may not be detected and treated.

Improved diagnosis could help in defining the distribution of the disease and also in improving control measures through more accurate detection and treatment of active infections. The need for more sensitive and specific diagnostic tools has led to the development of an antigen-detection ELISA by ILRAD [2]. The validation of this ELISA was conducted by different African Laboratories under coordination of IAEA. The general evaluation of this ELISA was not positive due to insufficient sensitivity and specificity of the system. It was therefore suggested to validate an antibody-detection ELISA system developed by IAEA at Seibersdorf. The first evaluation of that newly developed system was carried out in1998 and the results indicated some problems in terms of reagent stability (coating antigen or reference sera). As a partial solution to that, precoated plates have been delivered by IAEA. Here we are reporting on the effectiveness of an antibody-detection ELISA system using plates pre-coated with denatured trypanosomal antigen.

2. MATERIALS AND METHODS

2.1. Biological reagents

Reference sera were provided by IAEA and identified as C++ (high positive), C+ (moderate positive, C- (negative) for both *T. congolense* and *T. vivax*. Polystyrene 96-well flat bottom microplates, (Dynex Immulon 1, USA) were provided by IAEA. The plates were coated with *T. congolense* and *T. vivax* denatured antigens.

2.2. Field sera

A total of 240 cattle sera were used in the study. The sera were divided into 3 groups according to their geographical origin (Fig. 1).

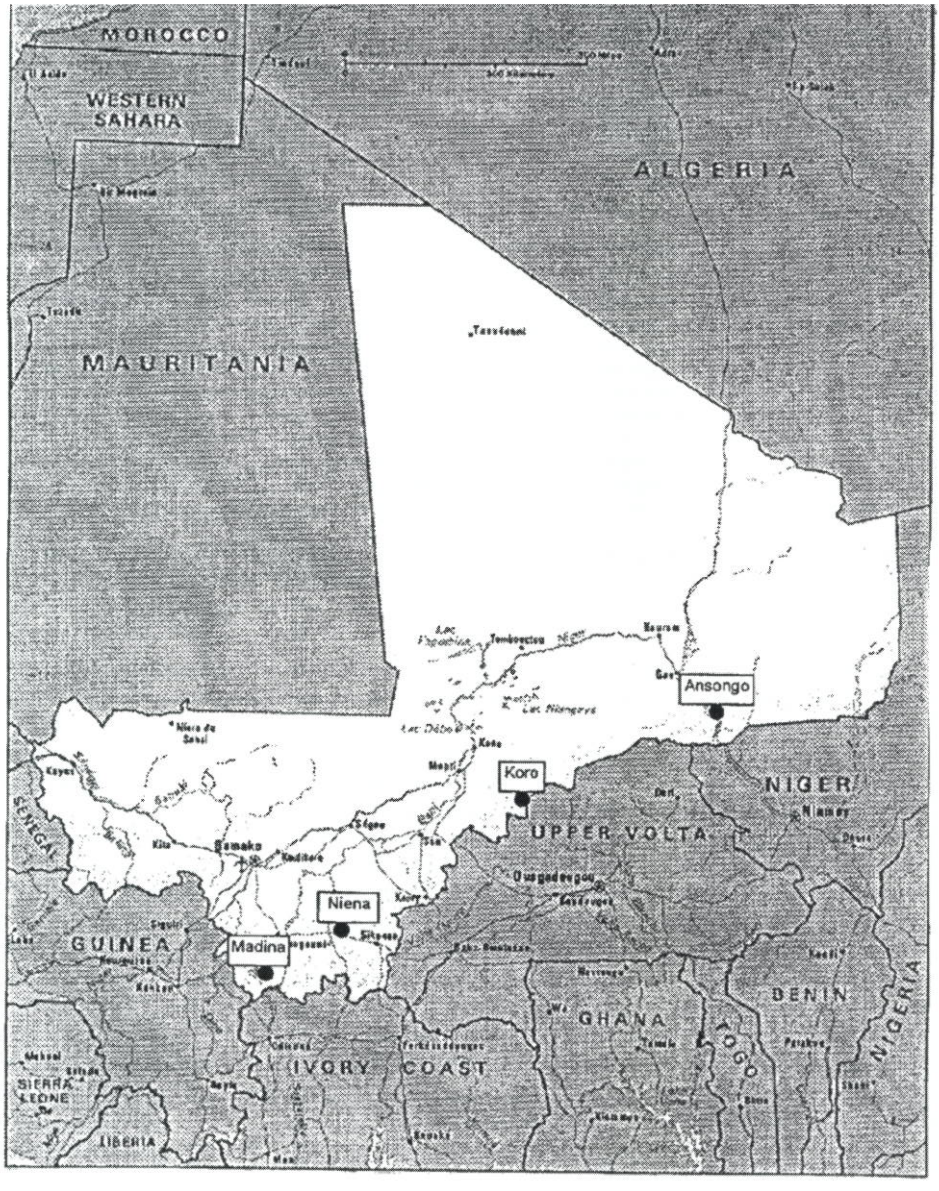

FIG. 1. Map of Mali indicating the various study areas.

2.2.1. Sera from a tsetse and tick-borne disease free area

The sera (n=40) had been collected during Rinderpest sero-epidemiological surveys in Koro (15°40' N and 00°30' E), stored at –20°C and catalogued at the serum bank of LCV. It is generally accepted that tsetse flies and ticks of veterinary importance are virtually absent. However, no examination for parasites was done in that geographical area.

2.2.2. Sera from a tsetse free, but tick infested area

The sera (n=40) had been collected during the Rinderpest sero-epidemiological survey in Ansongo (N= 15°40' and E= 00°30'), stored at –20°C and kept in the serum bank of LCV. Tsetse flies were apparently absent, but ticks of veterinary importance were present due to the proximity of the

river Niger and its tributaries. No parasitological examination was done for the purpose of parasite detection.

2.2.3. Sera from tsetse and tick-borne disease endemic areas

Serum samples were collected in the ranch of Madina-Diassa (10°49' N and 7°44' W) and in the village of Niéna (11°25' N and 6° 23' W), both located in the south of the country.

The sera were stored at –20°C in the serum bank of LCV. For all the serum donors, parasite examination by BCT was carried out and depending on the results, 160 sera were selected and four subgroups were established:

- sera from *T. congolense* positive animals,
- sera from *T. vivax* positive animals,
- sera from BCT-negative animals with PCV<27,
- sera from BCT-negative animals with PCV ≥27.

2.3. Antibody-detection ELISA

The antibody-detection ELISA (Ab-ELISA) technique was carried out as described in the FAO/IAEA test protocol [3].

2.4. Data analysis

Data interpretation was carried out using the EDI programme (Version 2.2, October 1997). The test sera demonstrating mean PP values equal to or greater than the calculated threshold PP value were considered to be positive. Test sera demonstrating mean PP values less than calculated threshold PP value were considered to be negative.

3. RESULTS

3.1. Stability of reagents

Reference sera were defrosted (from –20°C) on day 1 and tested in both the *T. congolense* and *T. vivax* system (Tables I and II). Stability of the reference sera was tested by storing them at +4°C after defrosting and testing them once during the next three days.

TABLE I. OPTICAL DENSITY (OD) VALUES OF *T. CONGOLENSE* REFERENCE SERA

Reference serum	Day 1	Day 2	Day 3	Day 4
C++	1.242	0.093	0.119	0.268
C+	0.537	0.047	0.069	0.104
C–	0.142	0.04	0.038	0.046

TABLE II. OPTICAL DENSITY (OD) VALUES OF *T. VIVAX* REFERENCE SERA

Reference serum	Day 1	Day 2	Day 3	Day 4
C++	0.897	0.664	0.784	1.077
C+	0.350	0.352	0.344	0.766
C–	0.132	0.137	0.135	0.233

The results showed that the reference sera when used immediately after defrosting produced OD values as expected for both the *T. congolense* and *T. vivax* systems. During the subsequent runs (days 2 and 3), the same sera stored at +4°C showed unexpectedly low OD values for both *T. congolense*, and *T. vivax* (Figs 2 and 3). At day 4, the replacement of distilled water by deionized water improved the OD's in the *T. vivax* system, but not in the *T. congolense* system.

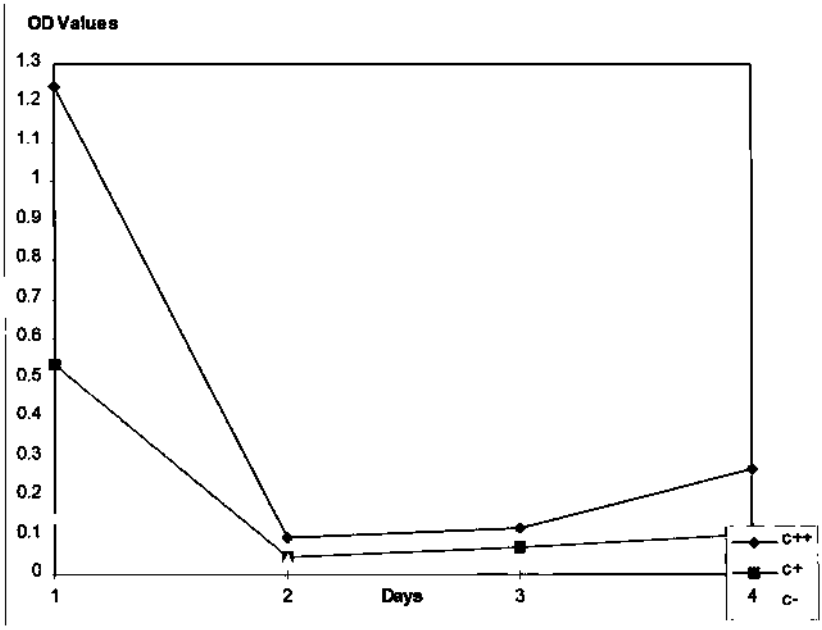

FIG. 2. Stability of the control sera for the T. congolense *coated antibody-detection ELISA.*

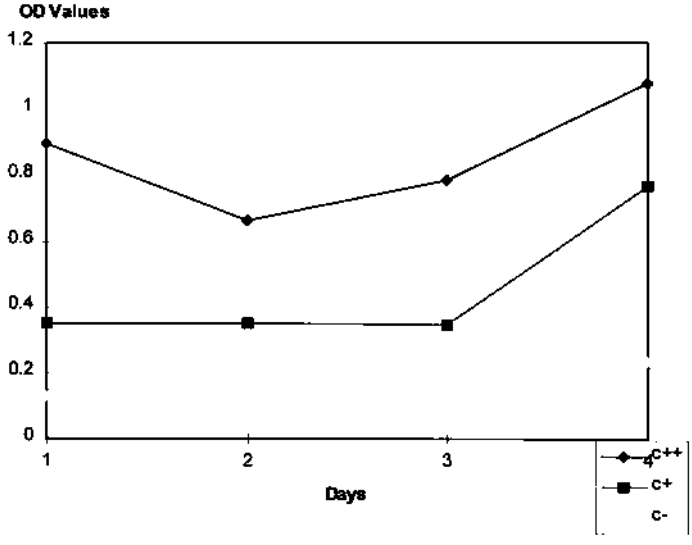

FIG. 3. Stability of the control sera for the T. vivax *coated antibody-detection ELISA.*

3.2. Analysis of serum samples using *T. congolense* coated plates

The antibody detection ELISA (Ab-ELISA) showed a maximal sensitivity (100%) using the BCT-positive population. The specificity as measured on cattle sera from a tsetse free area was 87.5-95%. In the BCT-negative populations from a trypanosomosis endemic area, antibody prevalence was high (52.5-70%). The test has showed good sensitivity and an acceptable specificity. Using the test results a clear difference can be made between serum samples from tsetse free and tsetse infested areas.

TABLE III. RESULTS OF SERUM ANALYSIS USING PLATES COATED WITH *T. CONGOLENSE* ANTIGEN

Group	n	Mean PCV	ELISA positive	Mean OD
Sera from a tsetse and tick-borne disease free area	40	ND	2 (5%)	0.069
Sera from a tsetse free but tick infested area	40	ND	5 (12.5%)	0.081
Sera from tsetse and tick-borne diseases endemic area, BCT- negative (PCV>27)	40	35.2	21 (52.5%)	0.183
Sera from tsetse and tick-borne diseases endemic area, BCT- negative (PCV<27)	40	21.16	28 (70%)	0.300
Sera from tsetse and tick-borne diseases endemic area, BCT-positive	40	23.7	40 (100%)	0.450

n = number of sera; PCV = packed red cell volume; ELISA = enzyme-linked immunosorbent assay; OD = optical density; ND = not done; Mean OD of the C++ in the tests was 0.9-1.4.

3.3. Analysis of serum samples using *T. vivax* coated plates

The Ab-ELISA showed a high sensitivity in BCT-positive animals (100%), but relatively low specificity (37.5-57.5%) as measured on populations from tsetse-free areas. The mean OD in that population was very low as compared to that in the BCT-positive population and indeed close to the threshold.

TABLE IV. RESULTS OF SERUM ANALYSIS USING PLATES COATED WITH *T. VIVAX* ANTIGEN

Group	n	Mean PCV	ELISA positive	Mean OD
Sera from a tsetse and tick-borne disease free area	40	ND	17 (42.5%)	0.216
Sera from a tsetse free, but tick infested area	40	ND	25 (62.5%)	0.255
Sera from tsetse and tick-borne disease endemic areas, BCT-negative (PCV>27)	40	35.2	33 (82.5%)	0.859
Sera from tsetse and tick-borne disease endemic areas, BCT-negative (PCV<27)	40	21.16	40 (100%)	1.318
Sera from tsetse and tick-borne disease endemic areas, BCT-positive	40	28.6	40 (100%)	1.053

n = number of sera; PCV = packed red cell volume; ELISA = enzyme-linked immunosorbent assay; OD = optical density; ND = not done; Mean OD of the C++ in the tests was 0.9-1.5.

4. DISCUSSION

As in previous studies, our present work has revealed a problem in the stability of the reference sera provided by the Joint FAO/IAEA Division. This was mainly the case with the C++ reference serum for *T. congolense*, which persistently gave low OD values despite the use of de-ionized water for solution preparation. Field sera stored in the LCV serum bank for several months and used in this study showed a good stability. The production procedure of reference sera should be reviewed in order to find an objective explanation for this phenomenon. After adopting the use of de-ionized water for solution preparation and replacing the reference C++ serum with a local field serum, the evaluation of the *T. congolense* ELISA system gave rather encouraging results. It showed a maximum sensitivity (100%) in the BCT-positive population, while the specificity measured on a cattle population from a tsetse free area was 87.5-95%. In BCT-negative populations from a trypanosomosis endemic area, the prevalence of trypanosomal antibodies was found to be high (52.5-70%). The test has shown a good sensitivity and an acceptable specificity. The results showed that a clear difference can be made between tsetse free and tsetse infested areas. The *T. congolense* Ab-ELISA may, therefore, be useful in prevalence studies, but not for individual diagnosis.

The ELISA system coated with *T. vivax* antigen showed a high sensitivity in BCT-positive animals (100%), but a relatively low sensitivity (37.5-57.5%) as measured on populations from tsetse free areas. This means that about 50% of the animals from tsetse free areas were positive to *T. vivax*. Cattle from these regions do not transhume in tsetse infested areas and are very far from the tsetse belt (500-1000 km). If *T. vivax* were to be found in these regions it should be sporadically. The mean OD in that population was very low as compared to that in BCT-positive populations and indeed close to the threshold value. Consequently, a better differentiation between populations from trypanosomosis endemic areas on the one hand and populations from trypanosomosis free areas on the other hand could be obtained by establishing a threshold value using serum samples collected locally from parasitologically negative populations.

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EPIDÉMIOLOGIE DE LA TRYPANOSOMOSE ANIMALE DANS LA RÉGION DES NIAYES DU SÉNÉGAL

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Abstract

EPIDEMIOLOGY OF ANIMAL TRYPANOSOMOSIS IN THE NIAYES REGION OF SENEGAL.

A study was performed in the Niayes region of Senegal to verify the return of tsetse and animal trypanosomosis 25 years after successful eradication of the disease and the vector from the region. Entomological investigations using tsetse traps demonstrated that a large part of the study area has been reinvaded by tsetse flies. Parasitological investigations of blood samples collected from cattle from the region showed rather high prevalence levels of *Trypanosoma vivax*, but did not indicate the presence of *T. congolense* or *T. brucei*. A serological survey of cattle using ELISA tests to detect antibodies directed against *T. congolense* and *T. vivax* showed high antibody prevalence levels for the two trypanosome species. Finally, the study permitted to establish the presence of antibodies directed against *T. congolense* and *T. vivax* in serum samples collected from the field using ELISA tests developed by the International Atomic Energy Agency (IAEA) in Vienna. The specificity of each of the two tests was found to be 95%. It is suggested that the two serological tests are used in combination with parasitological techniques in the diagnosis and epidemiology of bovine trypanosomosis.

Résumé

EPIDÉMIOLOGIE DE LA TRYPANOSOMOSE ANIMALE DANS LA RÉGION DES NIAYES DU SÉNÉGAL.

Cette étude a été faite dans la région des Niayes du Sénégal, pour établir le retour ou non des glossines et des trypanosomoses animales, plus de 25 ans après une campagne d'éradication des tsé-tsé de la région. Des investigations entomologiques menées à l'aide de pièges à glossines montrent qu'une bonne partie des zones étudiées a été ré envahie. Des examens parasitologiques de sang de bovins vivant dans la région indiquent des taux de prévalence de la trypanosomose à *Trypanosoma vivax* assez élevés, mais n'ont pas permis de visualiser *T. congolense* ni *T. brucei*. Un criblage sérologique réalisé sur les bovins avec des tests ELISA de détection d'anticorps anti *T. congolense* et anti *T. vivax* donnent des taux de séro-prévalence élevés pour ces deux espèces de trypanosomes. Enfin, l'étude a permis d'évaluer avec ces sérums de terrain un trousseau ELISA-anticorps pour le diagnostic de la trypanosomose à *T. congolense* et à *T. vivax*, mis au point par les laboratoires de l'Agence Internationale de l'Energie Atomique (AIEA) de Vienne. Une spécificité égale à 95 % a été trouvée pour chacun des deux tests. Les auteurs suggèrent l'utilisation de ces tests sérologiques en association avec des techniques parasitologiques de diagnostic dans les études épidémiologiques concernant les trypanosomoses bovines.

1. INTRODUCTION

La région des Niayes du Sénégal est une bande de terres d'une superficie de 10 000 hectares, qui s'étend sur environ 300 km le long de la côte atlantique Ouest du Sénégal, de la banlieue Nord de Dakar à l'ancienne province du Gandiole située peu avant Saint Louis.

La fertilité des terres de cette région en a fait le lieu d'une intense activité agropastorale: culture maraîchère et fruitière, élevage de bovins (N'Dama, Zébus et croisement Zébu x N'Dama), ovins, caprins et volailles. Le développement de ces activités était cependant limité par la présence des glossines (*Glossina palpalis gambiensis*) qui transmettaient localement *Trypanosoma brucei, T. vivax* et *T. congolense*.

Pour lever cette contrainte, une campagne d'éradication des glossines a été menée dans la région par le Laboratoire national de l'Elevage et de Recherches vétérinaires de Dakar, de 1970 à 1972 [1]. Le succès de cette campagne avait débouché sur un important essor économique: augmentation des superficies cultivées, implantation de fermes laitières avec importation de vaches européennes à haut rendement, consolidation des effectifs des troupeaux villageois de bovins, multiplication des élevages avicoles (poulets de chair et poules pondeuses), naissance de petites unités de fabrique d'aliments pour bétail, ouverture de dépôts de médicaments vétérinaires, etc.

Une alerte devait cependant survenir en juillet 1990 avec la capture d'un mâle de *Glossina palpalis gambiensis* dans un village de la région et le diagnostic de deux cas de trypanosomose bovine à *T. congolense* dans les élevages traditionnels du même village. Par la suite, des sondages entomologiques ont été effectués, ainsi que des examens parasitologiques sur les bovins. Ces investigations, ponctuelles et espacées, n'avaient pas permis de tirer des conclusions précises.

Cette étude a pour objectif d'obtenir des précisions sur l'ampleur de la ré-infestation éventuelle des Niayes par les glossines, les espèces de trypanosomes présentes chez les vecteurs, chez les animaux domestiques et éventuellement au niveau de la faune sauvage. Cela permettrait une meilleure compréhension de l'épidémiologie de la trypanosomose animale dans cette région. Des propositions de mesures à prendre pour une lutte efficace pourraient alors être faites.

2. MATÉRIEL ET MÉTHODES

2.1. Investigation entomologique

Des sondages entomologiques consistant en la pose de pièges "Challier-Laveissière" [2] ont été effectués dans la région. Les glossines capturées ont été identifiées puis triées selon le sexe et ensuite comptées.

2.2. Investigation parasitologique et sérologique

Deux grandes zones de la région, allant du sud vers le nord de Dakar, ont été parcourues au cours de quatre missions effectuées de septembre 1998 à juin 1999. Les villages visités avoisinent des gîtes potentiels de glossines, et les animaux domestiques prélevés se rendent tous les jours en ces lieux pour le pâturage et l'abreuvement.

- Zone Niayes-Sud ou zone Bambilor-Pout située à 40-60 km au nord de Dakar. Les bovins prélevés sont ceux des villages de Keur Ndiaye Lo, Diaksaw Peulh, Ndiakhirate et Palal. Dans cette zone 198 bovins de race *Diakore* (croisement Zébu x N'Dama) ont été saignés pour la recherche de trypanosomes par la technique du BCT [3] et pour la récolte de sérums en vue du diagnostic sérologique. Parmi ces sérums un échantillon de 115 prélèvements a été testé par la technique ELISA.
- Zone Niayes-Centre ou zone Tivaouane-Mboro: elle se situe au nord de la zone précédente, à 100-150 km de Dakar. Les prélèvements effectués dans cette zone ont concerné les bovins du village de Noto Gouye Djama et ceux des environs immédiats de Mboro. Un total de 650 prélèvements de sang ont été faits. La lecture de l'hématocrite et l'examen au BCT n'ont pas pu s'effectuer dans cette zone en raison de coupures régulières d'électricité. Ce travail reste cependant à faire, de même que l'examen parasitologique des équidés, des ovins et caprins. Par contre, les sérums de ces 650 bovins ont été récoltés; 40 parmi eux ont été testés en ELISA. Les sérums non encore analysés sont conservés à –20°C et seront testés lorsque des données parasitologiques seront obtenues sur les bovins de cette zone.

Par ailleurs, 40 sérums de bovins Zébus du Centre de Recherches Zootechniques (CRZ) de Dahra, situé dans le Nord-Est du Sénégal indemne de glossines, ont été obtenus de la banque du programme "Anaplasmose" du Laboratoire National d'Elevage et de Recherches Vétérinaires et analysés comme échantillon de la population négative.

Ces épreuves ELISA indirecte de détection d'anticorps ont été réalisées à partir de plaques pré-sensibilisées respectivement avec un antigène de *Trypanosoma congolense* et avec un antigène de *T. vivax*. Les plaques pré-sensibilisées, ainsi que les autres réactifs biologiques et chimiques nécessaires à l'exécution des tests ont été fournis par l'AIEA de Vienne sous la forme d'un trousseau complet de diagnostic [4, 5].

3. RÉSULTATS

3.1. Sondage entomologique

Au total 61 glossines (*G. palpalis gambiensis*) ont été capturées dans les deux zones, avec un plus grand nombre dans la zone Niayes-Sud. En outre, le nombre de mâles capturés est plus important que celui des femelles (Tableau I).

TABLEAU I. RÉSULTATS DU SONDAGE ENTOMOLOGIQUE EFFECTUÉ DANS LES DEUX ZONES VISITEÉS

Zone de capture	Nombre et sexe des glossines capturées (*G. palpalis gambiensis*)		
	Mâles	Femelles	Total
Niayes-Sud	22	16	38
Niayes Centre	14	9	23
Total	36	25	61

3.2. Diagnostic parasitologique des trypanosomoses bovines par la technique BCT

Les résultats du BCT indiquent la présence de *Trypanosoma vivax* chez les bovins des quatre villages visités dans la zone Niayes-Sud. Des taux de prévalence faible (2%) à très élevé (20%) ont été trouvés, avec une prévalence moyenne de 10 %. Nous n'avons pas rencontré de cas de *T. brucei* ni de *T. congolense* (Tableau II).

TABLEAU II. RÉSULTATS DES EXAMENS PARASITOLOGIQUES EFFECTUÉS AU BCT SUR LES BOVINS VISITÉS DANS LA ZONE NIAYES-SUD

Nombre de bovins positifs à *T. vivax** et prévalences dans les villages visités					
	Keur Ndiaye Lo	Diaksaw Peulh	Palal	Ndiakhirate	Total
Nombre de bovins examinés	50	50	48	50	198
Nombre de bovins positifs	1	3	4	10	18
Prévalence %	2.0	6.0	8.3	20.0	9.09

*Aucun cas de *Trypanosoma congolense* ni de *T. brucei* n'a été détecté.

3.3. Résultats de l'évaluation des deux tests ELISA

3.3.1. Résultats des témoins de référence

Avec le test à *T.congolense* comme avec celui à *T. vivax*, les sérums témoins de référence fort (C++) et modéré (C+) ont quasi constamment donné des réactions positives très franches. Le témoin de référence négatif (C-), sans jamais être positif, a donné assez souvent des réactions supérieures à la limite qui lui est attribuée. Le témoin Conjugué seul (Cc) a donné dans l'ensemble de bons résultats (Tableau III).

TABLEAU III. FREQUENCE (%) DES RESULTATS OBTENUS AVEC LES TEMOINS DE REFERENCE

Témoins de référence	Test *T. congolense*			Test *T. vivax*		
	PP < L.I.	PP. N.	PP > L.S.	PP < L.I.	PP. N.	PP > L.S.
C++	5	95	0	5	90	5
C+	30	40	30	5	60	35
C-	2	30	60	10	25	65
Cc	0	70	30	0	85	15

PP = Pourcentage de positivité ; L.I. = Limite Inférieure acceptable; N. = dans les limites normales admises; L. S. = Limite Supérieure acceptable.

3.3.2. Etablissement des seuils de positivité et détermination de la spécificité des tests

Le principe retenu pour établir les seuils de positivité est, pour chaque test, d'ajouter 2 écarts types à la moyenne des pourcentages de positivité obtenus par les sérums de la zone indemne de glossine. Les calculs effectués à partir des résultats des 80 cupules des 40 sérums du CRZ-Dahra (Tableau IV) ont donné les seuils de positivité suivants :

- Test *T. congolense* : seuil de positivité : PP > 40 %
- Test *T. vivax* : seuil de positivité : PP > 30 %

S'agissant de la spécificité de ces deux tests ELISA, dans les deux cas, seulement 5 % de l'échantillon de la population négative dépassent les seuils de positivité retenus (Tableau IV). Cela représente une spécificité de 95 %, qui fait de ces tests des outils de diagnostic assez rigoureux.

TABLEAU IV. COMPARAISON DES RESULTATS DES SERUMS DE LA ZONE INDEMNE AVEC CEUX DU SERUM DE REFERENCE NEGATIF

Niveaux des PP	Fréquence (%) des différents niveaux de pourcentages de positivité (PP)			
	Test *T. congolense*		Test *T. vivax*	
	Sérums Zone indemne	Référence négatif	Sérums Zone indemne	Référence négatif
0 – 10 %	7.5	30.0	20.0	20.0
11 – 20	22.5	45.0	55.0	55.0
21 – 30	42.5	25.0	20.0	25.0
31 – 40	22.5	0	5.0	0
41 – 50	5.0	0	0	0
Total	100	100	100	100

Seuils de positivité = Test *T. congolense* : PP > 40% ; Test *T. vivax* : PP > 30 % .

3.4. Diagnostic sérologique des trypanosomoses bovines par la technique ELISA indirecte

3.4.1. Résultats du test ELISA utilisant l'antigène T. congolense

Les épreuves réalisées avec cet antigène montrent des cas de séropositivité dans les deux zones visitées dans les Niayes, avec une prévalence légèrement plus élevée dans la zone centrale (Tableau V).

3.4.2. Résultats du test ELISA utilisant l'antigène T. vivax.

Les résultats obtenus avec ce test montrent une séro-prévalence très élevée dans les deux zones : près de 50 % dans la zone Sud, et plus de 30 % dans la zone Centre (Tableau V).

TABLEAU V. DISTRIBUTION DES POURCENTAGES DE POSITIVITE (PP) OBTENUS DANS LES DEUX TESTS ELISA AVEC LES SERUMS DE BOVINS DES NIAYES ET DU CRZ DAHRA

Niveaux des PP	Fréquence (%) des différents niveaux de pourcentage de positivité (PP)					
	Niayes-Sud (n = 115)		Niayes-Centre (n = 40)		CRZ Dahra (n = 40)	
	Test *T. c.*	Test *T.v.*	Test *T. c.*	Test *T. v.*	Test *T. c.*	Test *T. v.*
0 - 10	3.5	1.7	10.0	10.0	7.5	20.0
11 - 20	24.3	22.6	25.0	25.0	22.5	55.0
21 - 30	42.6	26.1	35.0	32.5	42.5	20.0
31 - 40	20.0	8.7	17.5	22.5	22.5	5.0
41 - 50	7.8	4.3	7.5	10.0	5.0	0
51 - 60	1.7	7.8	2.5	0	0	0
61 - 70	0	5.2	0	0	0	0
71 - 80	0	2.6	2.5	0	0	0
81 - 90	0	3.5	0	0	0	0
91 - 100	0	2.6	0	0	0	0
PP> 100	0	14.8	0	0	0	0

T. c. = *T. congolense*; *T. v.* = *T. vivax*
Seuils de positivité = Test *T. congolense* : PP > 40% ; Test *T. vivax* : PP > 30 %.

4. DISCUSSION

Les captures de glossines faites dans les deux zones montrent bien le retour de *Glossina palpalis gambiensis* dans la région. Des différences sont toutefois notées entre les deux zones, avec un nombre de mouches plus important dans la partie Sud de la région et surtout leur absence virtuelle dans les gîtes des environs immédiats du village de Mboro en zone Niayes-Centre. En effet, les captures faites dans cette zone relèvent toutes des alentours de Noto, village limitrophe de la zone Niayes-Sud. Ces différences pourraient être liées à l'habitat plus adapté dans la partie Sud de la région. On peut aussi penser que la ré invasion partirait de la banlieue de Dakar et progresserait vers le nord. En effet, c'est dans cette banlieue que se trouvent les grands Abattoirs de Dakar, et les nombreux animaux qui y sont convoyés tous les jours pourraient être des supports pour véhiculer les glossines.

Les examens parasitologiques n'ont décelé aucun cas de *T. congolense* ni de *T. brucei*. Mais cela ne permet pas d'affirmer l'absence effective de ces deux espèces de trypanosomes. Les villages visités et les échantillons examinés sont en nombre insuffisant pour une telle conclusion. Il est nécessaire de poursuivre les examens parasitologiques des bovins, d'étendre ces examens aux autres animaux domestiques (moutons, chèvres, chevaux, ânes), à la faune sauvage accessible (Rongeurs), et de procéder à des dissections de glossines. Les résultats de ces investigations devraient faire la lumière sur cette question. Pour l'instant, une certitude de grande importance est à retenir: la trypanosomose bovine à *T. vivax* est à nouveau présente dans la région des Niayes, avec un risque réel de propagation du fait de la présence confirmée de la mouche tsé-tsé. Une telle propagation, en atteignant les fermes laitières, anéantirait à coup sûr la plupart des efforts d'investissement consentis dans la région.

Concernant l'évaluation des deux tests sérologiques utilisés, la constance des résultats des sérums de référence positifs (C++ et C+) indique que ces réactifs peuvent garder pendant longtemps leurs anticorps si la conservation des aliquots est correcte, notamment à –20° C. Le contrôle conjugué seul (Cc) a également montré une stabilité et une spécificité satisfaisantes et, partant, une fiabilité certaine. Quant au «bruit de fond» du sérum de référence négatif (C–), il pourrait résulter d'une mauvaise qualité de l'eau dé-ionisée utilisée pour la préparation des tampons ou de lavages défectueux des plaques de travail. S'agissant des seuils de positivité, un plus grand nombre de sérums de la zone indemne aurait été sans doute préférable pour les calculs. Les seuils obtenus semblent néanmoins fiables au vu des résultats des 40 sérums de cette zone indemne, et aussi quand on les compare aux résultats du sérum de référence négatif (tableau IV).

Les sérums des bovins de la région des Niayes soumis au test ELISA utilisant l'antigène *T. congolense* indiquent une séro-prévalence plus forte dans la zone Niayes-Centre. Cela pourrait sembler paradoxal, puisque la population apparente de glossines est moins importante dans cette partie centrale que dans la zone Niayes-Sud. Les glossines de cette partie des Niayes ont bien pu s'adapter à des gîtes atypiques, par suite de la déforestation qui s'amplifie. Un comportement similaire de *Glossina palpalis gambiensis* a déjà été signalé par Touré dans la région en 1972 [6]. S'il en est ainsi, les mouches échapperaient aux piégeages effectués uniquement dans leurs gîtes traditionnels. Dans tous les cas, le test ELISA semble indiquer la présence de *T. congolense* sur toute la moitié de la région des Niayes. Ce serait là une situation sérieuse, qui mériterait une grande attention. Les épreuves ELISA réalisées avec l'antigène *T. vivax* confirment quant à elles les résultats des examens parasitologiques et montrent que la trypanosomose bovine due à *T. vivax* sévit à nouveau dans la région des Niayes à un niveau redevenu enzootique.

En conclusion, on peut donc affirmer que la ré invasion de la région des Niayes par la mouche tsé-tsé et la présence de la trypanosomose chez les bovins des troupeaux villageois sont aujourd'hui effectives. Cela est visible à travers les captures de glossines, les taux de prévalence obtenus aux examens parasitologiques, la séro-prévalence en ELISA indirecte.

Ce programme de recherche devrait se poursuivre: la dispersion réelle des glossines dans la région, les espèces de trypanosomes présentes, les espèces animales atteintes, les circuits de transmission du parasite sont à connaître avec précision. Des recommandations pourront alors être faites en vue d'une lutte efficace contre les trypanosomoses et leurs vecteurs dans cette région.

S'agissant des deux tests ELISA utilisés, ils se sont montrés tous les deux assez spécifiques et ont permis de distinguer nettement les bovins de la zone Nord du Sénégal indemne de glossines et ceux de la région des Niayes infestée. La combinaison de ces tests avec la technique parasitologique du BCT pourrait rendre des services appréciables dans l'épidémiologie de la trypanosomose animale.

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PREVALENCE OF *TRYPANOSOMA VIVAX* IN CATTLE IN CENTRAL SUDAN

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Abstract

PREVALENCE OF *TRYPANOSOMA VIVAX* IN CATTLE IN CENTRAL SUDAN.

The study was conducted to validate an antibody-detection ELISA test (AbELISA) using pre-coated ELISA plates with crude antigen preparation of *Trypanosoma vivax* and to study the prevalence of *T. vivax* infection in central Sudan. A total of 704 blood samples were collected from cattle in central Sudan, a known endemic area of *T. vivax* infection. Additionally, 74 blood samples were collected from northern Sudan (Atbra town), an area presumed to be *T. vivax*-free. Sera were collected during the period September1998 to May 1999 during three different seasons (summer, autumn and winter). Under the existing laboratory conditions, the test showed a clear distinction between different controls i.e. strong positive control (C++), weak positive control (C+), negative control (C–) and the conjugate control (Cc). A percent positivity of 25% was taken as a cut-off value to determine the positivity or negativity of the test. The acceptable optical density range of strong positive control (C++) was 0.65-1.22. Lower and upper percent positivity limits for different controls were also determined.

The study showed that *T. vivax* is endemic in central Sudan with 1.4% prevalence based on parasitological examination and 29.26% on AbELISA. The infection rate was significantly higher during the autumn and winter than in summer. Young cattle showed significantly lower infection rates than adults as indicated by both the parasitological and the AbELISA test. In relation to husbandry practice, migratory cattle showed significantly higher rates of prevalence than resident cattle. There was no significant difference in average packed red cell volume (PCV) values between ELISA positive and ELISA negative animals. Calves of less than one year of age showed significantly lower PCV values when belonging to migratory herds than to resident herds.

1. INTRODUCTION

Cattle population in the Sudan was estimated at about 33 million head [1]. They are an important source of milk meat and hides and contribute to the national income with US$ 32 million. Cattle contribute 76% and 65% of milk and meat to the national requirements, respectively [2]. Cattle husbandry is mainly practiced by nomadic pastoralists in Western and Eastern Sudan. In Central Sudan where irrigated agricultural schemes are localized, cattle are mainly kept for milk production and owned by sedentary farmers and agro-pastoralists.

Parasitic infections, especially trypanosomosis, are one of the major constraints hindering cattle husbandry in the Sudan. Cattle in irrigated agricultural schemes migrate to areas of high tsetse challenge. On the other hand, some herds get infected when they get come into contact with nomadic cattle grazing post-harvest agricultural products. *Trypanosoma vivax*, *T. evansi* and *T. theileri* have been reported the most prevalent trypanosome species in Central Sudan [3, 4, 5]. Diagnosis of these infections is mainly based on clinical signs and/or parasitological confirmation of infection. These techniques are not able to detect every infection, as the disease is characterized by chronicity. Serological detection has mainly been carried out for scientific reasons [6]. On the other hand, antigen-detection ELISA (AgELISA) has been used to detect *T. evansi* infections in camels in the Sudan [7].

This work was carried out to validate an indirect-ELISA for detection of *T. vivax* antibodies (AbELISA) in cattle sera and to study the prevalence of *T. vivax* in cattle of Central Sudan mainly in the Suki and Gezira Agricultural Schemes.

2. MATERIALS AND METHODS

2.1. Survey area

The survey was conducted in Central Sudan within the territories of the irrigated agricultural schemes of Suki (approximately 400 km south of Khartoum) and Gezira (approximately 200 km south of Khartoum). The area lies between 13°30'-14°30' N and 33°30'-14°00' E. A total of 704 cattle were sampled from September 1998 to March 1999. An additional 74 cattle were sampled from Atbra town

in Northern Sudan (approximately 340 km north of Khartoum). The town lies at 17°30' N and 34° E and is considered a trypanosomosis free area (Fig. 1).

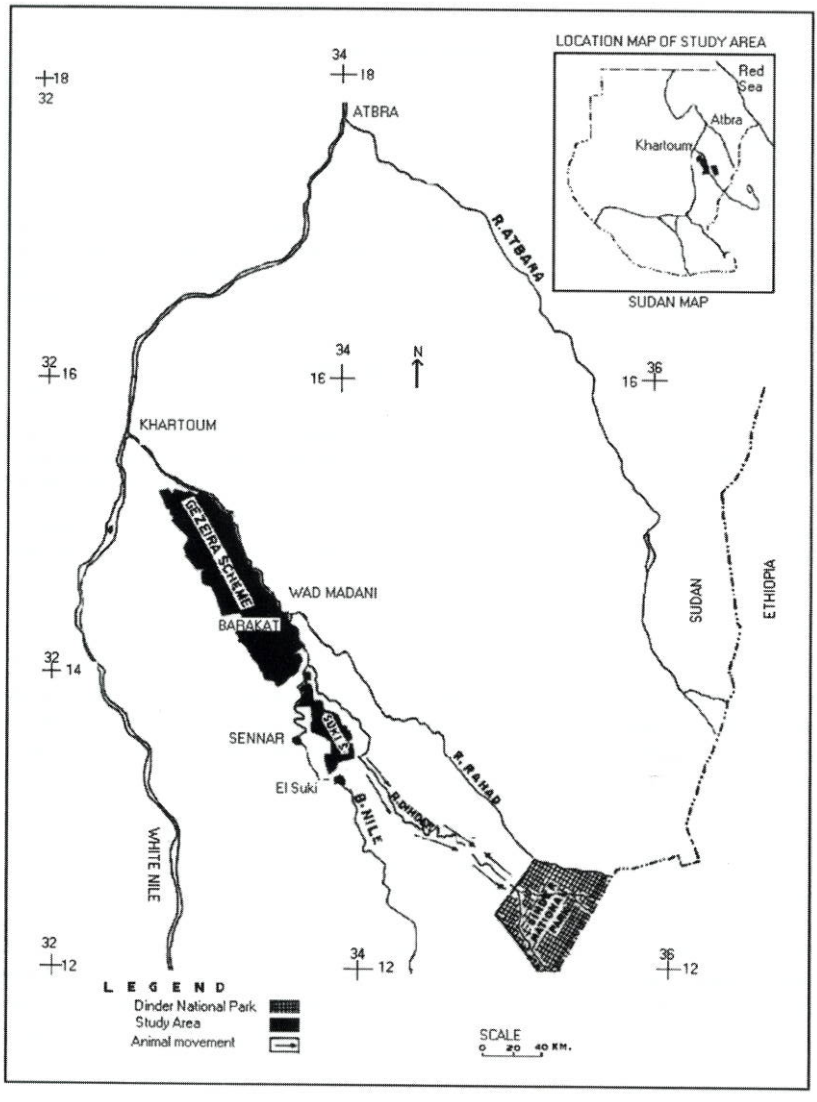

FIG. 1. A map of central Sudan showing the areas where cattle were sampled for Trypanosoma vivax *detection.*

2.2. Animals

Cattle from the *T. vivax* endemic areas were either Kenana or Friesian crossbreds. The cattle in the disease-free area were of the Butana breed and had been kept at a governmental farm since 1946. All three breeds are reared for milk production.

2.3. Samples

Cattle were selected at random and were bled by jugular vein puncture into plain and heparinized vacutainer tubes (Becton-Dickinson, NJ, USA). Samples were centrifuged in a microcentrifuge (Hawksley and Sons Ltd., USA). Packed red cell volumes (PCV) were estimated and buffy coat preparations (BCT) were examined for the presence of trypanosomes [8]. Positive cattle were treated with Ethidium® (Homidium bromide, Laprovet, France). Serum was separated from blood collected in plain vacutainer tubes and samples were transported on ice to Khartoum. They were kept at –20°C until analysis by ELISA.

2.4. Indirect-ELISA for detection of *T. vivax* antibodies

Reagents and equipment for AbELISA were supplied by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, Austria. The procedure was carried out as described in the Bench Protocol version-iTAB (TvAGd) prototype 1.0 (November 1998) of the Animal Production and Health Subprogramme [9]. Optical densities were read at 450 nm using Titertek Multiskan Plus MK II reader (Flow Laboratories, Switzerland). Readings of ELISA plates were calculated using the software program EDI version 2.1.

2.5. Data analysis

Results were analyzed using SAS for Windows (Release 6.12). Differences were considered significant when $p \leq 0.05$.

3. RESULTS

3.1. Determination of cut-off points

To determine an upper limit for negative AbELISA results, the mean absorbance value was determined for samples of cattle from a *T. vivax* free area. The cattle were apparently healthy without a known history of movement and/or trypanosome infection and had been kept in a breeding station since 1946. The animals showed a mean PCV value of 32 ± 5.44% and were parasitologically negative for *T. vivax*. The mean percent positivity (PP) of 12.6 ± 12.86% and the optical density (OD) value of 0.086 ± 0.075 were multiplied by 2 to determine a cut-off value with a 99% confidence limit for a positive test. The specificity and sensitivity of the test for the *T. vivax* free area is shown in Table I.

TABLE I. ANTIBODY ELISA RESULTS FOR CATTLE FROM A DISEASE FREE AREA (ATBRA TOWN)

	Examined by Buffy Coat Technique (BCT)		
Examined by AbELISA	Positive	Negative	Total
Positive	0	2	2
Negative	0	72	72
Total	0	74	74

ELISA sensitivity: 100%; ELISA specificity: 72/74 × 100 = 97%

A positive test was obtained at PP and OD of 25% and 0.172, respectively. The frequency distribution of the PP and OD values showed a skewed distribution to the right (Figs 2a and 2b).

The internal quality control values of 22 plates tested (mean OD ± 2SD) are shown in Fig 3.

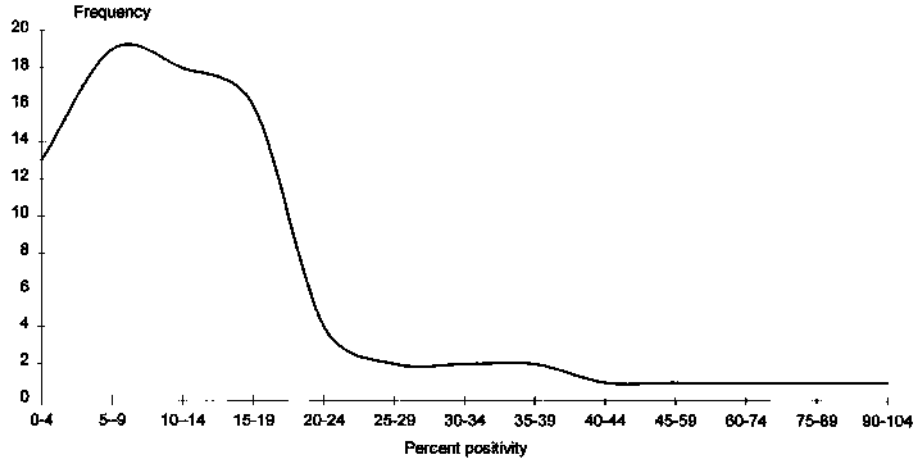

FIG. 2a. Frequency distribution of percent positivity values of cattle sera from disease-free area (Atbra)

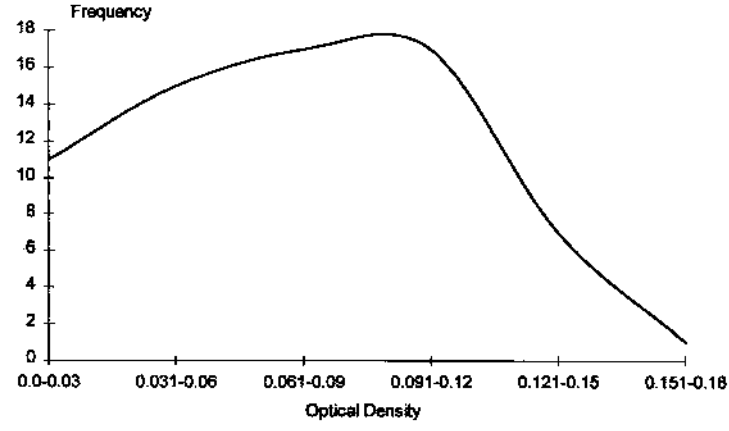

FIG. 2b. Frequency distribution of optical density values of cattle sera from disease-free area (Atbra)

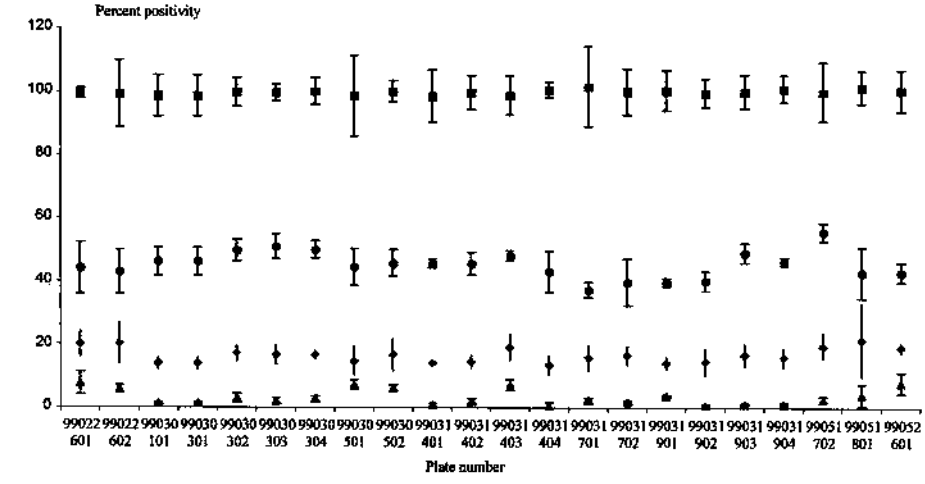

FIG. 3. Data chart of internal quality controls (mean ± 2STD's) of the indirect ELISA (TvAGd).

The acceptance criteria for control data in our laboratory conditions were performed from 20 plates out of 22 plates. Two plates were rejected as they showed the highest and the lowest average for OD values for the strong control positive (C++). The acceptance criteria for lower and upper control limits (LCL and UCL) are shown in Table II.

TABLE II. ACCEPTANCE CRITERIA FOR UPPER AND LOWER CONTROLS LIMITS FOR *T. VIVAX* INFECTION IN CATTLE IN THE SUDAN

	LCL	UCL
C++ (OD Values)	0.65	1.22
C++ (PP values)	93%	105%
C+ (PP values)	36%	56%
C- (PP values)	12%	24%
Cc (PP values)	0%	9%

LCL = lower control limit; UCL = upper control limit; OD = optical density; PP = percent positivity; C++ = strong positive control; C+ = weak positive control; C- = negative control; Cc = conjugate control.

3.2. Overall prevalence of *T. vivax* infection

Table III summarizes the results of 704 blood samples examined for parasites and tested for the presence of antibodies using antibody-detection ELISA (AbELISA).

TABLE III. PREVALENCE OF *T. VIVAX* INFECTION IN CATTLE IN CENTRAL SUDAN

	Examined by Buffy Coat Technique (BCT)		
Examined by AbELISA	Positive	Negative	Total
Positive	9	197	206 (29.3%)
Negative	1	497	498
Total	10 (1.4%)	694	704

ELISA sensitivity 9/10 × 100 = 90%; ELISA specificity 497/694 × 100 = 71.6%

3.3. Prevalence to *T. vivax* according to season

The prevalence rate of *T. vivax* showed significant differences between seasons as indicated by both ELISA and BCT. There was an increasing proportion of cattle with patent infections during winter when detected by BCT, while the same trend was observed during autumn (July-October) and winter (October-February) when detected by AbELISA (Table IV).

TABLE IV. SEASONAL PREVALENCE (%) OF *T. VIVAX* INFECTION IN CATTLE IN CENTRAL SUDAN (SUKI AREA)

Time period	No. examined	Test	
		BCT	AbELISA
Summer	40	0.0	7.5
Autumn	284	0.0	30.3
Winter	204	4.9	47.6
Total	528	1.9	35.2

BCT = buffy coat technique; AbELISA = antibody-detection enzyme linked immunoassay technique.

3.4. Prevalence of *T. vivax* according to age

The prevalence of *T. vivax* of each age group is shown in Table V. The percentage of cattle with demonstrable circulating antibodies increased significantly with age, while parasitaemia did not show a similar trend.

TABLE V. PREVALENCE (%) OF *T. VIVAX* WITHIN DIFFERENT AGE GROUPS OF CATTLE

Age (year)	No. examined	Test	
		BCT	AbELISA
0-1	194	1.6	6.7
1-2	122	1.6	15.6
2-4	104	1.9	21.0
>4	364	1.0	44.0
Total	784	1.3	27.0

BCT = buffy coat technique; AbELISA = antibody-detection enzyme linked immunoassay technique.

3.5. Prevalence according to geographical location

The prevalence was significantly higher in Suki area than in Gezira as determined by the presence of parasitaemia and circulating antibodies (Table VI). Results are presented for the winter season as serum samples were only collected from the Gezira area during the winter.

TABLE VI. PREVALENCE (%)DURING WINTER OF *T. VIVAX* IN CATTLE ACCORDING TO GEOGRAPHICAL LOCATION

Location	No. examined	Test	
		BCT	AbELISA
Suki	205	4.9	47.3
Gezira	176	0	11.4
Total	381	2.6	30.7

BCT = buffy coat technique; AbELISA = antibody-detection enzyme linked immunoassay technique.

3.6. Prevalence according to type of husbandry

Migratory cattle showed significantly higher infection rates by both serological and parasitological methods as compared to resident cattle (Table VII).

TABLE VII. PREVALENCE (%) OF *T. VIVAX* IN CATTLE ACCORDING TO HUSBANDRY PRACTICE

Husbandry Type	No. examined	Test	
		BCT	AbELISA
Migratory	449	2.2	39.2
Resident	335	0	11.3
Total	784	1.3	27.3

BCT = buffy coat technique; AbELISA = antibody-detection enzyme linked immunoassay technique.

3.7. Packed red cell volume (PCV) percent and infection rate of *T. vivax*

Although PCV values showed a negative correlation with BCT (–0.094) and AbELISA (–0.015), PCV values not significantly different between AbELISA positive (average PCV of infected cattle: 28%) and AbELISA negative (average PCV of non-infected cattle: 29%) animals. The frequency distribution curve of PCV values is shown in Fig. 4. A similar trend was observed in the average PCV values of resident (mean 29%) and migratory (mean 28%) cattle. On the other hand, cattle of less than one year of age showed a significantly higher PCV value in resident (mean 30%) than in migratory animals (mean 27.5%).

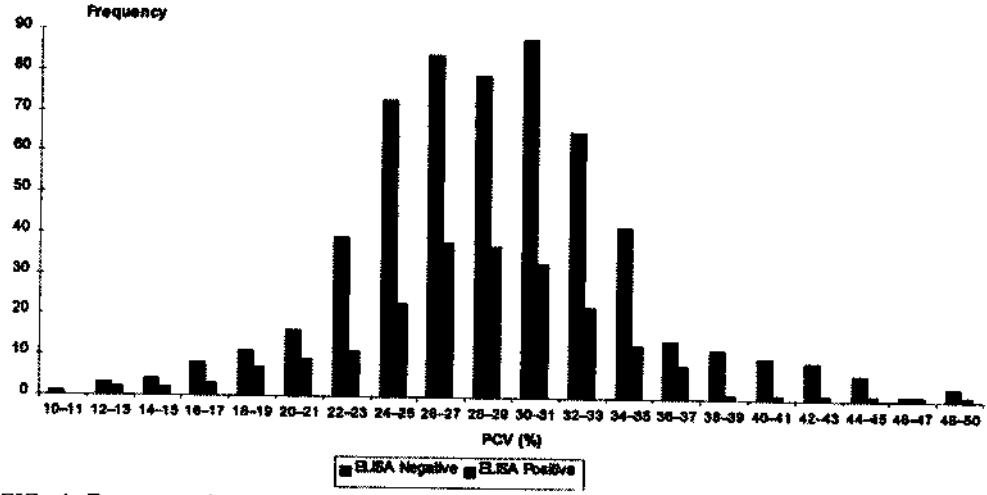

FIG. 4. Frequency distribution of packed red cell volume (PCV) values of AbELISA postive and negative cattle.

4. DISCUSSION

The results of internal quality assurance showed a lower optical density range and lower average optical density values of the strong positive control (C++) than those mentioned in the fact sheet of the FAO/IAEA manual. Nevertheless, the distinction between controls (C++, C+, C– and Cc) could easily be made under the laboratory conditions in the Sudan. Consequently, we obtained a wider range for upper and lower control limits as acceptance criteria for the test. The high sensitivity and specificity values obtained for sera from both a disease-free area and a disease endemic area indicated the high probability of the test to diagnose positive and negative animals.

The results of the test in dairy cattle of Suki and Gezira areas showed that infection with *T. vivax* is endemic in both areas with a higher infection rate in Suki. Infections were more prevalent during autumn (July-October) and winter (November-February) when assessed by AbELISA. Patent infections using the BCT were only noticed during the winter season. This infection pattern could be attributed to a higher tsetse challenge and the presence of biting flies namely *Tabanus* spp. during these two seasons [4]. The cattle owners in Suki area used to move along the Dinder River during early autumn reaching the boundaries of Dinder Game Park, where animals might have acquired infections through tsetse flies [10, 11] or through contact with the cattle of Fulani or other tribes entering the tsetse belt of Khor Yabos area [12]. During winter, cattle herds gathered in the agricultural schemes where infections could be spread by tabanid flies shown to be more abundant during early winter (dry season) in that area [4, 13, 14].

Patent parasitaemia was very low and no significant differences were detected between age groups. On the other hand, antibodies increased significantly with age. This might indicate that younger cattle were less susceptible to *T. vivax* infection or were exposed to a lesser degree to infection due to fly feeding preferences. On the other hand, it might also be due to adults experiencing frequent infections or due to the chronic nature of *T. vivax* infections. Significant age resistance has been recorded in cattle for *T. congolense* [15, 16, 17] and in camels for *T. evansi* [7].

Infection rates were found to be higher in the Suki area and in migratory cattle. This indicated that migratory cattle were subjected to a higher infection challenge than resident cattle in spite of the extra (veterinary) care they received as milk producing animals. The infections in resident cattle of Gezira and Suki area could be linked to the movement of migratory cattle from high challenge areas to the irrigated schemes for grazing. Disease transmission was facilitated by the presence of different species of biting flies such as *Tabanus* and *Stomoxys* spp., which were present in the man-made agricultural schemes being suitable habitats for insect breeding, i.e. water ponds and small forests in which cattle tend to rest at midday.

Packed red cell volume (PCV) values did not show a significant difference between infected, non-infected, migratory and resident cattle, which agrees with findings by other authors in the Sudan [5, 18, 19, 20]. Consequently, PCV values are not a reliable measure for the diagnosis of *T. vivax* infections especially not in adult cattle where infections are more prevalent. On the other hand, calves of less than one year of age originating from Suki area showed a significantly lower PCV value than calves from resident cattle. This might be related to the poor nutritional level of the calves rather than to *T. vivax* infections, as the calves in Suki area were not given enough milk.

As trypanosomosis is one of the most serious problems in the Sudan, more research studies and serological surveys are needed to determine the magnitude of the problem. So far a limited number of studies have been conducted in the area [4, 11, 12, 19, 20, 21]. A previous study using antigen-detection ELISA to assess trypanosomosis in camels [7] revealed the importance of serological monitoring. The present study of the disease in cattle provided similar results, which could be used for planning strategic control measures of bovine trypanosomosis in the Sudan.

ACKNOWLEDGEMENTS

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UTILISATION OF THE BUFFY COAT TECHNIQUE AND AN ANTIBODY-DETECTION ELISA AS TOOLS FOR ASSESSING THE IMPACT OF TRYPANOSOMOSIS ON HEALTH AND PRODUCTIVITY OF N'DAMA CATTLE

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Abstract

UTILISATION OF THE BUFFY COAT TECHNIQUE AND AN ANTIBODY-DETECTION ELISA AS TOOLS FOR ASSESSING THE IMPACT OF TRYPANOSOMOSIS ON HEALTH AND PRODUCTIVITY OF N'DAMA CATTLE.

The buffy coat technique (BCT), a parasitological test, and an indirect antibody ELISA (Ab-ELISA) were used to detect trypanosome infections in blood and serum samples, respectively, collected on N'Dama cattle exposed to natural high tsetse challenge. These two diagnostic tools were also utilized to assess trypanosomal status in sequentially collected blood and serum samples from two groups composed of 5 N'Dama cattle each experimentally challenged with *Trypanosoma congolense* and *T. vivax*. In both studies, packed red cell volume (PCV) and live weight were measured. The specificity of the Ab-ELISA was computed by testing approximately 70 serum samples obtained from a cattle population kept under zero tsetse challenge. The specificity was found to be 95.8% for *T. vivax* and 97.1% for *T. congolense*.

In the field study, 3.9% (12/310) of blood samples was parasitologically positive. In corresponding serum samples the prevalence of positive trypanosome sero-reactors was 54.8% (170/310). However, antibodies against trypanosomes persisted in serum when blood samples were no longer parasitologically positive. In both blood and serum samples, *T. vivax* was found to be the main infecting species. The sensitivity of the Ab-ELISA for *T. vivax* was 81.8%. Due to the extremely low numbers of *T. congolense* infection (only one), as detected by BCT, the sensitivity for that trypanosome species was not computed.

In the experimentally challenged cattle, 80% (24/30) and 33.3% (10/30) of blood samples were BCT positive for *T. congolense* and *T. vivax*, respectively. Antibodies in corresponding sera were present in 69% (20/29) and 96.3% (26/27) of animals challenged with *T. congolense* and *T. vivax*, respectively. The serological assay for *T. congolense* antibody detection exhibited high cross-reactivity with *T. vivax* antigens, as assessed in sera collected from *T. vivax* infected animals.

In the field study, cattle showing the presence of antibodies against *T. congolense* and/or *T. vivax* had significantly lower mean PCV values and daily weight gain in comparison with animals negative for antibodies and/or parasites. A significant positive correlation was observed between Ab-ELISA PP values and PCV in the 160 animals positive for trypanosomal antibodies. Moreover, in these cattle, daily weight gain was positively correlated with PCV value.

In experimentally infected cattle, a more severe effect of trypanosome infection was noticed in *T. congolense* infected N'Dama in comparison with animals infected with *T. vivax*, as assessed by lower PCV values and daily weight gain in the former.

In conclusion, the sensitivity and specificity of the *T. vivax* antibody detection assay appears to be satisfactory. Conversely, performance of the *T. congolense* Ab-ELISA needs to be improved. *Trypanosoma vivax* infection had limited impact on health and productivity of N'Dama cattle in both field and experimental studies. Therefore, it is recommended that selection for trypanotolerance should be based on the ability of N'Dama cattle to control the effects of *T. congolense* rather than *T. vivax* infections.

1. INTRODUCTION

The N'Dama breed is commonly considered to be trypanotolerant [1]. However, trypanotolerant N'Dama cattle can nevertheless suffer from trypanosomosis when submitted to high levels of tsetse challenge [2]. Moreover, within the N'Dama breed, the degree of trypanotolerance can vary between individuals and the impact of infection can vary according to the species of infecting trypanosome: *Trypanosoma congolense* having a greater detrimental effect on health and productivity under West African conditions than *T. vivax* [3, 4].

Major characteristics of trypanotolerance is the ability of infected animals to control parasitaemia and trypanosome induced anaemia. In both tolerant and susceptible animals, parasitaemia often fluctuates below the detection limit of the standard diagnostic tests, such as the buffy coat technique (BCT) [5]. Consequently, a number of infections go undetected. Therefore, it is imperative to utilise other and/or complementary diagnostic tests to assess more accurately the infection status of the animal. Thus breeding programmes can be facilitated aiming at selection of more tolerant and productive animals within the N'Dama breed. Furthermore, the application of highly sensitive and specific diagnostic tools, such as ELISA for either antibody and/or antigen detection and the PCR technique, which can demonstrate active infection and contact with trypanosomes, will enhance the understanding of the impact of trypanosomosis on health and production of trypanotolerant cattle.

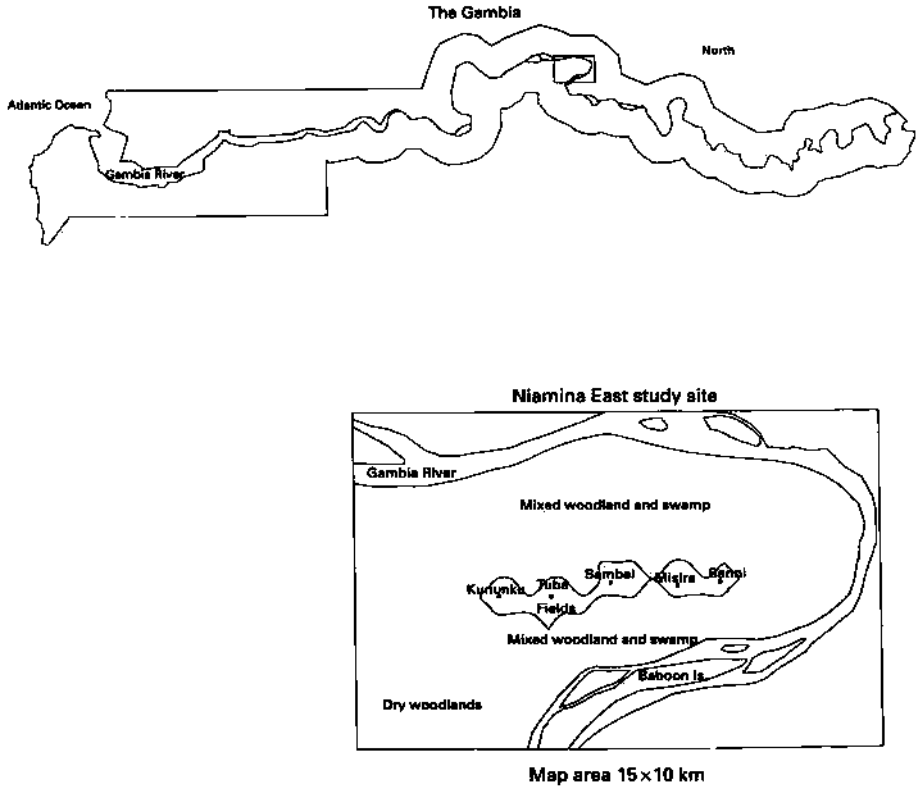

FIG. 1. Map of The Gambia showing the Niamina East study area.

2. MATERIALS AND METHODS

Two studies were conducted with the following objectives:

Study 1.

- assess the productivity of trypanosome-infected N'Dama weaners by measuring weight gain.
- identify the trypanosome status of animals as assessed by number of parasites by BCT and presence of anti-trypanosomal antibodies using an antibody-detection ELISA (Ab-ELISA) as measures of trypanotolerance.

Study 2.

- monitor the impact of *T. congolense* and *T. vivax* infection on health and live weight of N'Dama cattle.
- assess the complementarity of the BCT and Ab-ELISA for detection of trypanosome infection status.

2.1. Experimental animals

2.1.1. Study 1

The study period started in January 1998 and ended in August 1998. Fifty ear-tagged N'Dama cattle, aged 12 to 18 months (mean age of 14 months) at the start of the study, were randomly selected from weaners that were already involved in a selection scheme for trypanotolerance and productivity.

All animals were born and reared at the International Trypanotolerance Centre (ITC) stations (Kerr Seringe station situated at the coast, Keneba and Sololo stations located 160 and 312 km from

the coast, respectively). The cattle, after being weaned, were transferred to the study area in Niamina East (14°58' W, 13°51' N), situated approximately 220 km from the Atlantic coast. This area has seasonally high challenges of *Glossina morsitans submorsitans* Newstead and *G. palpalis gambiensis* Vanderplank. Vegetation is open Sudano savannah woodland interspersed with rice fields and swamps towards the river [6]. The climate of the study area is characterised by unimodal rainfall from July to October with a mean annual rainfall of 900 mm, followed by a cool early dry season from November to February and a hot late dry season from March to June [7].

2.1.2. Study 2

A herd of 25 N'Dama bulls, aged 3-4 years at the start of the study period, were used. These animals had been previously exposed to trypanosome infections in the field for more than a year.

The experiment was carried out at the ITC Station at Kerr Seringe and lasted seven months, i.e. from January to July 1997. Tsetse challenge at the study site is considered to be zero [8]. The experimental herd was randomly divided into five groups of five animals each. Animals were fed daily with 4 kg of groundnut hay and 1 kg of concentrate consisting of equal proportions of sesame cake and rice bran.

2.2. Interventions

2.2.1. Study 1

All animals were vaccinated against black quarter, haemorrhagic septicaemia and anthrax.

In order to limit losses (experienced in the past) caused by trypanosomosis and to reduce interference of stress factors, i.e. transportation and weaning, all animals were injected intramuscularly with a trypanocidal prophylactic drug (isometamedium chloride, 1 mg kg^{-1} body weight (bw) upon arrival in the study area.

Prior to the start of the study, cattle were dosed orally with fenbendazole (7.5 mg kg^{-1} bw) and treated intramuscularly with imidocarb diproprionate (3 mg kg^{-1} bw) in order to minimise the impact of gastrointestinal helminth and tick-borne diseases, respectively, on PCV and weight gain. Animals were dosed orally with 7.5 mg kg^{-1} bw of albendazole at the beginning of the rainy season, i.e. June 1996.

All animals were allowed to graze from 09.00 a.m. to 06.00 p.m. and watered twice daily, in the morning and around midday. During the night, animals were tethered individually.

2.2.2. Study 2

All animals were vaccinated against black quarter, haemorrhagic septicaemia and anthrax. Prior to the experimental infections the animals were dosed orally with fenbendazole (7.5 mg kg^{-1} bw) and treated intramuscularly with imidocarb diproprionate (3 mg kg^{-1} bw).

Since cattle were from a tsetse-infested area, they were screened for trypanosomes by microscopic examination of jugular blood samples using the BCT prior to the start of the study period. Eight of them were found positive. All animals, irrespective of their trypanosome status, were treated intramuscularly with 7.5 mg kg^{-1} bw of diminazene aceturate in mid November 1996.

2.3. Sampling methods

2.3.1. Study 1

Samples of jugular blood were collected monthly in EDTA-vacutainer tubes. The blood samples from EDTA tubes were micro-centrifuged in plain capillary tubes and the PCV values measured. The buffy coat from centrifuged preparations was examined by the dark ground technique/phase contrast method to detect trypanosomes [5]. Any animal with packed red cell volume (PCV) equal to or less than 20% and trypanosome BCT positive was treated intramuscularly with diminazene aceturate (7 mg kg^{-1} bw).

Serum samples were obtained from jugular blood samples collected in siliconized vacutainer tubes. These sera were stored at –20°C until tested for anti-trypanosomal antibodies. In some occasions blood and serum samples were not available (Table I).

TABLE I. MONTHLY DISTRIBUTION OF NUMBERS OF BLOOD SAMPLES BCT POSITIVE AND SERUM SAMPLES Ab-ELISA POSITIVE

Month	BCT positive			Ab-ELISA positive		
	T. c.	*T. v.*	*T. v.* & *T. c.*	*T. c.*	*T. v.*	*T. c.* & *T. v.*
January (n = 23)	0	1	0	0	10	1
February (n = 43)	0	6	0	0	20	5
March (n = 42)	0	3	0	0	20	5
April (n = 47)	0	0	0	1	25	2
May (n = 46)	0	0	0	1	22	5
June (n = 45)	0	0	0	0	15	5
July (n = 44)	0	1	0	3	19	2
August (n = 20)	1	0	0	1	4	4
Total (n = 310)	1	11	0	6	135	29

BCT = buffy coat technique; T. c. = *T. congolense*; T. v. = *T. vivax*; n = number of samples tested.

All animals were weighed monthly using an electronic scale.

According to trypanosome status, as assessed by BCT and Ab-ELISA, cattle were allocated to four classes as follows:

- Class 1: negative for BCT and Ab-ELISA;
- Class 2: BCT positive only;
- Class 3: BCT negative, but Ab-ELISA positive;
- Class 4: BCT and Ab-ELISA positive.

2.3.2. Study 2

Procedures for blood and serum collection, body weight and PCV measurements were similar to those described for Study 1. Trypanosome status was also assessed according to the techniques used in Study 1.

Any trypanosome-challenged animal with PCV value equal to or less than 20% and trypanosome BCT positive was treated intramuscularly with diminazene aceturate (7 mg kg-1 bw).

Blood and serum samples were tested for the presence of trypanosomes and trypanosomal antibodies 30 days before infection (D_{-30}) on the day of infection (D_0) and thereafter every 15 days until 90 days after infection (D_{90} p.i.). In this study the Ab-ELISA test and BCT results were considered in parallel. Using these criteria, an animal was considered as positive when either one of the two tests was positive. The sensitivity of the Ab-ELISA was computed from results obtained on serum samples collected from D_{15} to D_{90} p.i.

The specificity of the Ab-ELISA was calculated for each trypanosome species, i.e. *T. vivax* and *T. congolense*, by testing approximately 70 serum samples obtained from cattle kept under zero tsetse challenge.

2.4. Experimental infections

T. congolense and *T. vivax* stocks used in the study were isolated from naturally infected cattle originating from the same area as the experimental animals. An earlier study indicated that *T. congolense* and *T. vivax* populations occurring in this area are pathogenic for N'Dama cattle, as assessed by overt clinical signs of trypanosomosis and mortality occurrence in animals infected with either trypanosome species [2]. Each trypanosome stock was passaged by syringe once in goats and a stabilate was prepared [9] when the *T. congolense* and *T. vivax* parasitaemia levels reached a score of 6 in the caprine blood samples [10]. Stabilates were cryopreserved at –80°C until use.

On D_0 animals were intradermally inoculated with 1 ml of stabilate containing at least 10^4 motile parasites ml^{-1} [10] either with *T. congolense* (group 1) or with *T. vivax* (group 2). Each inoculated dose was equally distributed over three areas on the flank. On D_{14} one animal in group 2, previously challenged with *T. vivax* and found aparasitaemic, was challenged with the same

trypanosome stabilate as used for the primary infection. Micro-organism concentration per inoculating dose and technique used were similar to the initial experimental infection. Cattle in group 3 were used as uninfected controls.

In order to avoid mechanical transmission of *T. vivax* [11] the animals were maintained in a fly-proof stable previously treated with insecticide starting one week prior to infection and during the entire study period.

2.5. Statistical analysis

The analysis of variance (ANOVA) was performed to identify differences between trypanosome-infected classes, as previously described (Study 1) and between cattle groups (Study 2) in mean PCV values and daily weight gain (D_wg). D_wg was computed for each animal by regression of monthly weight on time. Optical density (OD) values and computed percent positivity (PP) values are proportional to the amount of antibodies present in serum samples in the indirect ELISA systems [12]. Due to the low number of animals in class 2 (Study 1), no comparison was performed between cattle allocated to this class and animals in other classes.

Correlation analysis was used to measure the time trend of PCV and D_wg with Ab-ELISA PP values and between PCV and D_wg on data collected in Study 1.

3. RESULTS

3.1. Parasitological and serological findings

3.1.1. Study 1

Overall infection rate detected by the BCT in centrifuged blood samples was 3.87% (12/310). *T. vivax* was the predominant trypanosome species observed [91.67% (11/12)]. In the serum samples of the same animals, 81.82% (9/11) had antibodies directed against *T. vivax*. Only one blood sample [8.33% (1/12)] was BCT positive for *T. congolense*. Serum from this animal showed antibodies to both trypanosome species. The animal was treated with diminazene aceturate.

Out of a total of 310 serum samples tested, 54.84% (170/310) showed trypanosomal antibodies. The monthly prevalence of trypanosome species (as detected by the BCT) and trypanosomal antibodies (as detected by ELISA) showed that in the months that no parasites were detected, antibodies to *T. vivax* and *T. congolense* were present in the corresponding sera (Table I).

3.1.2. Study 2

A higher percentage of positives was obtained with the BCT than with the Ab-ELISA in animals infected with *T. congolense*; while the reverse was true for cattle infected with *T. vivax*.

Eighty percent (24/30) of samples that were from animals in group 1 were found to be BCT positive whereas only 33.33% (10/30) of the blood samples of cattle in group 2 were positive by BCT.

Results obtained by Ab-ELISA, on the other hand, indicated that 68.96% (20/29) of the samples from group 1 had antibodies to *T. congolense*. However, these samples cross-reacted in all cases (30/30) when tested against the *T. vivax* antigen (Table II).

Sera from *T. vivax* infected animals (group 2) showed *T. vivax* antibodies in 96.30% (26/27) of the cases. Only in 3.33% (1/30) of the cases they cross-reacted with *T. congolense* antigen (Table II).

The agreement between the two tests was 40.74% (11/27) in the case of *T. vivax* and 48% (14/29) in the case of *T. congolense* infections.

All cattle were, at one time, Ab-ELISA or BCT or Ab-ELISA and BCT positive, with the exception of one animal in group 2 which was never BCT positive.

Parallel testing determined the overall positive results to be 96.30% (26/27) and 100% (29/29) for *T. vivax* and *T. congolense*, respectively (Table III). Only one of the samples collected from *T. vivax* challenged N'Dama cattle was negative in both tests.

TABLE II. NUMBERS OF BLOOD SAMPLES PARASITOLOGICALLY POSITIVE (BCT) AND SERA FOUND POSITIVE FOR ANTIBODIES (Ab-ELISA) ACCORDING TO TRYPANOSOME CHALLENGE SPECIES.

Trypanosome challenge	Diagnostic test		Trypanosome detection (days post infection)							
			–30	0	15	30	45	60	75	90
T. congolense	BCT	*T.c.*	0/5	0/5	5/5	5/5	5/5	2/5	4/5	3/5
		%	0	0	100	100	100	40	80	60
		T.v.	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		%	0	0	0	0	0	0	0	0
	Ab-E	*T.c.*	1/3	1/2	1/5	2/5	2/4	5/5	5/5	5/5
		%	33.3	50	20	40	50	100	100	100
		T.v.	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
		%	100	100	100	100	100	100	100	100
T. vivax	BCT	*T.c.*	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		%	0	0	0	0	0	0	0	0
		T.v.	0/5	0/5	2/5	3/5	1/5	1/5	2/5	1/5
		%	0	0	40	60	20	20	40	20
	Ab-E	*T.c.*	2/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5
		%	40	0	0	20	0	0	0	0
		T.v.	0/5	1/4	4/4	4/5	5/5	5/5	4/4	4/4
		%	0	25	100	80	100	100	100	100

BCT= buffy coat technique; Ab-E= antibody-detection ELISA.

TABLE III. PARALLEL TESTING FOR AGREEMENT OF THE BCT AND Ab-ELISA TESTS

	T. congolense				*T. vivax*		
	BCT				BCT		
		+	–			+	–
	+	14	6		+	10	16
Ab-E				Ab-E			
	-	9	0		-	0	1

BCT= buffy coat technique; Ab-E= antibody-detection ELISA.

3.2. PCV and daily weight gain (D_wg)

3.2.1. Study 1

Data on PCV values, when subjected to analysis of variance (ANOVA), showed a significant difference between animals within herds ($p<0.0001$), between herds ($p<0.0001$) and between visits ($p<0.0001$) (data not shown). This was unrelated to trypanosome infection ($p< 0.1260$), as determined by BCT. Animals negative by BCT, but positive for anti-trypanosomal antibodies had significantly different ($p<0.0291$) average PCV values from cattle that tested negative by both BCT and ELISA. Differences in mean PCV levels between classes of animals, defined according to their trypanosome infection status, are shown in Table III. A significant difference ($p<0.0001$) in mean PCV values was observed between animals that tested negative in both tests (class 1) and animals BCT negative but Ab-ELISA positive (class 3). Furthermore, a significant difference ($p<0.0204$) in mean PCV values occurred between classes 1 and 4, i.e. between animals negative and positive in both tests.

ANOVA was also used to test differences in mean D_wg between classes. Animals in class 1 had a significantly higher mean D_wg than cattle in class 3 ($p<0.0083$) or those in class 4 ($p<0.0038$). Cattle in class 3 showed a higher mean D_wg in comparison with animals in class 4 ($p<0.0482$) (Table IV). The statistical analysis also revealed there was no herd effect on D_wg ($p<0.0954$). Conversely, there were significant differences in D_wg between animals within herds ($p<0.0001$),

visits ($p<0.0001$), parasitologically positive and negative animals ($p<0.0001$) and animals showing presence of anti-trypanosomal antibodies and antibody-negative animals ($p<0.0001$) (Table IV).

TABLE IV. PCV VALUES (MEAN ± S.E.) AND DAILY WEIGHT GAIN (D_wg) (MEAN ± S.E.) IN CATTLE ACCORDING TO TRYPANOSOME STATUS AS DETERMINED BY BCT AND Ab-ELISA.

Infection status	Mean PCV	Mean D_wg (g/day)
Class 1	26.4 ± 0.3 a b (n = 138)	92.7 ± 4.2 a c (n = 138)
Class 2	27.0 ± 2.4 (n = 2)	106.3 ± 35.1 (n = 2)
Class 3	23.7 ± 0.3 a (n = 160)	77.4 ± 3.9 a b (n = 160)
Class 4	23.8 ± 1.1 b (n = 10)	45.4 ± 15.7 b c (n = 10)

Class 1 = BCT and Ab-ELISA negative; Class 2 = BCT positive only; Class 3 = Ab-ELISA positive only; Class 4 = BCT and Ab-ELISA positive; PCV = packed red cell volume; D_wg = daily weight gain; n = number of animals. Means in the same column which have the same letter are significantly different ($p<0.01$ or greater).

Correlation between PCV and ELISA (PP) values was tested in parasitologically negative and positive animals showing presence of anti-trypanosomal antibodies. A significant negative correlation was observed in the 160 animals that tested negative for BCT ($p<0.0032$), but not in the 10 animals diagnosed BCT positive ($p<0.4413$). This was also true for animals presenting antibodies against *T. vivax* only (Table V). In respect to D_wg, no correlation was found between D_wg and ELISA (PP) values in animals that were BCT positive ($p<0.0628$) or BCT negative ($p<0.9362$) (Table V). In animals testing BCT negative, but *T. congolense* antibody positive, no correlation between PCV and ELISA (PP) values was found. This might have been due to the small number of samples ($p<0.3784$) (Table V).

TABLE V. CORRELATION BETWEEN PCV AND Ab-ELISA PP VALUES; DAILYWEIGHT GAIN (D_wg) AND Ab-ELISA PP VALUES IN ANIMALS FOUND TO BE EITHER BCT POSITIVE OR BCT NEGATIVE BUT POSITIVE FOR *T. VIVAX.* ANTIBODIES OR *T. CONGOLENSE* ANTIBODIES OR ANTIBODIES TO BOTH SPECIES OF TRYPANOSOMES.

Ab-ELISA positive		PCV		D_wg	
		BCT-ve	BCT+ve	BCT-ve	BCT+ve
T. vivax	r	–0.3335	0.4830	–0.1717	–0.5061
	p	<0.0001	<0.2722	<0.0516	0.2426
	n	129	7	129	7
T. congolense	r	0.4435	-	0.4525	-
	p	<0.3784		<0.3676	
	n	6	0	6	0
T. congolense and *T. vivax*	r	–0.1996	–0.8146	–0.2295	0.5477
	p	<0.3387	<0.3939	<0.2697	<0.6310
	n	25	3	25	3
Total	r	–0.2313	0.2753	–0.1475	0.0292
	p	<0.0032	<0.4413	<0.0628	<0.9362
	n	160	10	160	10

BCT -ve = BCT negative; BCT +ve = BCT positive; r = correlation coefficient; p = statistical significance; n = number of observations.

There was a significant positive correlation between D_wg and PCV in animals found positive for antibodies, but negative for parasites (Table VI).

3.2.2. Study 2

The average-PCV of animals in group 1 dropped by 23.3% following infection as compared to the initial average value on D_0, but recovered towards the end of the study. Conversely, there was no decline in mean PCV in animals of group 2 (Table VII). Mean PCV and D_wg values in animals infected with *T. congolense* were significantly lower ($p<0.0001$) than those in cattle infected with *T. vivax* (Table VIII).

TABLE VI. CORRELATION BETWEEN PCV AND DAILY WEIGHT GAIN (D_wg) IN ANIMALS THAT WERE EITHER BCT POSITIVE OR NEGATIVE BUT POSITIVE FOR *T. VIVAX* ANTIBODIES, *T. CONGOLENSE* ANTIBODIES OR HAD ANTIBODIES TO BOTH TRYPANOSOME SPECIES.

		Correlated variables: PCV and D_wg	
Ab-ELISA positive		BCT-ve	BCT+ve
T. vivax	r	0.2703	–0.3334
	p	<0.0019	<0.4650
	n	129	7
T. congolense	r	–0.0479	-
	p	<0.9282	-
	n	6	0
T. congolense and *T. vivax*	r	0.5952	0.0391
	p	<0.0017	<0.9751
	n	25	3
Total	r	0.3154	–0.1877
	p	<0.0001	<0.6035
	n	160	10

BCT–ve = BCT negative; BCT+ve = BCT positive; r = correlation coefficient; p = statistical significance; n = number of observations.

TABLE VII. PCV VALUES (MEAN ± S.E.) IN CATTLE CHALLENGED WITH *T. CONGOLENSE* (GROUP 1) AND THOSE CHALLENGED WITH *T. VIVAX* (GROUP 2).

Month	Mean PCV (%)	
	Group 1 (n = 5)	Group 2 (n = 5)
Jan	26.6 ± 3.29	26.4 ± 1.34
Feb	25.0 ± 3.24	26.4 ± 1.67
Mar	20.4 ± 2.07	25.0 ± 1.0
Apr	21.4 ± 2.70	26.4 ± 1.67
May	23.6 ± 2.70	29.0 ± 1.87
Jun	24.8 ± 2.59	28.0 ± 2.0

TABLE VIII. PCV PERCENTAGE (MEAN ± S.E.) AND DAILY WEIGHT GAIN (D_wg) (MEAN ± S.E.) OVERALL THE STUDY PERIOD IN CATTLE ACCORDING TO TRYPANOSOME SPECIES CHALLENGE.

Cattle group	Trypanosome challenge	PCV	D_wg
1 n = 30	*T. congolense*	24.2 ± 0.3a	16.3 ± 26.5b
2 n = 30	*T. vivax*	26.7 ± 0.3a	179.5 ± 54.8b

n = number of animals multiplied by the number of observations per animal. Means in the same column followed by the same letter are significantly different (p<0.001 or greater).

In cattle in group 1, there was a significant difference in mean PCV (p<0.0001) and mean D_wg (p<0.0015) values between the pre-infection and post infection periods. This was not the case for cattle infected with *T. vivax*.

The data of weight gain were subjected to a regression analysis for individual animals computed on monthly weight on time. While animals in group 1 had a mean weight gain of 16 grams/day, mean weight gain of cattle in group 2 was 179 grams/day. This difference was statistically significant (p< 0.0201).

3.3. Test specificity and sensitivity

3.3.1. Study 1

The specificity of the indirect ELISA to detect *T. vivax* antibodies was 95.77% (68/71), while that of the indirect ELISA to detect *T. congolense* antibodies was 97.06% (66/68).

The sensitivity of the *T. vivax* Ab-detection ELISA was 81.82% (9/11), while that of the *T. congolense* Ab-detection ELISA was not computed as only one sample was BCT positive.

3.3.2. Study 2

The sensitivity of the *T. vivax* Ab-ELISA was shown to be 96.30% (26/27) and that of the *T. congolense* Ab-ELISA was 68.96% (20/29).

4. DISCUSSION

The specificity of the ELISA to detect antibodies against *T. vivax* and *T. congolense* in both studies was satisfactory, i.e. > 95%.

With a sensitivity between 81 and 96% and a specificity of over 97%, the *T. vivax* Ab-detection ELISA can be recommended as a suitable test for antibody detection. However, the *T. congolense* Ab-detection ELISA needs to be improved, since the sensitivity was 69%, the specificity was 97% and cross reactivity was observed with *T. vivax* antigens in the experimentally infected animals (group 2).

Observations derived from the field study indicated that a positive correlation between PCV and daily weight gain existed. This finding corroborates previous work carried out in cattle populations in Gabon and the Democratic Republic of Congo [13].

In the indirect ELISA test system a positive correlation exists between PP values and the amount of antibodies present in the host [12]. In addition, generally high antibody levels are observed during active trypanosome infections. A constant decline in circulating antibodies occurs over time following parasite clearance as a result of treatment or self-cure. Results from our field investigations revealed a negative correlation between PCV and PP values. This suggests that high antibody levels may be indicative of an active trypanosome infection and/or recent contact with the parasite. However, anti-trypanosomal antibodies were detectable for a period of 3 months after having detected trypanosomes in blood samples using the BCT. Similar observations were made by Bocquentin et al. [14]. Consequently, a better understanding of the epidemiological situation and the impact of trypanosomosis on productivity in N'Dama cattle can be obtained by combining the two tests.

Furthermore, the study provided evidence of the capacity of the N'Dama breed to be productive under tsetse challenge, as assessed by the observed weight gain. It should be noted, however, that the low infection rate, as detected by BCT, could have contributed to minimising the impact of trypanosomosis on cattle productivity.

In addition, more than 90% of the infections detected by BCT was identified as *T. vivax*. It has been shown that N'Dama cattle suffer less negative effects, i.e. anaemia and weight loss [2, 3] when infected with *T. vivax* than when infected with *T. congolense*. This observation could have contributed to the limited impact of the trypanosome infections. Moreover, only one N'Dama infected with *T. congolense* required trypanocidal treatment.

The negative correlation between PP values and PCV levels seems to indicate that *T. vivax* may affect the health of N'Dama cattle. However, no negative correlation was detected between Ab-ELISA PP values and daily weight gains. Trail *et al.* reported a significant positive correlation between PCV levels and daily weight gain [13]. A similar observation was made in the present study. Furthermore, results of PCV value and daily weight gain changes obtained from the experimental infection (study 2) support the observation from the field study on the limited impact of *T. vivax* infections on health and productivity of N'Dama cattle in comparison to *T. congolense* infections. It is unlikely that the *T. vivax* stock used in the experimental infection was of a low virulence, as a decrease in PCV values was observed when expanding the *T. vivax* stock in goats.

Considering the limited impact of *T. vivax* on health and productivity of N'Dama cattle, it can be concluded that a selection programme for the assessment of trypanotolerance should be based on the ability to control the effects of *T. congolense* rather than *T. vivax* infections.

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VALIDATION IN UGANDA OF ANTIBODY-DETECTION ELISA'S USING PLATES PRECOATED WITH DENATURED *TRYPANOSOMA CONGOLENSE* AND *TRYPANOSOMA VIVAX* ANTIGEN

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Abstract

VALIDATION IN UGANDA OF ANTIBODY-DETECTION ELISA'S USING PLATES PRECOATED WITH DENATURED *TRYPANOSOMA CONGOLENSE* AND *TRYPANOSOMA VIVAX* ANTIGEN.

Indirect enzyme-linked immunosorbent assays (ELISA) for detection of trypanosomal antibodies using denatured *Trypanosoma congolense* and *Trypanosoma vivax* antigens were evaluated in Uganda. A total of 400 bovine sera were analyzed, consisting of 120 parasitologically negative samples from a tsetse-free area, 120 parasitologically positive samples, 80 samples from an area of low tsetse challenge (Kiboga) and 80 samples from of an area of medium to high tsetse challenge (Arua) of unknown disease status. Using the modified ROC analysis, cut-off points of 35% and 25% positivity were determined for the *T.congolense* and *T.vivax* assay, respectively. The *T. congolense* assay had a diagnostic specificity of 74%, a sensitivity of 52.5% for infections due to all trypanosome species, 81% for homogenous infections and 48% for heterogenous infections. The *T. vivax* assay had a diagnostic specificity of 81.3%, sensitivity of 81.3% for infections due to all trypanosome species, 76.5% for homogenous infections and 81.3% for heterogenous infections. The Buffy Coat Technique (BCT) revealed trypanosomes in none of the samples from Kiboga and in 15% from Arua. In contrast, the *T. congolense* assay revealed a sero-prevalence of 52.5% in Kiboga and 30% in Arua while the *T. vivax* assay revealed 21.3% in Kiboga and 46.3% in Arua. The *T. vivax* assay had a higher negative predictive value (78%) than the *T. congolense* assay (47%) in the area of low tsetse challenge (Kiboga) and a higher positive predictive value (75%) than the *T. congolense* assay (66%) in the area of medium to high tsetse challenge (Arua). The *T.vivax* assay appears to be more efficient than the *T. congolense* assay and it is potentially useful in determining the distribution bovine of trypanosomosis and targeting appropriate control measures in different areas of Uganda.

1. INTRODUCTION

Animal trypanosomosis presents special problems with regard to diagnosis. The clinical signs are not pathognomic and the standard techniques for the detection of trypanosomes are not sufficiently sensitive. Antibody detection and antigen detection ELISA tests have been developed to overcome these shortcomings. However, recent findings show that antigen detection ELISA's have poor sensitivities though display high specificities [1]. On the other hand, standardization of the antibody-detection tests is rather difficult, reproducibility of results is not optimal [2], the antigens used are not properly defined [3], serum samples and antigen used are bound to deteriorate if not strictly maintained in a cold-chain. Attempts have been described to circumvent the difficulties of ensuring strict cold-chain storage for serum samples by adaptation of antibody ELISA for use with dried blood spots on filter paper [4]. In an attempt to standardize procedures and improve stability, the FAO/IAEA has developed an antibody-detection ELISA using plates precoated with denatured *T. congolense* and *T. vivax* antigen. This paper reports on the results of the kit evaluation under laboratory conditions in Uganda.

2. MATERIALS AND METHODS

2.1. Samples

The negative sera (n = 120) had been collected from cattle in a tsetse-free area situated on the slopes of Mt. Elgon at an altitude of 1954 masl. The positive sera (n = 120) had been collected from cattle in trypanosomosis endemic areas in Busia, Tororo and Iganga districts of South-East Uganda. The samples of unknown disease status (n = 160) had been collected from cattle in Kiboga, an area of low tsetse challenge and Arua, an area of medium to high tsetse challenge. All serum samples were stored at –20°C at the Livestock Health Research Institute.

2.2. Reagents

The strong positive (C++), weak positive (C+) and negative (C-) reference sera, the conjugates, the substrate and microplates precoated with *T. congolense* and *T. vivax* denatured antigens were supplied by the Animal Production Unit of the FAO/IAEA Agriculture and Biotechnology Laboratory.

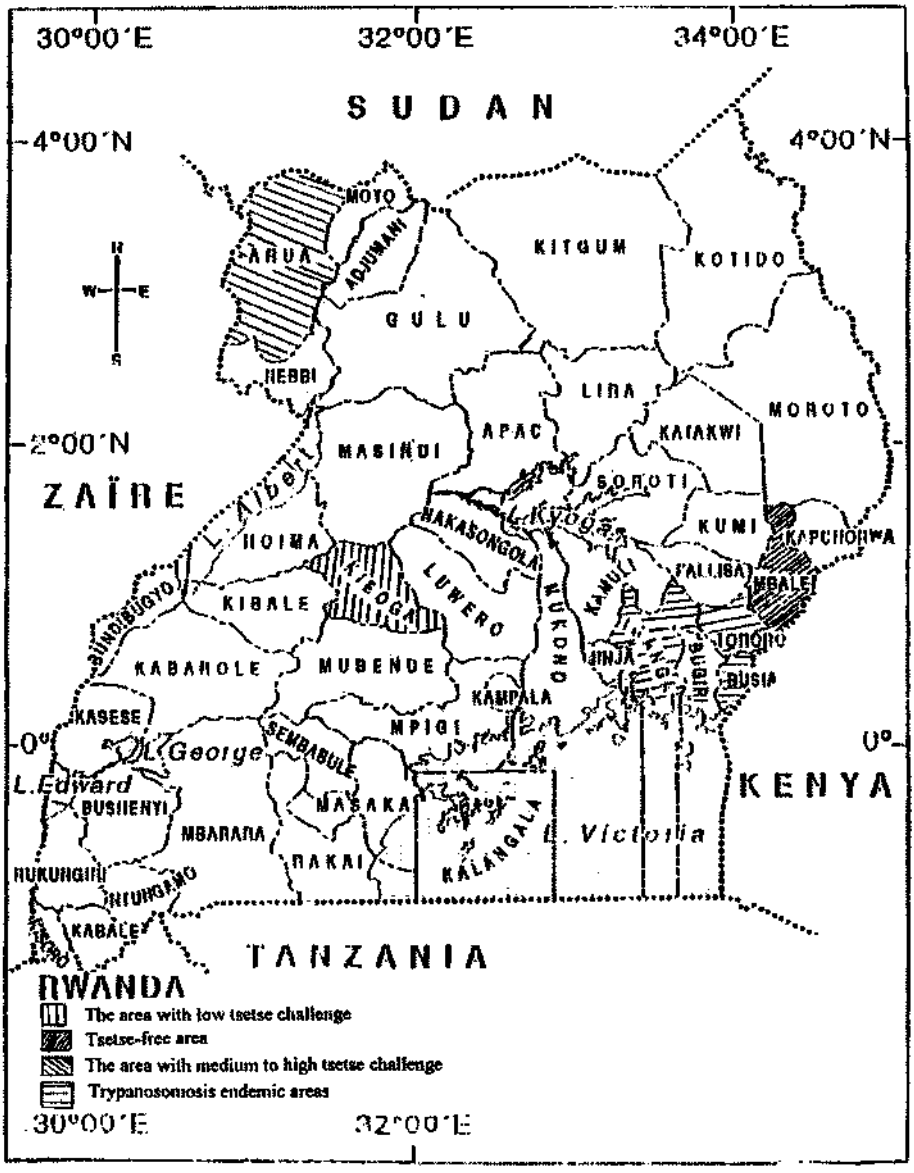

FIG. 1. Map of Uganda showing the tsetse free and tsetse infested areas (... = international boundary; —••— = district boundary)

All reference sera and conjugates were stored at –20°C and the substrates were kept at 4°C. Precoated microplates were stored in a dry and dark area at room temperature (25°C).

2.3. Sample analysis

Serum samples were analyzed using plates precoated with either *T. congolense* or *T. vivax* according to the protocol provided by the FAO/IAEA Agriculture and Biotechnology Laboratory for the trypanosomosis indirect ELISA.

2.4. Analysis of ELISA data

Absorbance values were expressed into percentage positivity (PP). PP values for quality assurance and acceptance of the test sera were calculated using formulae given in the FAO/IAEA protocol. Whereas the absorbance values from the analysis of all positive (n = 120) and negative samples (n = 120) with the *T. congolense* assay were accepted, absorbance values of the analysis of only 80 positive and 80 negative samples with the *T. vivax* assay were accepted. Controls were examined to see whether their fell within the desired control limits. The diagnostic sensitivities and specificities of both assays were estimated based on cut-off points determined using the modified receiver-operating characteristic (ROC) analysis [5]. For both assays, the negative predictive values were determined based on sera from cattle grazing under low tsetse challenge and the positive predictive values determined based on sera from cattle grazing under medium to high tsetse challenge.

3. RESULTS

Analysis of the internal quality control data of both the *T. congolense* and *T. vivax* assays under laboratory conditions in Uganda showed that the coefficient of variation (CV) for optical density (OD) values of the strong control sera (C++) for both the *T. congolense* and *T. vivax* assays were good since they were approximately 30%. The CV for the weak control sera (C+) and for the negative control sera (C–) for both assays were acceptable since they were about 50% but those for the background (Cc) for both assays were perfect (Table I). Generally, the internal quality control results were good since the OD values of the strong control sera (C++) for the *T. congolense* and *T. vivax* assays fell within the required control limits of 1.406 –0.936 and 1.507-0.938, respectively.

TABLE I. ANALYSIS OF THE PROFICIENCY OF THE INDIRECT ELISA'S USING DENATURED *T. CONGOLENSE* AND *T. VIVAX*

	T. congolense ELISA (10 microplates)				*T. vivax* ELISA (8 microplates)			
	C++	C+	C-	Cc	C++	C+	C-	Cc
Mean OD	1.257	0.783	0.200	0.037	1.227	0.736	0.354	0.033
STD	0.403	0.378	0.120	0.025	0.463	0.420	0.188	0.025
CV	32%	48%	60%	67%	37%	57%	53%	75%

ELISA = enzyme linked immunosorbent assay; OD = optimal density; STD = standard deviation; CV = coefficient of variation

The distribution of the ELISA percent positivity values for the negative and positive cattle populations are shown for the *T. congolense* and *T. vivax* assays in Figures 2 and 3, respectively. A substantial proportion of cattle in the positive population (Fig. 2) had values similar to those in the negative population, especially with heterogenous trypanosome infections (false negatives), while a few cattle in the negative population had values similar to those of the positive population (false positives). Furthermore, a few cattle in the positive population had values similar to those in negative population (false negatives) especially with homogenous trypanosome infections (Fig. 3) and likewise, a few cattle in the negative population had values similar to those in positive population (false positives). Based on the distribution of the percent positivity values of both the negative and positive cattle populations, the most suitable cut-off points were considered to be 35% for the *T. congolense* assay and 25% for the *T. vivax* assay in order to attain acceptable diagnostic sensitivity and specificity values. However, for the *T. congolense* assay, a cut-off point of 90% would give the best discrimination between the negative cattle population and the positive cattle population with homogenous trypanosome infections.

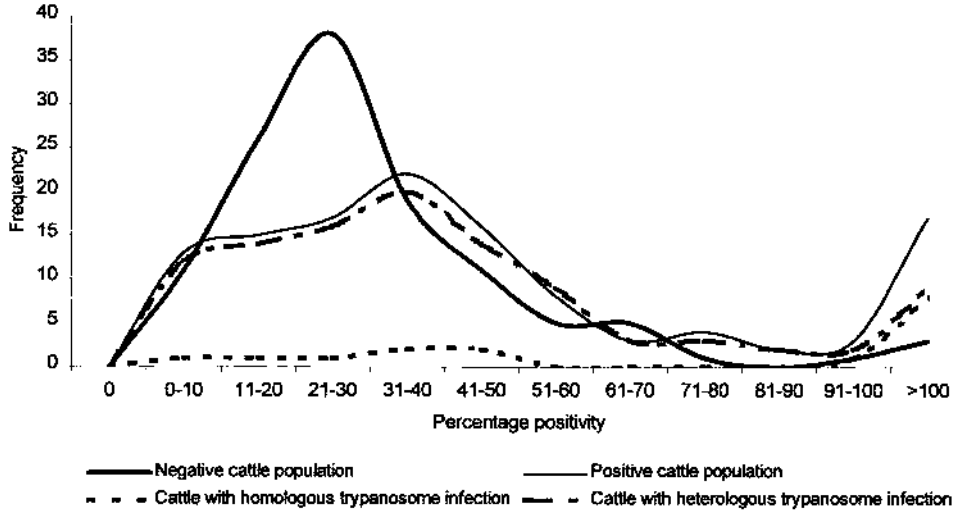

FIG. 2. Distribution of the Trypanosoma congolense *antibody-detection ELISA percentage positivity values for infected and uninfected cattle populations (cut-off point was 35%).*

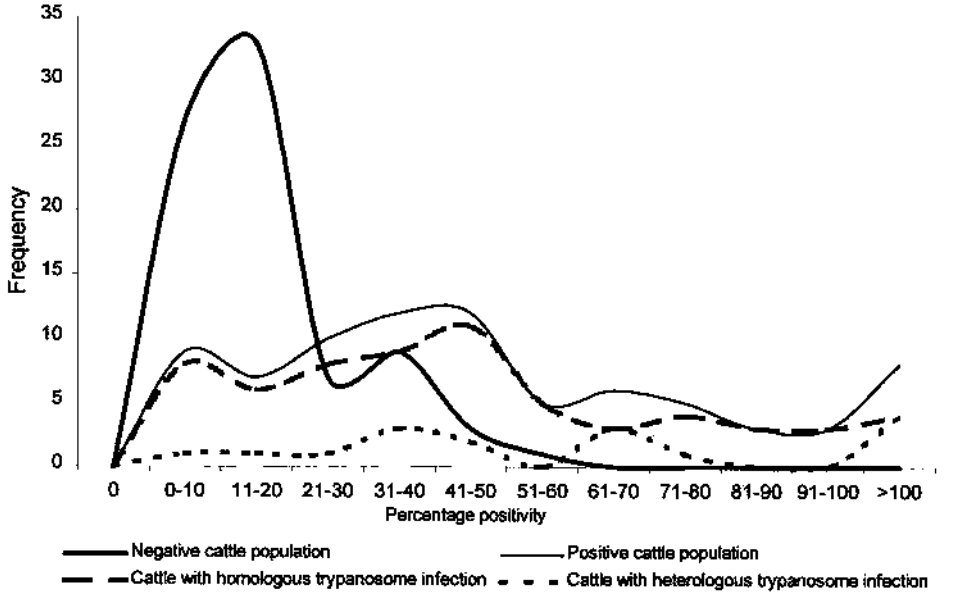

FIG. 3. Distribution of the T. vivax *antibody-detection ELISA percentage positivity values for infected and uninfected cattle populations (cut-off point was 25%).*

TABLE II. DIAGNOSTIC SENSITIVITY AND SPECIFICITY OF THE *T. CONGOLENSE* AND *T. VIVAX* ANTIBODY-DETECTION

Parameter	n	PP values using denatured *T. congolense* antigen (cut-off point: 35%)	n	PP values using denatured *T. vivax* antigen (cut-off point: 25%)
Diagnostic sensitivity (DSN)	120	52.5%	80	81.3%
DSN- homogenous infections	16	81%	64	76.5%
DSN- heterogenous infections	104	48%	16	81.3%
Diagnostic specificity	120	74%	80	81.3%
Positive predictive value[1]	80	66%	80	75%
Negative predictive value[2]	80	47%	80	78%

PP = percent positivity; [1] Positive Predictive value was determined using bovine sera from Arua – an area of moderate to high tsetse challenge with a trypanosome prevalence of 15% using the BCT; [2] Negative Predictive value was determined using bovine sera from Kiboga - an area of low tsetse challenge with a trypanosome prevalence of 0% using the BCT.

The cut-off points for the *T. congolense* and *T. vivax* assays were estimated using a ROC analysis (Figs 4 and 5, respectively). Cut-off points of 35% and 25% for the *T. congolense* and *T. vivax* assays, respectively, were obtained. A maximum binding ratios of 63.2% and 81.3% was obtained with the *T. congolense* and *T. vivax* assay, respectively. The sensitivity, specificity, positive predictive value and negative predictive value for the *T. vivax* assay were higher than those for the *T.*

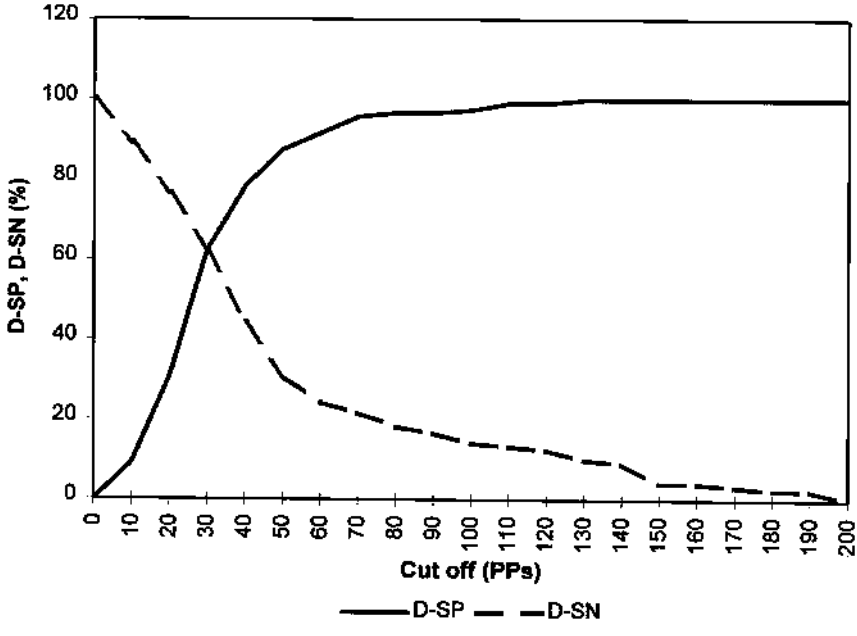

FIG. 4. Estimates of cut-off points (modified ROC analysis) of the Trypanosoma congolense *(TcAGd) ELISA based on 120 negative bovine serum samples (DSP=diagnostic specificity, DSN=diagnostic sensitivity).*

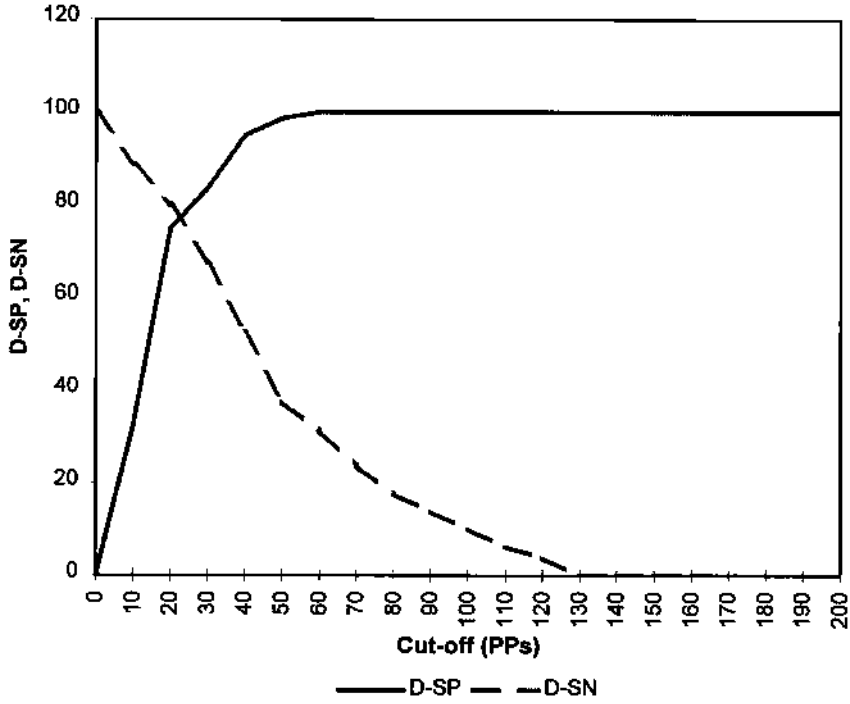

FIG.5. Estimates of cut-offs (modified ROC analysis) of the Trypanosoma vivax *(TvAGd) ELISA based on 80 negative and positive field bovine sera (DSP=diagnostic specificity, DSN=diagnostic sensitivity).*

congolense assay (Table II). Whereas the *T. vivax* assay had fairly high sensitivity for both homogenous and heterogenous trypanosome infections, the *T. congolense* assay had low sensitivity for heterogenous trypanosome infections but high sensitivity for homogenous trypanosome infections.

The distribution of percent positivity values for cattle in area of a medium to high tsetse challenge (Arua) and in the area of low tsetse challenge (Kiboga) using both *T. congolense and T. vivax* assays is shown in Figures 6 and 7, respectively. Both assays distinguished two distinct cattle populations (negative and infected) in the area of medium to high tsetse challenge, but only one cattle population in the area of low tsetse challenge.

4. DISCUSSION

The proficiency of the indirect *T. congolense* and *T. vivax* assays utilizing denatured antigen was examined under laboratory conditions in Uganda by analyzing the internal quality data. The results clearly demonstrated that the two assays are robust and can be used under laboratory conditions in Uganda following shipment without any specific precautions. Thus, denaturation of antigens and pre-coating of plates before transportation to Africa mitigates the problem of antigen deterioration.

A substantial proportion of cattle detected positive by BCT had percent positivity values similar to cattle coming from a tsetse-free area and negative by BCT. The majority of false negative results in the *T. congolense* assay were cattle, which had heterogenous trypanosome infections. This observation can be attributed to a low sensitivity of the *T. congolense* assay for infections due to *T. vivax* and *T. brucei*, although *T. congolense* antigen cross-reacts with antibodies against *T. vivax* and

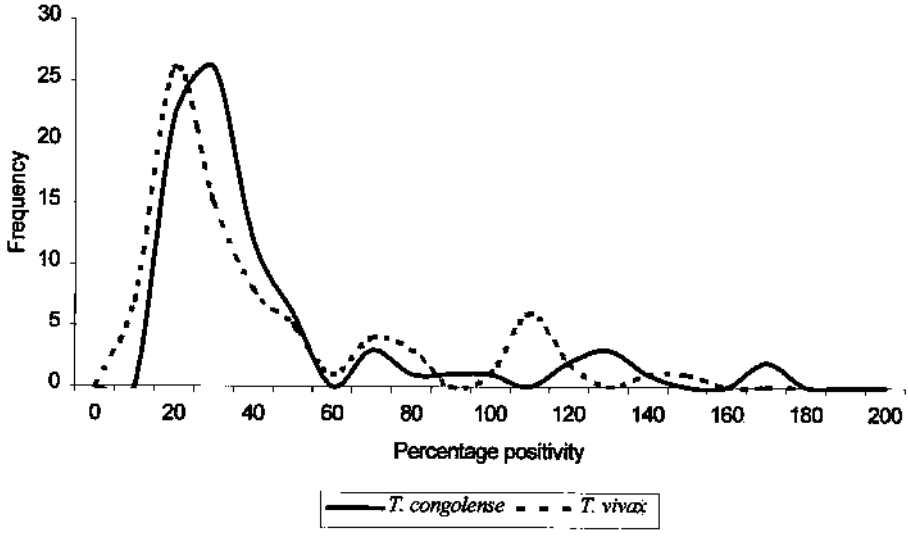

FIG. 6. Distribution of percentage positivity values of cattle from Arua (an area of medium to high trypanosome challenge) using the T. congolense *and* T. vivax *indirect ELISA.*

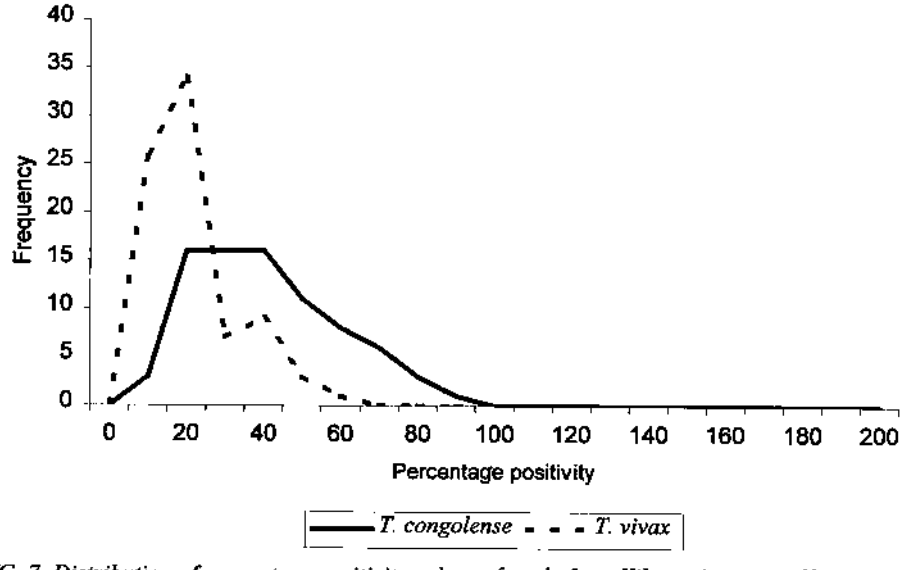

FIG. 7. Distribution of percentage positivity values of cattle from Kiboga (an area of low trypanosome challenge) using the T. congolense *and* T. vivax *indirect ELISA.*

T. brucei. In contrast, the majority of the false negative results in the *T. vivax* assay were from cattle which had homogenous trypanosome infections, while a large number of cattle with heterogenous infections were detected positive. This can be attributed to a marked difference in antibody response by individual cattle depending on the stage of infection, either early or late. Trypanosomal antibodies have been detected within 14 days post-infections [6]. The distribution of percent positivity values of the positive cattle population especially in the *T. vivax* assay showed three peaks probably corresponding to different sub-populations according to different stages of infections. Moreover, differences in immunogenicity of the different trypanosome species infecting animals has been observed to lead to a large proportion of infected animals to have low OD values [6]. Based on this observation, the probable reason why the *T. vivax* assay detected more heterogenous than homogenous infections is that the *T. congolense* and *T. brucei* isolates that infected cattle in the positive population were more immunogenic than the *T. vivax* parasites. Consequently, antibody production in the cattle infected with heterogenous infection was stimulated to a higher extent than in those with a homogenous infection. Similarly, more cattle that were infected with a homogenous infection had PP values similar to those of the negative cattle population than cattle infected with a heterogenous infection.

A proportion of cattle from a tsetse-free area and proven negative by BCT, had high PP values similar to those of infected cattle. In general cattle movement out of the tsetse-free area of Mt. Elgon is usually limited to animals taken for slaughter rather than for restocking, since these cattle can not survive in tsetse-infested area. On the other hand, it is possible that cattle from the tsetse-infested areas are moved to the tsetse-free areas. Thus, animals having maternal antibodies [7] or antibodies persisting following successful treatment could explain why some cattle in the negative cattle in the negative cattle population had fairly high PP values. Furthermore, antibodies against other parasitic diseases can cause false positive results, since the antigens used in the Indirect ELISA are ill-defined [3]. However, it was impossible to determine the origin of the cattle sampled in the tsetse-free area. It has been pointed out that a correct interpretation of assay results requires consideration of biological factors, including sex, breed, age, health and nutritional status of the animals [8]. Unfortunately, such information was not available.

The *T. vivax* assay had a higher maximum binding ratio (81.3%) than the *T. congolense* assay (63.2%) and had a higher sensitivity (81.3%) and specificity (81.3%). A sensitivity of 81.3% for the *T. vivax* assay is lower than that obtained with an indirect *T. brucei* assay (86.1%) by Hopkins and colleagues [4], while the specificity (of 81.3%) is also lower than that achieved by the same workers (95.2%). In addition, the *T. vivax* assay had a higher negative predictive value (75%) and positive predictive Value (78%) than the *T. congolense* assay.

Based on the distribution of PP values of cattle in the low tsetse challenge area and in the medium to high tsetse challenge area both assay seemed to detect antibodies against a common trypanosome antigen. Hence the distribution of PP values by both assay is similar.

In conclusion, the *T. vivax* assay appears to be more efficient than the *T. congolense* assay and is potentially useful in determining the distribution of bovine trypanosomosis and targeting appropriate control measures in different areas of Uganda.

ACKNOWLEDGEMENTS

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STUDY OF TRYPANOSOME AND ANAEMIA INDICATORS DURING THE ERADICATION OF TSETSE FLIES FROM UNGUJA ISLAND, UNITED REPUBLIC OF TANZANIA

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Abstract

STUDY OF TRYPANOSOME AND ANAEMIA INDICATORS DURING THE ERADICATION OF TSETSE FLIES FROM UNGUJA ISLAND, UNITED REPUBLIC OF TANZANIA.

A large number of cattle has been monitored regularly during a ten-year period on Unguja island, United Republic of Tanzania, as part of a number of consecutive programmes to initially control and eventually eradicate tsetse and trypanosomosis. Haematological and parasitological results were used among others to monitor and adjust the control and eradication programmes from 1988 to 1997. A Geographic Information System (GIS) was applied to the data set to assess changes of animal health parameters in time and space. Analysis of the data showed significant changes over the years in infection status and degree of anaemia. Moreover, differences in health status of cattle populations between adjacent geographic areas were detected. Regular monitoring using standardized protocols by a multidisciplinary team resulted eventually in the eradication of tsetse flies from the island.

1. INTRODUCTION

Zanzibar comprises two major islands, Pemba and Unguja, forming part of the United Republic of Tanzania. In an attempt to support a fast growing population and to become self sufficient in livestock products, the Ministry of Agriculture in Zanzibar has embarked on a programme to stimulate agricultural development and reduce the major constraints. At present the Government of Zanzibar relies heavily on the import of cattle from mainland Tanzania to provide sufficient meat for domestic consumption.

Tsetse transmitted trypanosomosis has been recognized as an important constraint to animal production on Unguja island, and control of the disease has become a priority for the Government of Zanzibar. Previously, more than 60% of the cattle population in some parts of the island was either permanently or seasonally at risk of becoming infected with trypanosomosis. Upgrading of the local East African Shorthorn through cross-breeding with exotic breeds was severely hampered by the higher susceptibility of the latter animals to the disease. It is anticipated that control of trypanosomosis will have a significant impact on the island economy resulting in self sufficiency of meat and milk production.

The trypanosomosis control programme attacked the problem both through vector and parasite control. Vector control included (a) the application of synthetic pyrethroids on living targets (livestock) via pour-on formulations or dip tanks (b) the use of insecticide impregnated screens, and (c) the use of the sterile insect technique (SIT). Parasite control included (i) therapeutic treatment of all infected animals (ii) prophylactic treatment of cattle with isometamidium chloride (iii) diagnostic monitoring and treatment of selected animals, i.e. imported livestock, sentinel cattle.

In any disease control programme it is essential to monitor progress on a continuous basis and to identify areas where the vector and/or the disease may persist. Consequently, entomological and parasitological surveys were conducted on a routine basis, but both methods have limitations with regard to diagnostic sensitivity. As an additional monitoring tool GIS was used to assist programme managers in evaluating progress and identifying areas of residual vector populations or disease transmission.

2. HISTORICAL BACKGROUND

2.1. The control activities

The first report of trypanosomosis on the island of Unguja was published in 1909 [1]. In 1923 a survey among cattle revealed a prevalence of 12.5% predominantly due to *Trypanosoma congolense* [2]. Transmission of the disease was attributed mainly to horseflies, *Tabanus* spp., since no tsetse flies had been captured on the island despite repeated searches. Tsetse flies, *Glossina* spp., were first caught on the island in 1945 around Chukwani area. In contrast, tsetse flies have never been caught on the neighboring island of Pemba and no cases of trypanosomosis in the local animal population have been reported in Pemba. During an island-wide trypanosomosis survey of Unguja conducted from 1948 to 1951 it was revealed that *Glossina austeni* was the only tsetse species present (Fig. 1) and that it probably had been established on Zanzibar for a very long period [3]. During the same period the infection rate of a population of 8000 cattle was found to be 17 %, predominantly due to *T. congolense* and occasionally due to *T. vivax* [4]. In the mid-sixties a small scale tsetse control programme was implemented in Mangapwani area by bush-clearing and spraying with Dieldrin®.

An extensive disease survey was initiated on the island during 1985 and 1986 as part of FAO project URT/81/017 resulting in the collection of 1881 thin and thick blood smears from a total cattle population of approximately 28,000 (Census 1978). The results showed that 19% of the samples were infected with trypanosomes, predominantly *T. congolense* [4]. High trypanosomosis prevalence rates were found in the Mangapwani/Fujoni area (64%), Pete area (40%) and Muyuni/Muungoni (43%) areas [4]. The percentage of cattle with PCV values below 25% was highest (66-100%) in the areas around Jozani forest in 1986 [4]. The first successful control efforts were initiated by FAO project TCP/URT/6758 in 1988 [5]. The area of Mangapwani in the northwestern part of the island was chosen as a suitable relatively isolated area of 20 km^2 for a pilot trial using pour-on formulations. A deltamethrin (1% w/v) formulation, Spot-On® (Schering-Plough, USA), was applied to all cattle (older than 6 months), donkeys and goats in the area. The tsetse population was monitored using three-dimensional sticky panels [5] and the cattle were examined for blood parasites using the microhaematocrit centrifuge technique [6]. Technical assistance was provided by the Joint FAO/IAEA Division to assess the location and density of *G. austeni* [5].

Mainly based on the Mangapwani results, UNDP funded two animal health projects which included tsetse and trypanosomosis control components. The UNDP/FAO Animal Disease Control Projects (URT/86/022 and URT/91/006) provided assistance to the Government of Zanzibar for a larger scale tsetse control programme using treatment of livestock with residual synthetic pyrethroids in those areas with sufficient number of livestock to ensure a good host-vector contact. Stationary insecticide impregnated screens were used in those areas with a low cattle density (<10 per km^2) like the Jozani forest. Although the tsetse population was successfully controlled, complete eradication was not achieved [7].

Subsequently, the International Atomic Energy Agency (IAEA) was requested by the Government of Tanzania to assist in the eradication of tsetse flies from Unguja island using the Sterile Insect Technique (SIT) as part of an integrated pest management plan [8]. The FAO project URT/91/006 was terminated in 1994 and the veterinary activities of this project were incorporated into the IAEA TC project URT/5/016, which had as its objective the eradication of *G. austeni* from Zanzibar. Applied research was initiated to improve tsetse fly rearing techniques and to understand tsetse population dynamics in Jozani forest [8]. Initially project URT/5/016 concentrated on the suppression of the wild tsetse population using Spot-On® applications, dipping of livestock in Decatix® (Cooper, Zimbabwe) and insecticide impregnated screens. The fly suppression was closely monitored by entomological and veterinary surveys and if required infected cattle were treated with diminazene aceturate (Berenil®, Hoechst, Germany). During a subsequent phase of the project large numbers of sterile tsetse flies were released by air and monitoring of the eradication process was intensified through veterinary and entomological surveys.

2.2. The eradication activities

Batches of male *G. austeni* immobilized by chilling at 4° C shortly after emergence were exposed to an ionizing radiation dose of 120 gray (Gy) of gamma rays from a ^{60}Co source or a ^{137}Cs source [9]. Details of colonization, sterilization, packing, handling, transport and release methods have been described elsewhere [9].

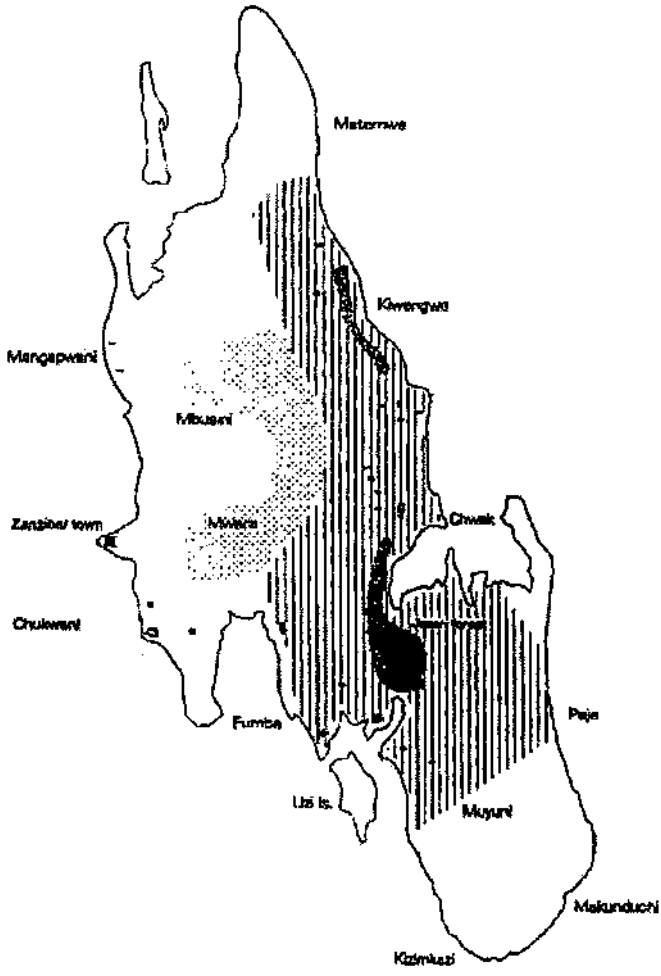


FIG. 1. Distribution of tsetse flies, Glossina austeni, *on the island of Unguja in 1952 [4]. Black area:* G. austeni *present in high numbers; hatched area:* G. austeni *frequently encountered; dotted area:* G. austeni *occasionally encountered.*

During an initial trial release 220,699 sterile *G. austeni* males were released in the northern part of the Jozani forest from November 1990 till February 1991 [10]. The first test releases of sterile tsetse males from fixed wing aircraft were initiated in August 1994 in the southern part of Unguja island. As of May 1995 aerial releases of sterile males exceeded 40,000 per week. The insects were released on a regular basis twice weekly over the southern part of the island resulting in a ratio of sterile to wild tsetse males of more than 15 to 1. During 1996 the average number of sterile males released weekly had increased to 70,000 tsetse flies and a ratio of sterile:wild tsetse males of >100:1 was achieved. As of March 1996 insects were released over the southern and central parts (Chejuu area) of the island and as of July 1996 tsetse flies were released over the entire island. Although the vast majority of tsetse were sterile males, approximately one percent of the released insects were females due to unavoidable sexing errors. The average survival time for sterile male *G. austeni* was estimated to be 7-9 days with a maximum of recorded survival time of 15 days [9]. However, female tsetse survive considerably longer than males with a mean expected life (for non-irradiated tsetse) between 2 and 3 months and a maximum not exceeding 5 months [11].

3. MATERIALS AND METHODS

3.1. Study area

Unguja island covers an area of approximately 1,650 km^2 and is separated from the East African coast by the 35 km wide Zanzibar channel. The rainfall pattern is bimodal with the main rainy season from March to June and a lesser rainy period in November/December. The current human population is about 400,000 growing at an annual rate of 3% [9].

For a long time it was assumed that no tsetse occurred on the island due to the fact that the single tsetse species, *G. austeni*, present on the island was difficult to catch using traditional tsetse traps [1]. Consequently, searching pupae proved the most reliable way to determine the tsetse distribution during an island wide survey in 1984 [4].

3.2. Cattle population

According to the livestock census of 1993 a total of 45,750 head of cattle were present on Unguja island (65,943 on Pemba island) together with 26,472 goats, 375 sheep and 494 donkeys [9]. The vast majority of cattle (97%) belong to the East African Zebu breed.

3.3. Sampling methods

Blood samples have been collected from cattle at random on a regular basis since 1988. A total of 15,363 samples were available for analysis from the period 1988 to 1993. To facilitate and standardize sampling procedures the island was divided into 38 blocks in 1994. Sentinel herds of approximately 30 female animals of 1-2 years of age were established in each block wherever possible in order to monitor the disease in livestock in a systematic way. During an initial visit in February 1994 blood samples were collected and the sentinel animals were treated with Berenil® at 7 mg/kg intramuscularly. Thereafter, samples were collected regularly from all sentinel animalsat 4 to 6 times per year resulting in a total of 16,767 samples being available for analysis (Table I). The samples were collected from the jugular vein using vacutainer tubes and needles and were examined for packed red cell volume (PCV) values as a indication of general health and a measure of anaemia. In addition, the samples were examined for the presence of trypanosomes by the MHCT [6] and/or the buffy coat technique (BCT) using a darkground/phasecontrast microscope [12]. Whenever sentinel animals were found positive for trypanosomes, they were treated the next day with a curative dose of Berenil® (7 mg/kg).

TABLE I. SAMPLING DATES AND NUMBER OF SENTINEL ANIMALS SAMPLED TO MONITOR THE STERILE INSECT TECHNIQUE PROGRAMME TO ERADICATE TSETSE FLIES FROM UNGUJA ISLAND

Month*	Year	Number of cattle sampled#
May	1994	1068
July	1994	1056
September	1994	1097
November	1994	1420
January/February	1995	1209
March/April	1995	987
June	1995	418
August/September	1995	959
October	1995	363
December	1995	1007
March/April	1996	297
May	1996	875
September	1996	314
December	1996	847
April/May	1997	850
August/September	1997	656
January-October	1998	2242
January/April/July/October	1999	1102

*Sentinel animals were selected, sampled and simultaneously treated with a trypanocidal drug in February 1994. However, the results of this initial bleeding were not included in the analysis.
The number includes sentinel animals bled in blocks 1-9, blocks 10-29 and blocks 30-38, being the Northern, middle and Southern part of Unguja island, respectively. Because of the large number of animals involved sampling visits were usually staggered in time (for example the animals in blocks 30-38 were bled and treated for the first time in November 1994).

To monitor the tsetse population panels made sticky with glue (a suspended free rotating version of the Chuka trap) proved to be most effective for catching *G. austeni* [9]. In 1995 approximately 200 of these devices were used as permanent monitoring sites and 40-80 additional ones were employed on a temporary basis (Fig. 2). During the twice weekly sterile insect release period, panels were checked on a regular basis. By November 1996 a total of 250 traps were placed in the Northern blocks (1-29) and 301 traps were present in the Southern part of the island (blocks 30-37).

3.4. Data analysis

Veterinary data were collected from animals located in specified blocks and geo-referenced afterwards. The data consisted of packed red cell volume (PCV) values which are a measure of anaemia and thus a useful indicator of disease and health status. Individual PCV values were regularly collected from sentinel animals from 1994 to 1996. Entomological data were geo-referenced as they were collected using a Global Positioning System [13]. Both sets of information were stored in an Access relational database. Arc/Info was used for spatial analysis and overlay of geo-referenced data sets [14] and Arcview for map production and display [15].

For statistical analysis, the raw geo-referenced PCV data were extracted into Microsoft Excel spreadsheets, from which they were imported into the Statistical Package for the Social Sciences (SPSS) for analysis [16]. Where a number of PCV values for individual animals were available from one year, they were aggregated into means. Differences between PCV values for 1994/5/6, and changes between years (94/95, 95/96, and 94/96), for animals inside and outside the control area were assessed using the default SPSS Analysis of Variance module (ANOVA) whereby all effects are assessed simultaneously for their contribution (unique method). That is, each effect is adjusted for all other covariates, main effects, and interaction terms in the model. In addition, any trends of PCV values in relation to the distance of the sample point from the control area boundary were evaluated using linear regression on the un-transformed values.

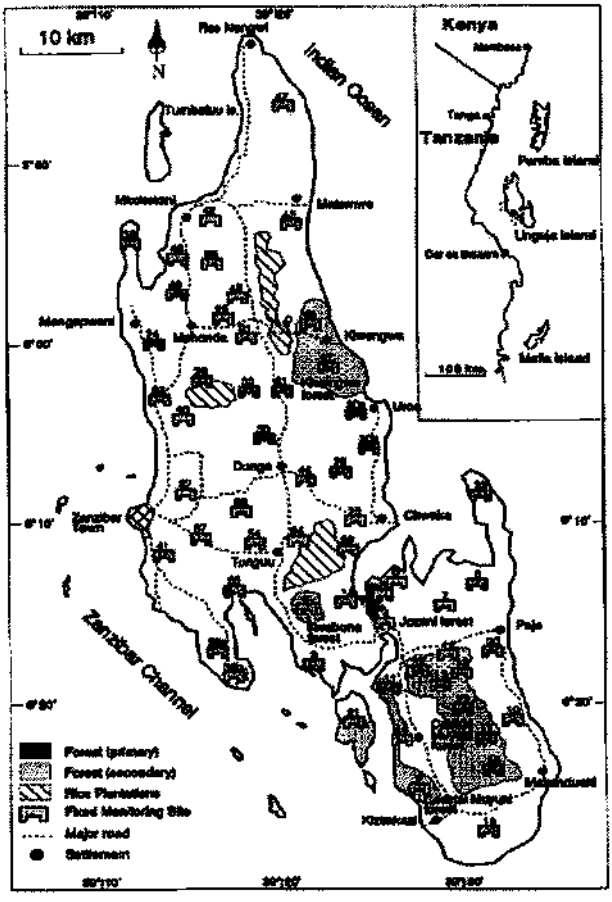


FIG. 2. Map of Unguja island showing the fixed entomological monitoring sites, which contained a total of 399 free-rotating blue-white leg-panel traps made sticky with glue (map courtesy of Marc Vreysen).

4. RESULTS

The average pre-control apparent density of the tsetse population in Mangapwani in 1987 was found to be 2.67 tsetse/target/day (0.14-3.66), while the pre-control trypanosomosis prevalence was 46% [5]. When comparing the trypanosomosis prevalence in 1989 (Fig. 3a) with the situation in 1991

(Fig. 3b) a sharp decrease can be noticed. This decrease was due to the systematic application of livestock with pour-on formulations containing residual synthetic pyrethroids. After five consecutive applications of the pour-on (Spot-On®) at 15 to 18 day intervals at a dose rate of 10 ml/100 kg body weight, the incidence of infection in sentinel cattle dropped to zero (Fig. 4). At the same time no tsetse flies were caught after the third application (day 47-54). On the other hand, 50% of the cattle monitored in the adjacent untreated area of Mwakaje became re-infected following trypanocidal drug [5].

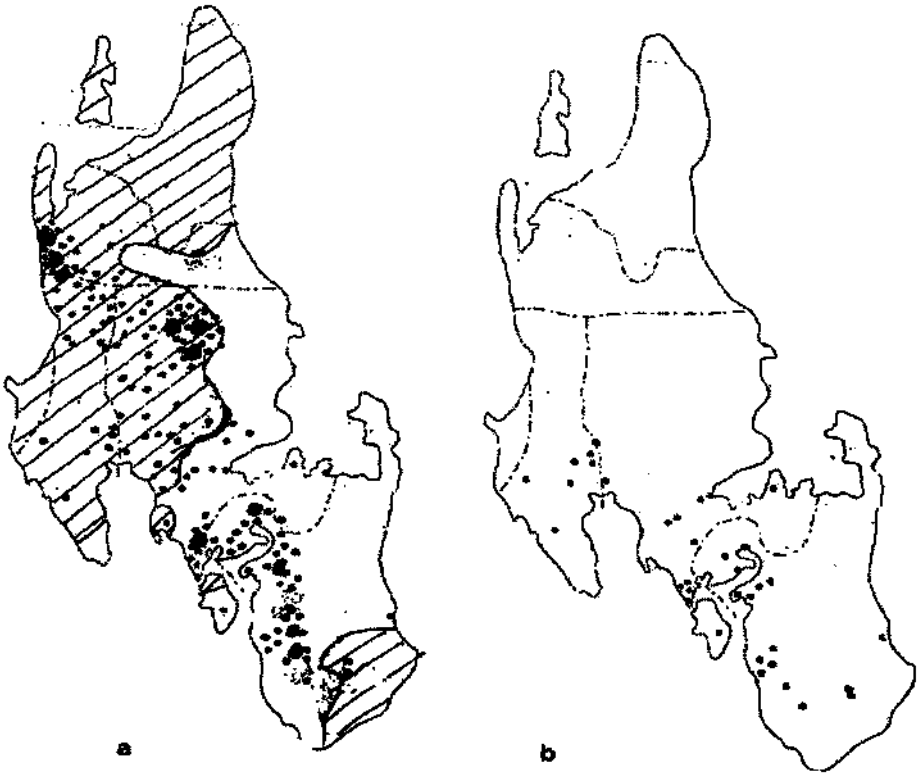
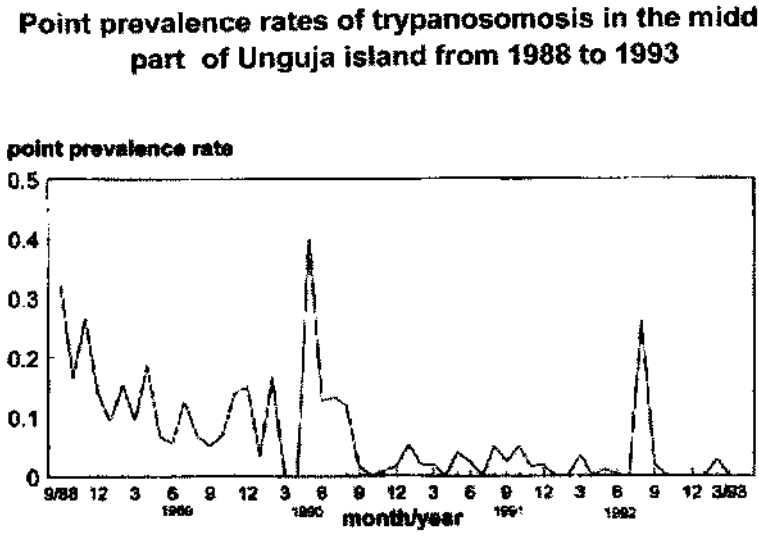


FIG. 3. Prevalence of cattle trypanosomosis (small dot represents a trypanosome-positive case; large dot indicates >30 % of the samples positive) in 1989 (a) and in 1991 (b) in the major livestock producing areas of Unguja island (as surveyed and prepared by Andreas Schönefeld and Detlef Höreth-Böntgen under FAO projects URT/6758 and URT/86/022). Hatching indicates the main livestock rearing areas.



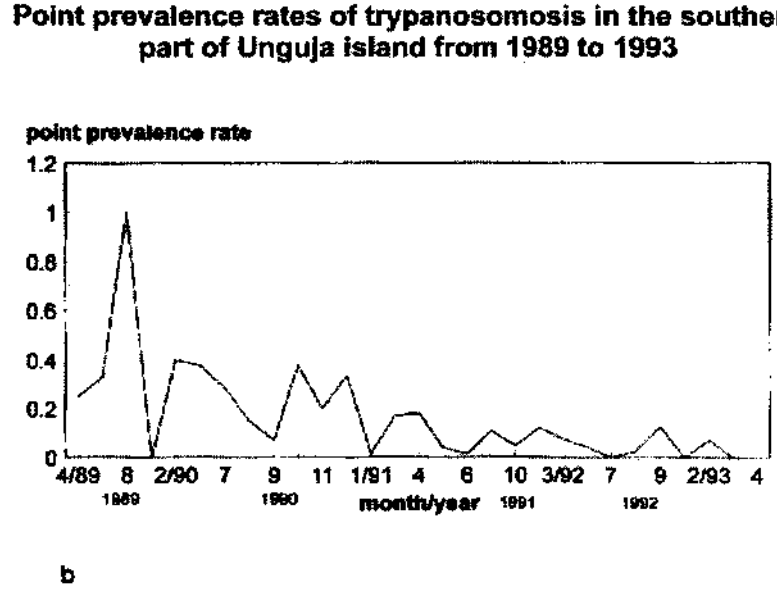


FIG. 4. Point prevalence rates of trypanosome infections in cattle sampled in the middle (a) and Southern (b) parts of Unguja island from 1988 to 1993. Data were obtained from FAO archives [5].

During the final visits of a sequential sampling exercise in 1994 in the Mangapwani area (block 9), no trypanosomes could be detected in the cattle using the BCT (Table II) [17]. On the other hand, blood samples collected from cattle near the forested area of Jozani (blocks 27 and 28), showed persistent parasitaemias indicating an average disease prevalence of 11% (Tables III and IV).

TABLE II. TRYPANOSOMOSIS SITUATION IN MANGAPWANI (BLOCK 9), UNGUJA ISLAND, FOLLOWING AN INTEGRATED CONTROL STRATEGY*

	Sampling visit**				
	First	Second	Third	Fourth	Total
Number of cattle sampled	47	33	70	81	231
Number of samples positive by BCT	1 T.c./T.v.	0	0	0	1

* = The strategy included the use of pour-on (deltamethrin) to control tsetse on cattle, artificial attractants (sticky targets and blue screens, blue cloth impregnated with insecticide) and treatment of infected cattle with a trypanocidal drug (diminazene aceturate); ** = At two-month-intervals in 1994; BCT = buffy coat technique; T.c./T.v. = mixed infection of *T. congolense* and *T. vivax*; Table has been adapted from [17].

TABLE III. RESULTS OF BLOOD SAMPLES COLLECTED FROM CATTLE IN NDIJANI (BLOCK 27), UNGUJA ISLAND

	Sampling visit*			
	First	Second	Third	Total
Number of cattle sampled	44	31	43	118
Number of samples positive by BCT	4 T.v.; 3 T.c./T.v.	0	2 T.v.; 1 T.c./T.v.	10

* = At two-month-intervals in 1994; BCT = buffy coat technique; T.v. = *T. vivax;* T.c./T.v. = mixed infection of *T. congolense* and *T. vivax;* Table has been adapted from [17].

T. vivax was the predominant trypanosome species detected in 87% of the positive blood samples, while *T. congolense* was found in the remainder of positive samples [17]. *T. brucei* has never been detected in bovine blood samples on the island, except during a short period from March to August 1992 when 107 of the 125 positive cases (of 807 animals sampled) supposedly contained this species.

TABLE IV. RESULTS OF BLOOD AND SERUM SAMPLES TESTED FROM KIKUNGWI (BLOCK 28), UNGUJA ISLAND

	Sampling visit*			
	First	Second	Third	Total
Number of cattle sampled	41	25	30	96
Number of samples positive by BCT	1 T.c.; 4 T.v.	2 T.v.	2 T.v.	9

* = At two-month-intervals in 1994; BCT = buffy coat technique; T.c. = *Trypanosoma congolense*; T.v. = *T. vivax;* Table has been adapted from [17].

The regular monitoring of sentinel animals during 1994 showed a low or zero infection rate in cattle in the northern part of the island (blocks 1-9), a moderate number of infections detected in the middle part (blocks 10-29) and the highest number of infections in animals located in the southern part (blocks 30-38), especially in areas close to the Jozani forest area (Fig. 5).

During the period of regular release of sterile insects in the years 1995 and 1996 the number of sentinel cattle detected positive for trypanosomes gradually decreased (Fig. 6a and b), except for a temporary, but pronounced increase from March to December 1995 in the southern blocks (Fig. 6b).

When analyzing average changes in PCV values of herds monitored at the various sampling points on the island and comparing the values for 1994 and 1996, it appeared that negative changes predominated in the southern part of the island. When the island was divided in two areas, one subjected to intensive tsetse control activities (inside the SIT aerial release target area), the other situated outside the SIT target area and subjected to minimal control activities, differences became more obvious. The percentage of animals with PCV values below 25% increased from 1995 to 1996 inside the SIT area and decreased outside the area (Table V).

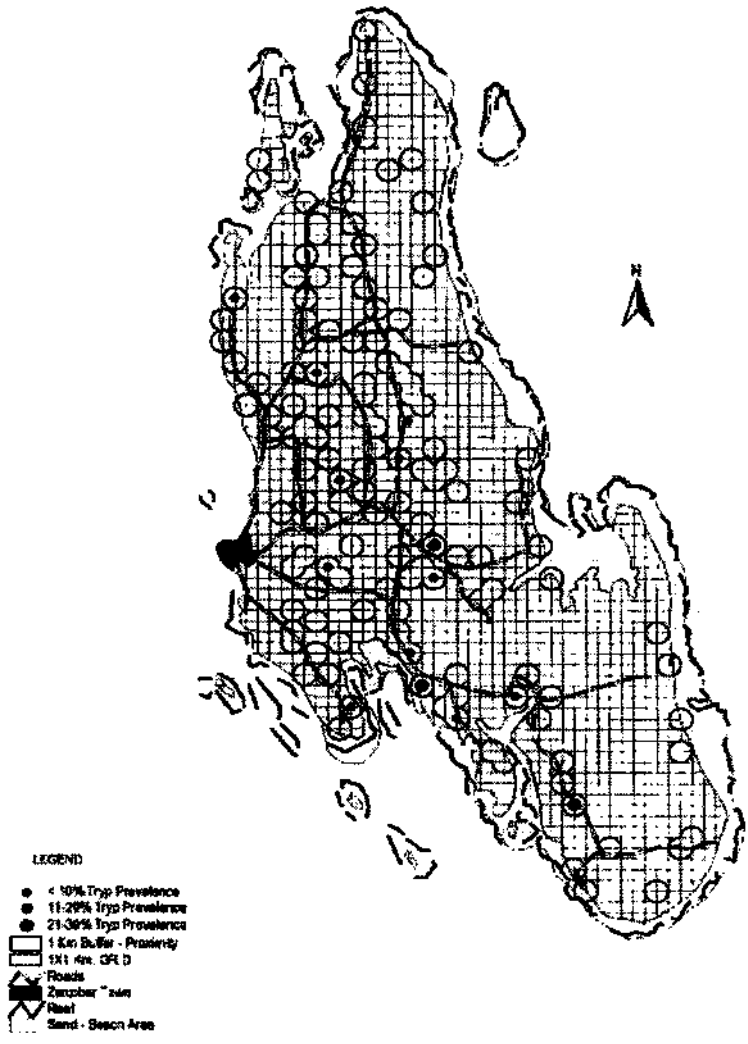
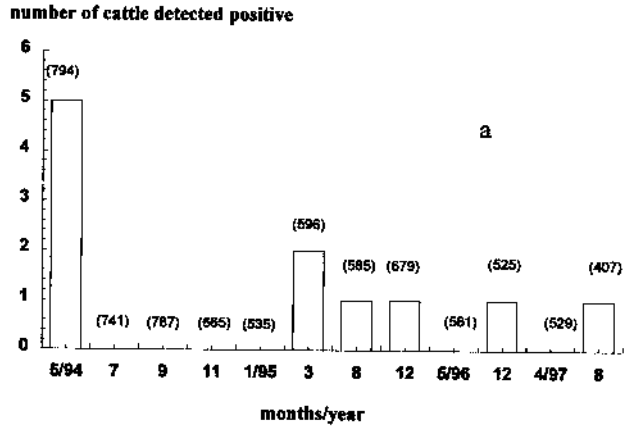


FIG. 5. Distribution of trypanosomosis prevalence in sentinel cattle sampled during the year 1994. Each square on the map represents 1 km^2. Proximity mapping based on geo-referenced place names was used to represent the data from neighboring sampling points (indicated as a circle with a 1 km buffer generated around the center).



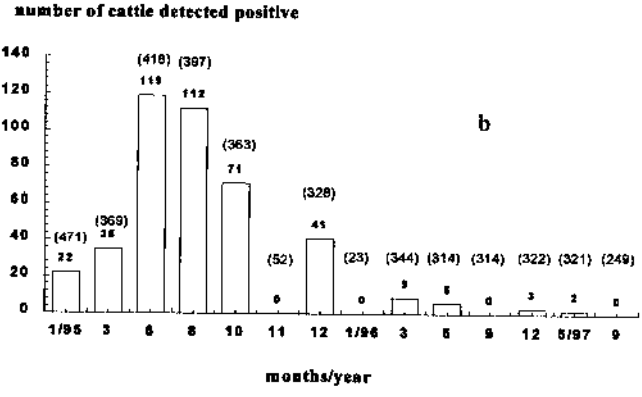


FIG. 6. Number of trypanosome-positive cases detected using the buffy coat technique [12] in a) the middle part (blocks 10-29) of Unguja island from May 1994 to August 1997 and b) the Southern part (blocks 30-38) from January 1995 to September 1997.

TABLE V. COMPARISON OF AVERAGE PACKED RED CELL VOLUME (PCV) VALUES BELOW AND ABOVE 25% INSIDE AND OUTSIDE THE STERILE TSETSE RELEASE AREA IN 1994, 1995 AND 1996

		Number of animals		Percentage	
		inside	outside	inside	outside
PCV in 1994	below 25 %	175	235	28.2	20.0
	above 25 %	446	939	71.8	80.0
PCV in 1995	below 25 %	73	229	14.9	18.1
	above 25 %	416	1036	85.1	81.9
PCV in 1996	below 25 %	126	90	36.4	13.9
	above 25 %	220	557	63.6	86.1

The analysis of PCV values in relation to the distance from the SIT release area showed a statistically significant relationship when comparing the values collected inside and outside the control area in 1994 and 1996 (Table VI). Moreover, PCV values outside the SIT area remained constant over the years, while small differences in PCV values were noticed inside the area small when comparing 1994, 1995 and 1996 (Table VI).

Regression analyses of both individual animal PCV values and PCV data averaged on a herd basis showed that the levels increased significantly with distance from the regular tsetse release site located in the southern part of Unguja island. This increase became more pronounced in 1996 (R square = .043;P<0.001) than it was in 1995 or 1994 (R square = .001 or .0028, respectively;P<0.001). Moreover, when the change in PCV of values in 1994 and values in 1996, was assessed in relation to the distance away from the sterile insect release area, it was found that the change was negative for animals within or near the release area and became more positive as animals were geographically located further away from the release area (Table VI).

TABLE VI. DIFFERENCES BETWEEN PCV VALUES OF CATTLE INSIDE AND OUTSIDE THE STERILE TSETSE RELEASE AREA IN 1994, 1995 AND 1996

Control Area		PCV '94	PCV '95	PCV '96	DIFF 94-96	DIFF 95-96	DIFF 94-95
Inside	**Mean**	**28.2027**	**29.8707**	**27.1748**	**-1.0211**	**-2.2233**	**1.6899**
	N	592	455	326	213	256	330
	Std. Deviation	4.8265	4.4780	4.9780	6.5498	5.7225	5.5028
Outside	**Mean**	**29.5482**	**29.5200**	**29.8598**	**.5022**	**.4419**	**.3762**
	N	1203	1299	667	225	324	1006
	Std. Deviation	4.9086	4.6318	4.3544	4.9352	4.7082	2.9159
F of Difference		30.141	1.965	75.642	7.608	37.854	30.909
Sig of Difference		**<0.001**	**NS**	**<0.001**	**0.006**	**<0.001**	**<0.001**
DF		1793,1	1752,1	436,1	436,1	578,1	1334,1

PCV = packed red cell volume; DIFF 94-96 = difference between average PCV values of 1994 and 1996; N= number of observations; Std. Deviation = standard deviation; F of Difference= Fraction of difference between means; Sig of Difference = significance of difference; DF = degrees of freedom.

5. DISCUSSION

The results show clearly that the integrated control programmes executed during the years 1986-1991 were effective in reducing the prevalence of trypanosomosis in cattle and in decreasing the tsetse population below detectable levels. However, the programmes were not able to eradicate the disease nor the vector. Following successful suppression of the insect population it was logical to attempt to eradicate the vector completely from the island. The geographical situation of Zanzibar made it ideally suitable for the application of the sterile insect technique originally applied to eradicate the screwworm fly, *Cochliomyia hominivorax* [18], but subsequently proposed for the control of a number of other insect and animal populations [19, 20]. The major advantages of SIT over other techniques are that it is environmentally friendly and targets a single species [21]. Moreover, if applied under the right preconditions, SIT should eradicate the vector. Although SIT has been tested previously during feasibility studies in Zimbabwe [22] and applications have been reported in Tanzania [23], Burkina Faso [24] and Nigeria [25], it proved in each case difficult to prevent reinvasion of cleared areas. However, following the eradication of tsetse flies reinvasion will very likely not pose a problem on the island of Unguja due to its geographical isolation.

During regular monitoring of sentinel animals as part of the tsetse eradication programme on Unguja island analysis of the data showed a temporary increase in trypanosomal infections during 1995 and a gradual decrease in PCV values as the release of large numbers of biting insects continued. It has been reported that the release of sterile male tsetse flies can produce an increase in disease transmission [26], which in turn has been linked to decreases in PCV values [27]. Furthermore, during the eradication programme on Unguja island relatively large numbers of sterile females have been released due to the unavoidable error when separating the two sexes. To reduce disease transmission, tsetse flies were fed by artificial membrane on blood containing a trypanocidal compound, isometamidium chloride (Samorin®, May & Baker, UK), before aerial release [28]. However, a recent report showed that a bloodmeal containing Samorin® did not prevent the establishment of infection in teneral tsetse when they were subsequently fed on mice infected with trypanosomes [29]. Moreover,

mechanical transmission of *T. vivax* will not be prevented by feeding tsetse flies a bloodmeal containing Samorin® before release. Consequently, the released female tsetse flies would be expected to act as potential disease vectors, thus, conceivably maintaining disease transmission.

Bloodmeal analysis of 308 samples showed that the preferred host of *G. austeni* was the bushpig (57.7% of the bloodmeals), but that 35.6% of the bloodmeals originated from ruminants (duiker in Kenya and cattle on Zanzibar) and 4.9% came from humans [30]. An updated survey following analysis of 100 additional samples showed a similar trend: 46.6%, 14.5%, 7.1% and 4.9% originated from bushpig, cattle, duiker and man, respectively [31]. The latter two reports indicate that although the majority of meals were taken from bushpigs, it can be presumed that a large number of *G. austeni* did feed on cattle.

In summary, the release of large numbers of insects during 1995 and 1996 and the transient increase in disease transmission in 1995 were accompanied by a gradual decrease in PCV values over time in the sentinel animals. Such a decrease in health status is certainly an undesirable side effect of the tsetse eradication programme, but can at present not be firmly attibuted to the large-scale insect release programme. It has been documented that under field conditions a decrease in PCV values can be linked to other factors than trypanosomosis such as poor nutritional status, ticks, tick-borne diseases and helminth infections [32]. Unfortunately, the nutritional status, worm load or tick infestation of the sentinel animals were not monitored. During a tsetse eradication campaign using the sterile insect technique it is important to monitor progress using entomological and veterinary techniques as described above. As a result of the present investigation, it is advisable to treat with a trypanocidal drug not only the animals, which are detected parasitologically positive, but also the cattle with a PCV below 25%.

ACKNOWLEDGEMENTS

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EVIDENCE REQUIRED FOR ESTABLISHING THE ABSENCE OF TSETSE AND TRYPANOSOMOSIS ASSOCIATED WITH TSETSE ERADICATION PROGRAMMES

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Abstract

EVIDENCE REQUIRED FOR ESTABLISHING THE ABSENCE OF TSETSE AND TRYPANOSOMOSIS ASSOCIATED WITH TSETSE ERADICATION PROGRAMMES.

This paper considers entomological and epidemiological criteria for establishing the absence of tsetse and trypanosomosis associated with tsetse eradication programmes. The sampling goal is simple – to maximise the probability of detecting tsetse flies in the field or trypanosomes in hosts, if they exist. Sampling strategies cannot guarantee the absence of tsetse and trypanosomes but they can be used to estimate the probability of their eradication. Because tsetse eradication programmes are targeted at defined areas, geographical information system (GIS) tools are very useful in guiding spatial sampling strategies. Two approaches to assess tsetse eradication are discussed. The first depends only on information on the sensitivity of tsetse trapping methods used. The second combines information on pre-eradication tsetse trapping and the proportion of time during which no tsetse have been trapped, assuming either a stable or declining (preferred) pre-eradication tsetse population. For establishing the absence of trypanosomosis in host populations, there are standard sampling techniques that can be adjusted for the sensitivity and specificity of the trypanosome detection methods used. Required sample sizes can be calculated for both direct trypanosome detection methods of 100% specificity or indirect tests with imperfect sensitivity and specificity. For the latter, both the sample size and the number of reactors (assumed to be false positive) are estimated for the required confidence level. These entomological and epidemiological methods were then applied to assess the eradication of tsetse and trypanosomosis from Unguja island of Zanzibar using the sterile insect technique (SIT). Pre- and post-eradication data collected were sufficient to establish with 95% confidence that both tsetse and trypanosomosis were eradicated.

1. INTRODUCTION

In planning and executing eradication programmes, the evidence required to confirm eradication must be quantitatively sound and agreed upon in advance. This minimises the probability of making the expensive and embarrassing error of declaring an area eradicated of tsetse and trypanosomosis when it is not. Secondly, it establishes an evidence-based end-point for eradication efforts, thus, minimising on-going programme expenditures if eradication has already occurred.

The goal of both entomological and epidemiological sampling in the end stage of an eradication programme is very simple - to maximise the probability of detecting any tsetse flies in the field or animals infected with trypanosomes, if they exist. There are a number of basic principles that must be kept in mind. First, in trying to detect the presence of tsetse flies or trypanosome infections, any declaration of absence cannot be guaranteed but must be qualified with a probability (confidence) statement. The techniques for establishing the absence of trypanosomes in animals are better defined and quantified than are those for establishing the absence of tsetse flies. Second, the lower the expected prevalence the larger the sample size required for a given confidence level. Third, test errors (sensitivity and specificity) need to be taken into account, either implicitly or explicitly, when estimating the confidence level. Fourth, more information and an increased sampling efficiency can often be achieved by stratified sampling on spatial ecological units.

As eradication occurs on an area basis, geographic information systems (GIS) can be an important tool for guiding the entomological and epidemiological sampling required to confirm eradication. GIS is particularly suited for: 1) identifying spatial differences in ecological suitability for tsetse [1, 2] and trypanosmosis risk [3], and 2) in defining spatial sampling units [4]. These features are crucial throughout and beyond eradication to ensure that the entire eradication area is being monitored and to target potential transmission hot-spots for intensive follow-up and monitoring.

In this paper, I present probability-based indicators to be used in assessing the absence of tsetse and trypanosomosis following a tsetse eradication programme. These criteria will then be applied in assessing the success of the tsetse eradication programme on Unguja island, Zanzibar.

2. ENTOMOLOGICAL CRITERIA

Standard, probability-based entomological criteria for confirming the eradication of tsetse have not been regularly applied to assess tsetse eradication programmes. This mirrors the lack of established criteria for the determination of species extinction by conservation biology organisations for endangered species [5].

In the ecological literature, there are two basic approaches followed to assess species extinction. The first, uses information only after extinction is assumed to occur to assess the number of negative sightings required to establish extinction at a given probability level [5]. This was the method used by staff associated with the Zanzibar tsetse eradication project [4] and requires that the probability of detecting tsetse flies, given the sampling methodologies used, is well known. The estimation of the number of negative samplings required is highly influenced by the probability of detection (sensitivity of the tsetse trapping method) (e.g. for a 95% confidence in eradication, 14 negative sampling weeks would be required if the probability of detection is 20%, 59 if it is 5% and 298 if it is 1%).

A second approach, which is more powerful, incorporates data from pre- and post-eradication samplings. Either a relatively small but stable pre-eradication [6] or a declining population [7] can be assumed. Sightings are considered to follow a Poisson process, which may be stationary or non-stationary. In this approach, the probability of eradication (extinction) for regularly monitored populations is estimated as a function of the frequency of pre-eradication sightings and the proportion of total time (pre- and post-eradication) during which no sightings have been made. For tsetse populations under an eradication programme, a declining (non-stationary) population is most appropriate. The assumptions underlying the method for relatively small stationary populations generally yields confidence levels that are considered too optimistic [6].

For either approach, it is crucial that structured sampling of the tsetse population across the entire area should begin in the pre-eradication period. An important reason is to establish the most sensitive and representative tsetse trapping methods. All tsetse trapping methods are biased for different segments of the population [8]. The key is to understand how to collect and interpret trapping data that can be used to estimate relative and apparent abundance of tsetse species. Trap locations, seasonal variations and trap efficiencies in different habitats need to be considered [9]. Of particular concern is to maintain the sensitivity of trapping methods for very small tsetse populations, as happens towards the end of an eradication programme. A constant probability of detecting tsetse flies is a strong assumption of all models used to assess the probability of eradication. Since a declaration of eradication when it has not occurred is a serious error, steps to decrease this error should be considered. These could include increasing efforts to catch tsetse flies (increased numbers and distribution of traps or targets) when tsetse numbers are declining and reducing confidence levels from 5% to 1% or less.

Eradication needs to be confirmed across an area. Thus, GIS tools will be essential to establish a spatial sampling structure for tsetse monitoring using this prior information and stratified random sampling strategies to ensure appropriate area-wide coverage, particularly if re-invasion is a possibility. Ecological suitability for tsetse and trypanosomosis risk will be important stratification gradients. During the eradication programme, tsetse flies are most likely to persist in areas of highest ecological suitability. Because less suitable areas will have much lower expected tsetse populations, more intensive sampling in these areas will be required to be assured that no tsetse are present for a given level of confidence.

Better eradication decisions can be made by utilising all available data. The techniques described above simply classify the presence or absence of tsetse flies. Additional information, such as tsetse counts and the age and sex distribution of the tsetse population, could provide more support for decision-making purposes (e.g. adjusting sterile: wild fly ratios in SIT or targetingcertain habitats for insecticide-based methods) throughout the eradication period. Tsetse counts (total and by age and sex classes) would be particularly valuable for developing empirically based estimates of the probability of tsetse eradication for different time periods after the last tsetse fly has been caught. The development of new methods and adaptations of existing methods [7] are required for this purpose.

3. EPIDEMIOLOGICAL CRITERIA

Because of the difficulties of confirming tsetse presence by tsetse monitoring, trypanosomosis prevalence surveys are a crucial adjunct and more sensitive indicator of the potential presence of tsetse vectors.

Simplifying approximations and tables for determining sample sizes required for the detection of infected or diseased animals with specific confidence were developed by Cannon and Roe [10]. Given that trypanosome prevalence will be quite low at the end stages of an eradication programme, sample sizes to detect the presence of infection will be quite large. These simple formulas [10] assume perfect test performance. This is not a reasonable assumption for current direct or indirect detection methods for trypanosomosis. Fortunately, Cameron and Baldock [11] have developed software to incorporate test sensitivity and specificity parameters into sample size estimates. They have also further extended this methodology to allow for stratified two-stage sampling [12]. This is particularly useful for varying sampling rates by herds or ecological zones. In addition to stratification on spatial risk, it is also important to consider temporal changes, particularly seasonal variations. In any longitudinal monitoring system, at least one of the repeated sampling times should be during the period of expected maximum prevalence to enhance the sensitivity of detection.

As noted above, diagnostic methods for the detection of trypanosome infections are imperfect. Direct microscopic examination has perfect specificity (with experienced technicians) but relatively low sensitivity. Antibody detection methods have much better sensitivity but imperfect specificity. Antigen detection methods have proved unreliable [13] and are no longer widely supported or used. PCR and other nucleic-acid detection methods are not yet available for widespread use and their performance and repeatability under field situations is insufficiently investigated [14].

Although individual test methods are imperfect, testing strategies can be developed that minimise the impact of test errors by increasing the probability of detecting true trypanosome infections (sensitivity) and decreasing the probability of misclassifying uninfected animals as positives (1-specificity). Because the impact of test errors vary with true prevalence, strategies can and should be adjusted as prevalence decreases during the programme. Potential adjustments include varying individual test methods, combining tests or adjusting sampling methods to target animals and areas with different levels of risk.

The sensitivity of direct microscopic methods can be increased by examining haematocrit tubes in duplicate. For indirect methods such as antibody ELISA, sensitivity can be increased by lowering the cut-off, but at the expense of decreasing specificity. An appropriate cut-off can be determined at different true prevalence levels depending on the relative costs of misclassifying positives and negatives (misclassifying positives is usually a serious error in eradication programmes). Using two or more tests in parallel or series [15] can also be used to make test strategies more sensitive or specific. In practice, sensitive tests are often used for initial screening and then a second, more specific, confirmatory test is applied. Available field evidence, such as whether reactors occur singly or in clusters, should also be considered to improve decision making. Sampling strategies can also play a role in detecting infections during eradication. For example, detection of low prevalence infections can be improved by testing all sick animals or animals with low PCV levels, by careful scrutiny of animals in areas with high transmission potential or by the use of naive sentinel animals. On the other hand, specificity of detecting recent infections, because of antibody persistence using the relatively sensitive antibody ELISA as a screening test, would be improved by targeting animals born after suppression at an age at which maternal antibodies should have declined (6 months).

Establishing freedom from trypanosomosis may also require sampling of other potential host reservoir species (e.g. pigs, wildlife). Because there is little experience in tsetse and trypanosomosis eradication, it is difficult to be sure what unintended changes in tsetse ecology or trypanosomosis transmission may occur in a certain circumstance. Other potential hosts should be characterised as to their potential for trypanosome infection and highest probability species sampled as per the instructions above. Methods used for the analysis of health risks associated with importing agricultural products and other probabilistic decision-making tools would be useful for this task.

4. CASE STUDY – CRITERIA FOR THE DECLARATION OF TSETSE AND TRYPANOSOMOSIS ERADICATION IN ZANZIBAR

To illustrate the criteria required to establish tsetse and trypanosomosis eradication, the eradication of tsetse and trypanosomosis from Unguja Island, Zanzibar, will be used as an example. The background and eradication programme [16] will be briefly described and then the methods used to declare tsetse and trypanosomosis eradication will be examined.

4.1. History of tsetse and Trypanosomosis control in Zanzibar

Zanzibar consists of two main islands Unguja and Pemba. Trypanosomosis was first reported on Unguja island by Bruce in 1909 (see [16]). Various surveys (1923, 1948-51) demonstrated trypanosomes to be prevalent on the island. Initially, trypanosomes were thought to be transmitted mechanically by biting flies as no tsetse had been trapped. *Glossina austeni* were first caught in 1945 [16]. In contrast, Pemba has remained trypanosome and tsetse free throughout.

Trypanosomosis and tsetse control activities were initiated on Unguja island in the 1980s with the assistance of the Food and Agriculture Organization of the United Nations (FAO). Unguja island covers approximately 1600 km^2 with a population of approximately 400,000 people and is separated from the Tanzanian mainland by the Zanzibar channel, which is at least 35 km wide. In 1993, a livestock census counted 45,750 cattle, 26,472 goats, 375 sheep and 494 donkeys [17]. Initial surveys estimated a trypanosome infection prevalence of 19%, mainly *Trypanosoma congolense*. Both tsetse flies and trypanosome infections were more prevalent in the south-east of Unguja, particularly around Jozani Forest (see Fig. 1). Synthetic pyrethroids were employed for tsetse control with success, both on cattle and on stationary targets in areas of low cattle density (<10 per km^2). Subsequently, the International Atomic Energy Agency (IAEA) was requested to assist in the eradication of *G. austeni* from Unguja island using the sterile insect technique (SIT).

Details of the tsetse eradication effort have been described elsewhere [17]. Briefly, emerging *G. austeni* males were immobilized by chilling at 4°C and sterilized by gamma radiation treatment. Trial releases of sterilized males occurred from November 1990 until February 1991. The eradication phase began in earnest in 1994 with the release of sterile male flies, first by ground releases and from August 1994 by airplane. Sterile males were released twice weekly and by May 1995 more than 40,000 sterile flies were released per week over the eradication area. At that time, the ratio of sterile to wild flies was estimated to be 15:1. During 1996, the average numbers of flies released weekly increased to 70,000 (ratio of sterile:wild males estimated to exceed 100:1). Release was extended to cover the entire island in July 1996 and continued until the end of 1997. The last wild tsetse flies were trapped in week 36 of 1996.

Because considerable tsetse trapping and animal sampling for trypanosomosis was conducted during and after the eradication programme in Zanzibar it is possible to investigate the entomological and epidemiological criteria outlined in Sections 2 and 3 in assessing the optimal methods for confidently predicting the eradication of tsetse and trypanosomosis from Zanzibar.

5. CRITERIA FOR DECLARATION OF TSETSE ERADICATION

During the pre-eradication phase, tsetse capture methods for *G. austeni* using sticky panels were tested and adapted, as *G. austeni* proved very difficult to trap with conventional techniques [18]. These studies provided information on the preferred tsetse habitats and distribution. Fifty-six tsetse monitoring sites were chosen to ensure both geographical coverage of all parts of Unguja island and to include a number of sites of optimal tsetse habitats (particularly around Jozani and other forests) (Fig. 1). Sticky panels were monitored on a weekly basis at each sampling site. Both released sterile and wild flies were trapped, allowing for the estimation of both the relative size of the wild tsetse population and the sterile:wild fly ratio. Since, in the latter phases of eradication, all wild flies were trapped at the two sampling sites in Jozani forest, data from these two sites was used to estimate this ratio. It was considered that a ratio of >50:1 and perhaps as high as 100:1 was required to ensure that virtually all emerging females bred with sterile males [4].

Wild tsetse fly catches over time are shown in Fig. 2 (adapted from [19]). Apparent population densities fluctuated for individual visits (as has been noted elsewhere, for example by [9]). These fluctuations were attributed to changes in fly behaviour [4]. Applying the methods presented in section

2 to these data allows for the estimation of the time from the last tsetse catch to when eradication can be assumed.

First, by ignoring previous tsetse catch data and assuming a 5% probability of tsetse detection [4], 59 negative trapping weeks would be required for 95% confidence (using the method in [5]: number of weeks = ln (α)/ln(1-probability of detection, where $\alpha = 0.05$)). Given that wild tsetse flies were last trapped in week 32 of 1996, it would have taken until week 46 of 1997 to make a declaration of eradication. With lower detection rates or higher confidence requirements larger follow-up periods would be needed. For example, for a 1% detection rate and 95% confidence level 298 negative trapping weeks would be required and for a 5% detection rate and 99% confidence level 90 negative trapping weeks would be required.

Second, using all available tsetse trapping data in Fig. 2, the two methods of Solow [6,7] can be applied. If a low but stable tsetse population in the latter stages of the eradication programme was assumed, the methods presented in Solow [6] would allow a very rapid declaration of eradication, by week 36 of 1996 with 95% confidence and by week 38 of 1996 with 99% confidence. For the more realistic assumption of a declining tsetse population, eradication would not be declared until week 39 of 1996 with 95% confidence and week 43 of 1996 with 99% confidence.

As noted earlier, probability-based methods for assessing the eradication of tsetse have not been regularly applied. In applying the methods above to the Zanzibar tsetse eradication data, three important lessons can be learned. The first is that the stable population assumption is not realistic and only the declining population approach should be applied. When the stable population approach was applied sequentially to all periods of negative catches in 1996, eradication would have been declared incorrectly between week 23 and week 32, prior to the last positive tsetse catch. The declining population approach was more conservative and did not make this error. The second lesson is that the time period over which tsetse populations are monitored is crucial. If tsetse-monitoring data only from 1996 and 1997 were used (rather than for the entire eradication programme period from August 1994 to December 1997), much longer follow-up periods would be required. The difference for the stable population assumption [6] was not so great, with the required follow-up period increasing by 5 weeks and 9 weeks for 95% and 99% confidence levels respectively. However, for the preferred declining population approach, required follow-up periods of negative catches were greatly increased, such that a declaration of eradication at the end of 1997 could be made with only 60% confidence. The third point is that both these methods only used the information on positive or negative sighting occurrences and not the actual numbers of tsetse flies caught. As can be seen in Fig. 2, the number of tsetse flies declined as the eradication programme progressed and this information would be useful for predicting eradication. These limitations highlight the need for further work on probability-based and/or model-based methods to assess when tsetse eradication occurs.

6. CRITERIA FOR DECLARATION OF TRYPANOSOMOSIS ERADICATION

The epidemiological sampling protocol designed to assess the presence of trypanosomosis post-tsetse-eradication was based on a combination of the identification of trypanosomes in the buffy coat (BCT) [20] and an antibody detection enzyme linked immunosorbent assay (Ab-ELISA) [21,22]. Pre-eradication cattle sampling protocols and prevalence estimates were described by Dwinger *et al.* [16]. To confirm the absence of trypanosomosis in 1998, a total of 3000 animals 6 months of age or older were sampled, 10 at each of 300 randomly-selected spatial sampling locations. The cattle population on Unguja island was assumed not to exceed 50,000. All samples were examined using the BCT and subsequently by Ab-ELISA. For the BCT, as no positives were found, this sample provided 95% confidence that trypanosomosis was absent based on the following assumptions– that trypanosomosis if present would be at a prevalence of at least 1% and that the sensitivity of the BCT as used in Zanzibar was at least 10% and the specificity was 100%. Correspondingly smaller sample sizes would be required if greater BCT sensitivity could be achieved by the methods described in section 3 (997 for 30% sensitivity, 598 for 50% and 427 for 70%; calculation using FreeCalc [11]). To increase confidence that trypanosomes were absent, a special study, in which 295 cattle in historically high-risk areas close to Jozani forest and north-east of Zanzibar town (see Fig. 1) were sampled, was also conducted. All were parasitologically negative.

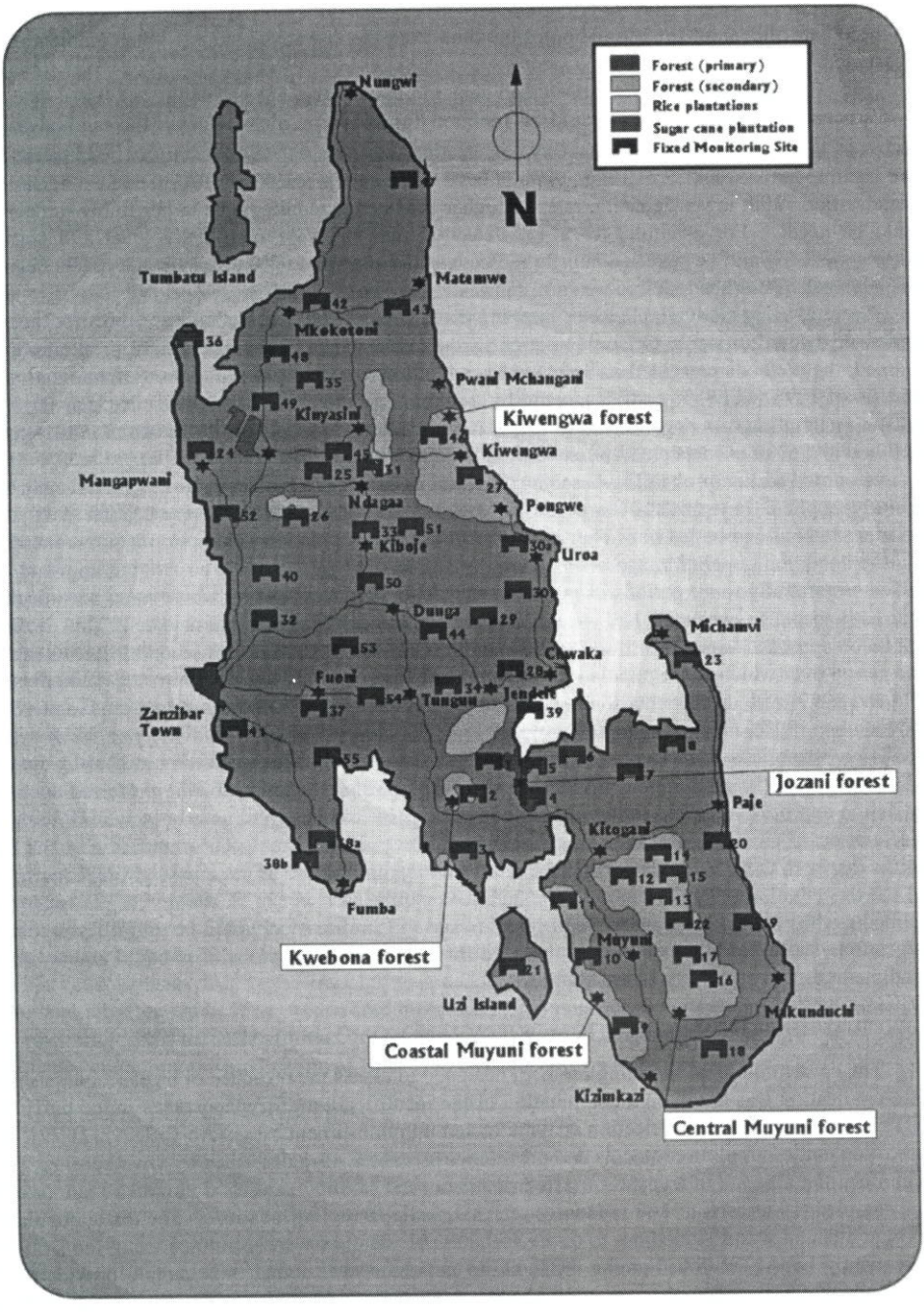

FIG. 1. Map of Unguja island showing main forest areas and distribution of tsetse trapping sites (courtesy M. Vreysen).

TABLE I. PROBABILITY* OF ABSENCE OF TRYPANOSOMOSIS OR PRESENCE AT 1% PREVALENCE FOR DIFFERENT REACTOR RATES USING TESTS OF VARYING SENSITIVITY AND SPECIFICITY FOR A SAMPLE OF 3,000 CATTLE FROM THE CATTLE POPULATION OF UNGUJA ISLAND, ZANZIBAR (ASSUMED CATTLE POPULATION OF 50,000)

Positive Reactors Detected	Test performance																	
	Se=95/Sp=98		Se=95/Sp=95		Se=95/Sp=90		Se=90/Sp=98		Se=90/Sp=95		Se=90/Sp=90		Se=50/Sp=98		Se=50/Sp=95		Se=50/Sp=90	
	p(0%)	p(1%)	p(0%)	p(1%)	p(0%)	p(1%)	p(0%)	p(1%)	p(0%)	p(1%)	p(0%)	p(1%)	p(0%)	p(1%)	p(0%)	p(1%)	p(0%)	p(1%)
0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0
20	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0
40	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0
60	0.52	0	1	0	1	0	0.52	0	1	0	1	0	0.52	0.05	1	0	1	0
80	0.01	0.21	1	0	1	0	0.01	0.26	1	0	1	0	0.01	0.77	1	0	1	0
100	0	0.91	1	0	1	0	0	0.94	1	0	1	0	0	1	1	0	1	0
120	0	1	1	0	1	0	0	1	1	0	1	0	0	1	1	0	1	0
140	0	1	0.81	0	1	0	0	1	0.81	0	1	0	0	1	0.81	0.03	1	0
160	0	1	0.21	0.10	1	0	0	1	0.21	0.12	1	0	0	1	0.21	0.41	1	0
180	0	1	0.01	0.61	1	0	0	1	0.01	0.66	1	0	0	1	0.01	0.91	1	0
200	0	1	0	0.96	1	0	0	1	0	0.97	1	0	0	1	0	1	1	0
300	0	1	0	1	0.51	0.07	0	1	0	1	0.51	0.08	0	1	0	1	0.51	0.25
400	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1
500	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1

* Estimated by the methods in reference [11].

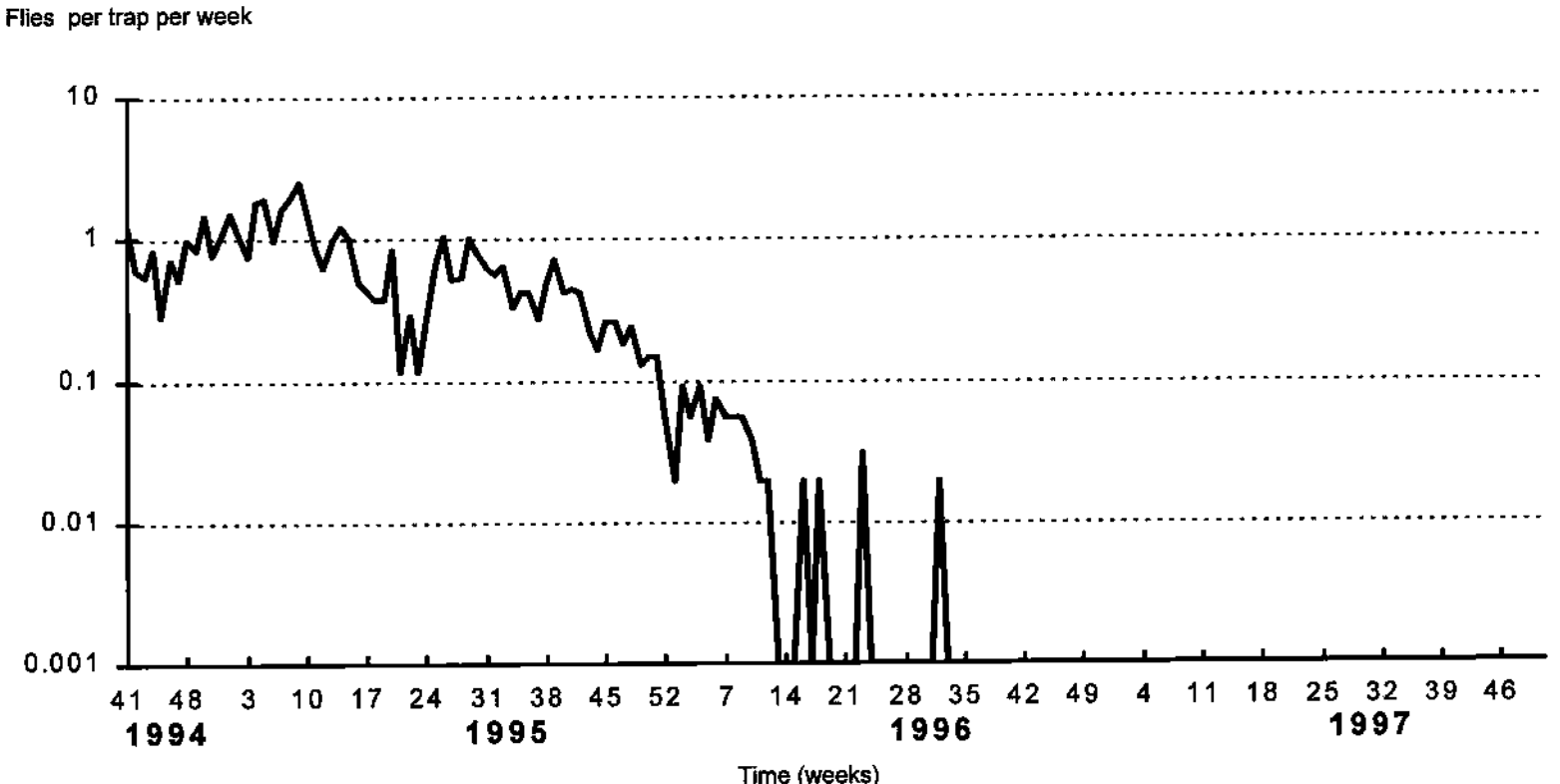

FIG. 2. Number of wild tsetse flies caught per trap per week during the tsetse eradication programme on Unguja island, Zanzibar, 1994-1997 (adapted from [19]).

For tests of imperfect specificity, such as the Ab-ELISA, false positive reactors are assumed to occur. For example, a 95% confidence of trypanosomosis absence could be achieved, given this sample size of 3000, with an Ab-ELISA test of sensitivity equal to 95% and specificity 98% with up to 72 reactors. Table I illustrates the relationship between the number of reactors and the probability of trypanosomosis absence and presence at 1% prevalence for Ab-ELISA tests of different sensitivity and specificity performances. Tests with poor sensitivity and specificity fail to discriminate between trypanosome-free and 1% prevalence states. Probability estimates depend most on test specificity, which can vary greatly from lab to lab (due to differences in water quality, for example). Serological results from the post-eradication sampling in Zanzibar are currently being finalized.

In conclusion, probability-based sampling strategies are available for assessing both tsetse and trypanosomosis absence as a result of tsetse eradication programmes. When applied to the available data, these methods supported with at least 95% confidence that both tsetse and trypanosomosis had been eradicated from Unguja island of Zanzibar as a result of the SIT programme.

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VALIDATION OF ANTIBODY ELISA FOR USE IN COMBINATION WITH PARASITOLOGICAL TESTS IN MONITORING TRYPANOSOMOSIS CONTROL PROGRAMMES

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Abstract

VALIDATION OF ANTIBODY ELISA FOR USE IN COMBINATION WITH PARASITOLOGICAL TESTS IN MONITORING TRYPANOSOMOSIS CONTROL PROGRAMMES.

The available techniques for trypanosomosis diagnosis, surveillance and control have failed to provide an effective control of the disease when used in isolation. However, when used in combination the techniques might provide a better alternative for a sustainable disease control strategy. Antigen-detection ELISA was validated and established at ADRI through support of the Joint FAO/IAEA Division, but still required perfection in order to meet the requirements for which it was intended (detection of current infection and species specificity). The primary objective of the present study was to evaluate the trypanosomal antibody-detection ELISA (Ab-ELISA) kit produced by the International Atomic Energy Agency (IAEA) Vienna, Austria, to supplement conventional diagnostic techniques in trypanosomosis surveillance and control.

Three hundred sixty cattle from selected herds in the Tanga region of Tanzania were bled to obtain buffy-coat and serum samples in a study of the prevalence of trypanosomosis using parasitological and serological techniques. The prevalence of trypanosomosis, by means of parasitological techniques, in Korogwe, Muheza, Tanga and Pangani districts of Tanga region, varied from 0 – 18.2%. The IAEA Ab-ELISA kit, showed a 97.2% specificity for *Trypanosoma congolense* and *T. vivax* antibodies, at a cut-off point of 30.0 percent positivity (PP), out of 160 sera from a trypanosomosis-negative population; and a 94.5% sensitivity for the same antibodies and cut-off point, out of 200 sera from trypanosomosis-buffy-coat-positive samples. Packed red cell volume (PCV) values for a trypanosomosis-negative population were much higher than those from cattle from trypanosomosis-endemic areas: 77.5% of samples from a disease-free area showed PCV values higher than 30.0, while only 15% of the samples from endemic areas were above 30.0.

1. INTRODUCTION

Trypanosomosis continues to be one of the most important diseases in the United Republic of Tanzania affecting not only livestock but man as well. About 4.4 million cattle are at risk of contracting trypanosomosis. Likewise, about 4.0 million people are at risk of contracting human trypanosomosis. The pathogenic trypanosomes are cyclically transmitted by tsetse flies of the genus *Glossina*. Indeed, control of the insect vector and the disease in mammals is highly essential in order to avoid or minimise losses in productivity either directly as a result of mortality and morbidity or indirectly through its impact on land use and rural development. Since the techniques available for trypanosomosis diagnosis, surveillance and control have failed to provide an effective control of the disease when used in isolation, integration of improved techniques for disease diagnosis with vector control methods, is a more suitable alternative for a sustainable disease control strategy. It is for the sole purpose of integrating novel improved techniques into trypanosomosis control strategies, that the IAEA Ab-detection ELISA kit was supplied to the Animal Disease Research Institute, for validation and possible integration of the test as a tool complementary to the available, but less sensitive diagnostic techniques.

2. MATERIALS AND METHODS

2.1. Parasitological diagnosis

One hundred sixty dairy cattle of pure Friesian or Ayrshire breeds and their crosses from Amani Tanga, about 1000 masl and reported to be free from tsetse flies and trypanosomosis, were bled from a peripheral ear vein for preparation of blood smears and buffy-coats for parasitological examination. In addition, 1100 cattle (Zebu, Boran, Friesian or Ayrshire crosses with Boran or Zebu breeds) from small to large-scale livestock farms in lowland (<200 masl), trypanosomosis-endemic areas in the Tanga region (Fig. 1), were similarly bled. The samples were examined by the buffy-coat technique [1]. Blood smears were air-dried and then fixed in methanol (for thin smears) or de-haemoglobinized by a brief immersion in distilled water (for thick smears) before colouring using a Giemsa stain.

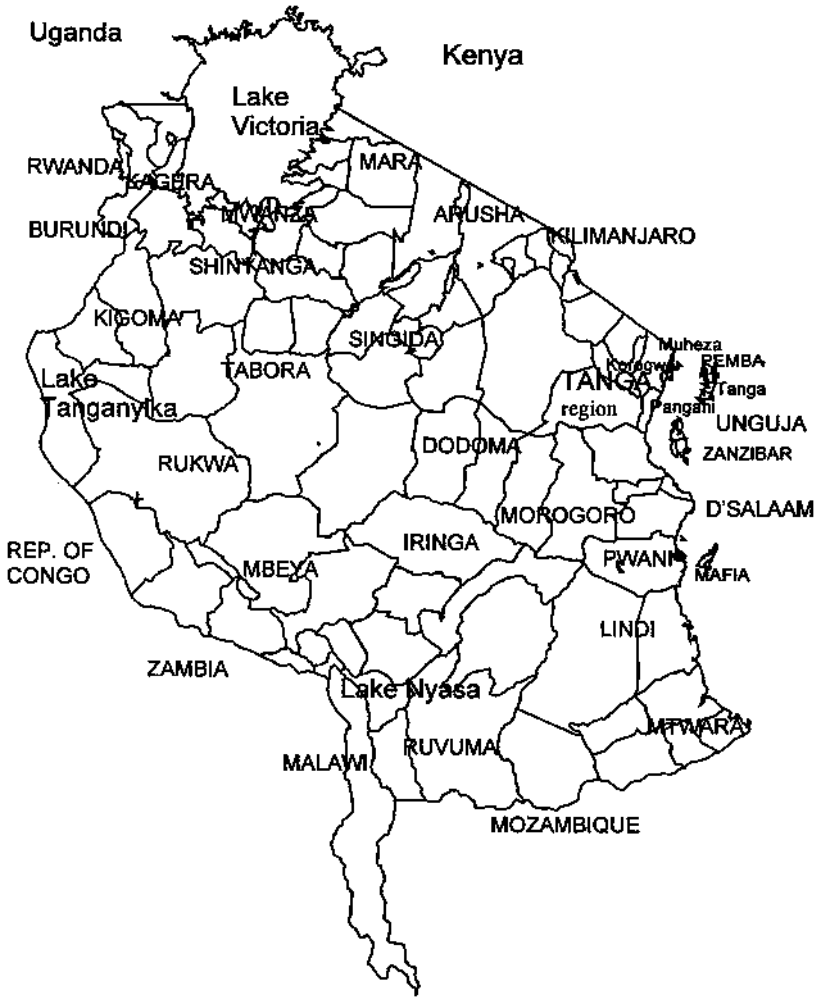

FIG.1. Antibody-detection ELISA study sites in Tanzania: Korogwe, Muheza, Pangani and Tanga districts, Tanga region.

2.2. Serological diagnosis

2.2.1. Sera from a trypanosomosis-negative population

Animals were also bled from the jugular vein as they were being sampled for buffy coats and blood smears. Jugular blood was used for the preparation of sera. Sera from parasitologically negative samples from Amani, Tanga, were used for the determination of the specificity of the Ab-detection ELISA. In addition, 200 sera from Pemba island of Zanzibar, with no records of tsetse flies or trypanosomosis [2], were used to compare the specificity of the ELISA, between negative populations of Pemba and mainland Tanzania. Pemba sera were obtained from a serum bank in the Parasitology Department of ADRI.

2.2.2. Sera from trypanosomosis-endemic areas

Sera of 200 parasitologically positive cattle out of the 1100 cattle sampled in the lowland areas of Tanga region, were used to determine the specificity of the ELISA to detect trypanosomal antibodies. Four species of tsetse flies of the genus *Glossina* are incriminated in the transmission of trypanosomosis and are of major economic importance in these areas: *Glossina morsitans, G. pallidipes, G. brevipalpis* and *G. austeni.*

2.3. Reagents and equipment

Reagents were supplied in kit-form by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, Austria. *Trypanosoma congolense* and *T. vivax* antigens were supplied pre-coated on Dynatech, Immulon I, 96-well microtitre plates. Optical density values were read using an ELISA reader (Multiskan Plus MK II) linked to a computer using the EDI version 2.2 software program. The full list of reagents and equipment, including a detailed description of the procedure for running the Indirect Trypanosome-Antibody ELISA is described in the FAO/IAEA Bench Protocol [3]. A sample was judged as positive or negative based on percentage positivity (Ratio of Replicate Optical Density (OD) Value of Test Serum to Median OD Value of Strong Positive Control x 100). A percentage positivity (PP) threshold of 30.0 was used for samples from Tanzania mainland and 45.0 for samples from Pemba island.

3. RESULTS

3.1. Parasitological results

3.1.1. Trypanosomosis-negative population

Out of 160 buffy-coat samples and blood smears examined, none was positive for trypanosomes or for any other haemoparasites. Ninety percent of the 160 cattle sampled showed PCV values of > 26.0% (Fig. 2).

3.1.2. Trypanosomosis-positive population

Following the examination of 1100 buffy-coat samples and blood smears, 200 (18.2%) were found positive for trypanosomes. Of the positive samples, 172 (86.0%) were single infections and 28 (14.0%) were multiple infections. *Trypanosoma congolense* was predominant (64.0%) among the single infections, followed by *T. vivax* (16.0%) and *T. brucei* (6.0%). Mixed infections consisting of *T. congolense/T. vivax* were predominant (Table I). Sixty five percent of the 200 parasitologically positive cattle showed PCV values of <26.0% (Fig. 3).

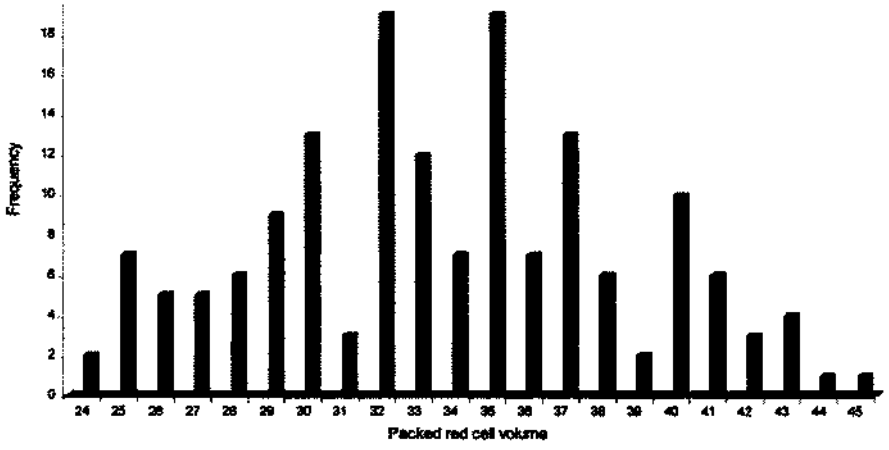

FIG. 2. Packed red cell volume values for a trypanosomosis-negative population of Amani, Tanga Region, Tanzania Mainland.

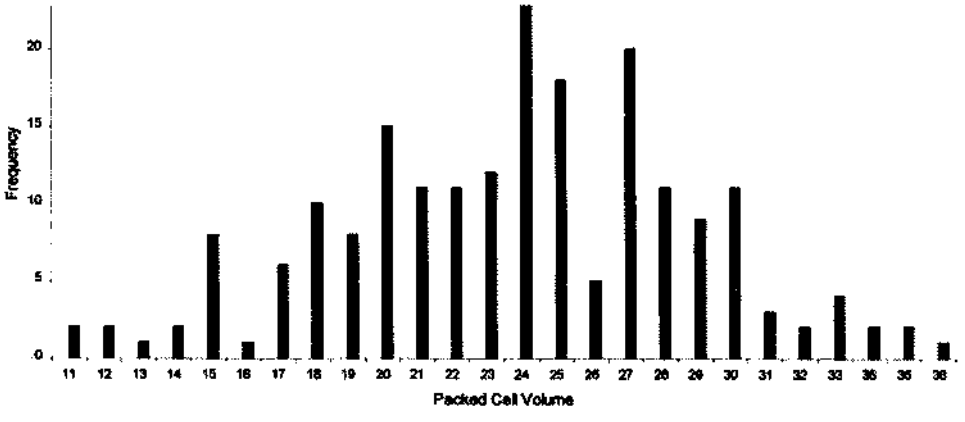

FIG. 3. Packed red cell volume values for a trypanosomosis-positive (by buffy coat technique) population, Tanga Region, Tanzania Mainland.

TABLE I. DIFFERENTIATION BY TRYPANOSOMAL SPECIES OF SAMPLES DETECTED POSITIVE BY THE BUFFY-COAT TECHNIQUE

	Trypanosomal Species	No. Positive	% Positive
Single infections	*Trypanosoma congolense*	128	64
	T. vivax	32	16
	T. brucei	12	6
Multiple infections	*T. congolense/T. vivax*	11	5.5
	T. brucei/T. vivax	8	4
	T. brucei/T. congolense	7	3.5
	T. brucei/T. congolense/T. vivax	2	1
	TOTAL	200	100

3.2. Serological results

3.2.1. Sera from a trypanosomosis-negative population

Out of 160 sera screened, antibodies directed against *T. congolense* and *T. vivax* were detected in 4.4% and 0.2% of the samples giving a specificity of 95.6% and 98.8%, respectively (Fig. 4a and 4b), at a cut-off point of 30 percent positivity (PP). Both species combined, showed a specificity of 97.2% (Fig. 4c). On the other hand, specificity of the test for the 200 sera from Pemba island of Zanzibar was 94.5% and 95.0% using *T. congolense* and *T. vivax* antigens, respectively (Fig. 5a and 5b), and 94.75% for both species combined, at a cut-off point of 45.0 PP (Fig. 5c).

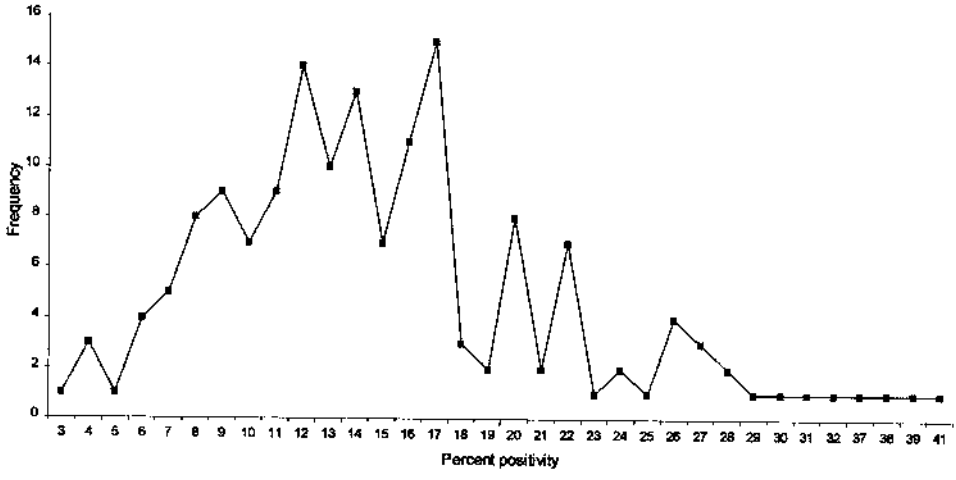

FIG. 4a. Trypanosoma congolense *antibody-detection ELISA for a trypanosomosis-negative population of Amani, Tanga Region, Tanzania Mainland.*

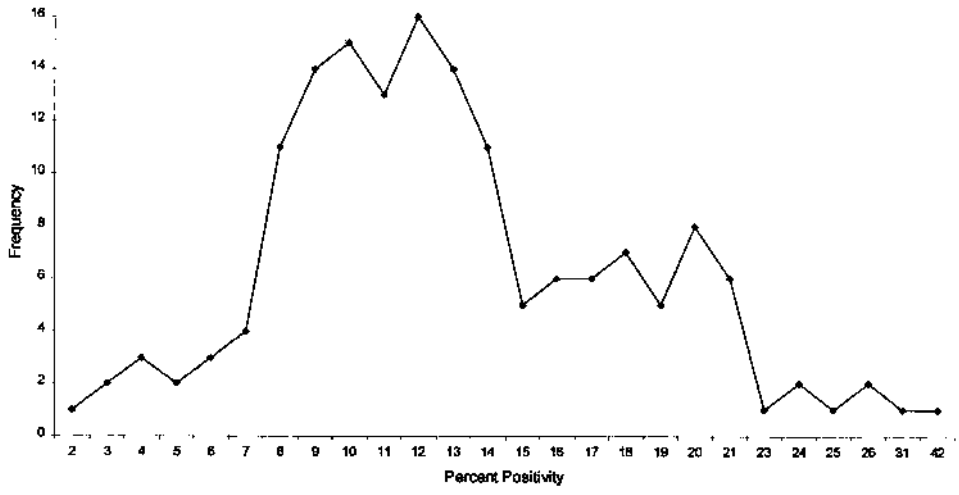

FIG. 4b. Antibody-detection ELISA results (using T. vivax *antigen) of a trypanosomosis-negative population of Amani, Tanga Region, Tanzania Mainland.*

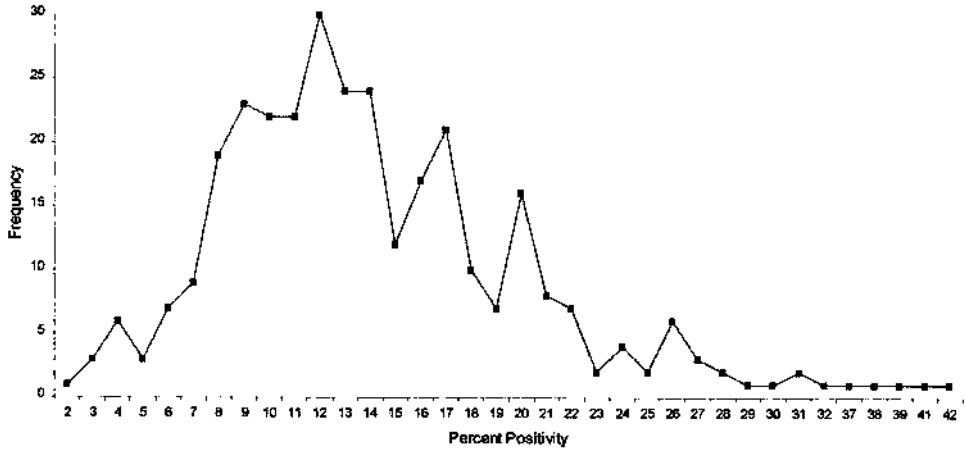

FIG. 4c. ELISA results of antibodies against Trypanosoma congolense *and* T. vivax *in a trypanosomosis-negative population of Amani, Tanga Region, Tanzania Mainland.*

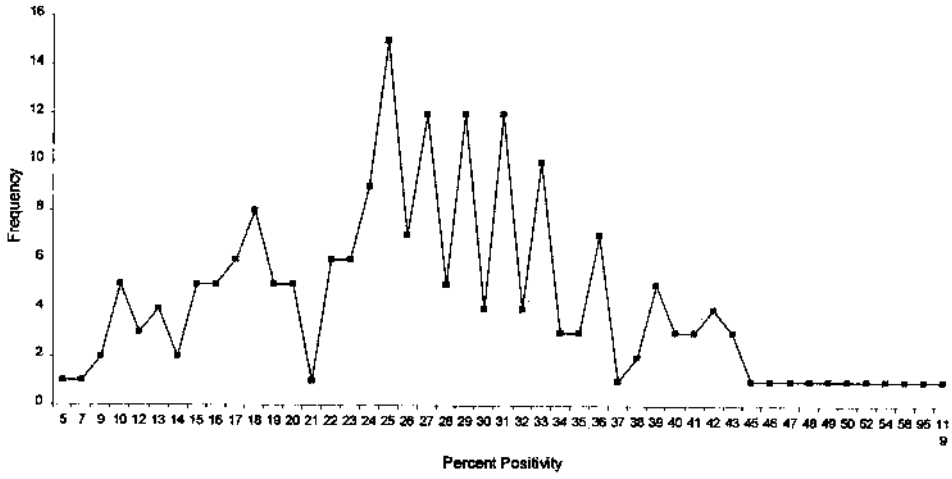

FIG. 5a. Trypanosoma congolense *antibody-detection ELISA for a trypanosomosis-negative population of Pemba Island of Zanzibar.*

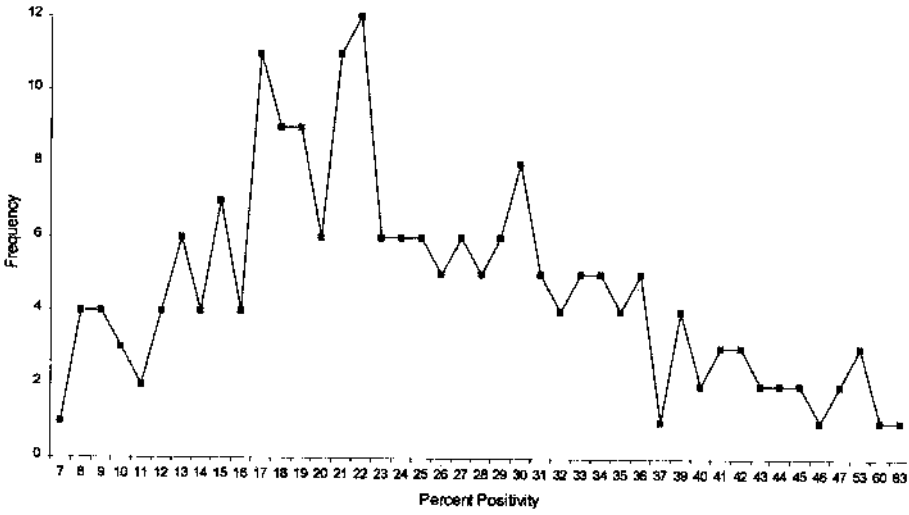

FIG. 5b. Antibody-detection ELISA results (using T. vivax *antigen) for a trypanosomosis-negative population of Pemba Island of Zanzibar.*

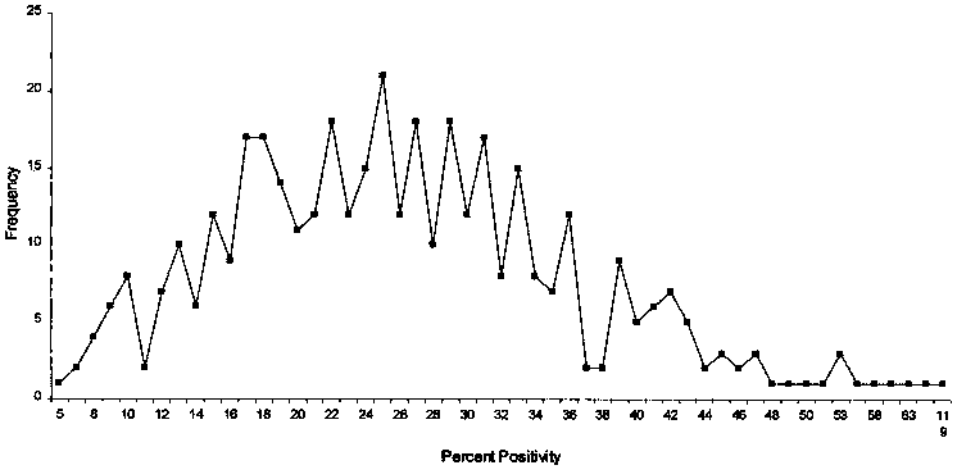

FIG. 5c. ELISA results of antibodies directed against Trypanosoma congolense *and* T. vivax *in a trypanosomosis-negative population of Pemba Island of Zanzibar.*

3.2.2. Sera from an area endemic for trypanosomosis

Of the 200 sera from parasitologically positive cattle screened for trypanosome-antibodies 95.5% showed presence of antibodies directed against *T. congolense* (Fig. 6a), while 93.5% were positive for *T. vivax* antibodies (Fig. 6b). The sensitivity of the test for both species at a 30.0 PP threshold was 94.5% (Fig. 6c).

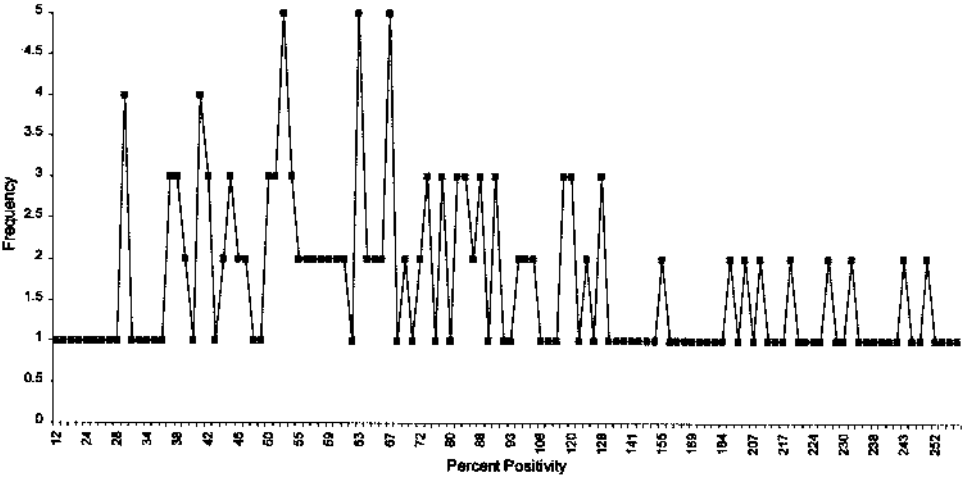

FIG. 6a. Trypanosoma congolense *antibody-detection ELISA for buffy-coat-positive samples, Tanga Region, Tanzania Mainland.*

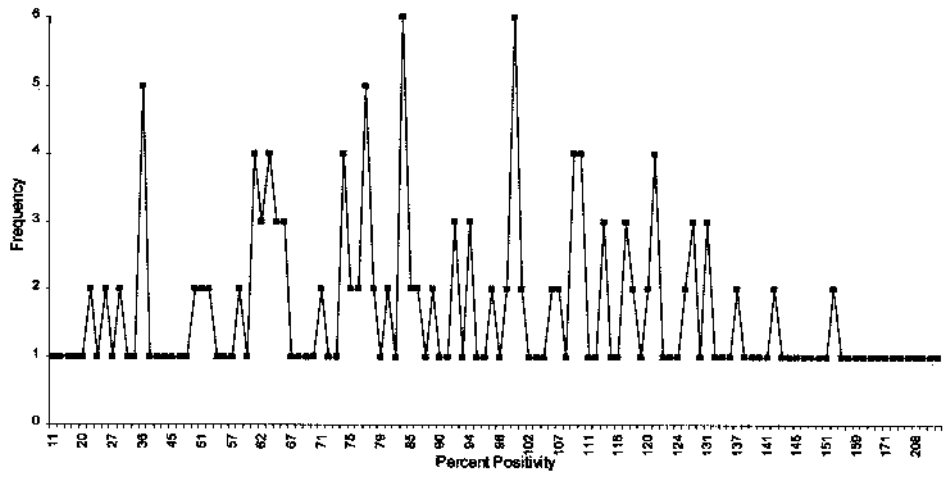

FIG. 6b. Antibody-detection ELISA results (using T. vivax *antigen) of parasitologically-positive samples, Tanga Region, Tanzania Mainland.*

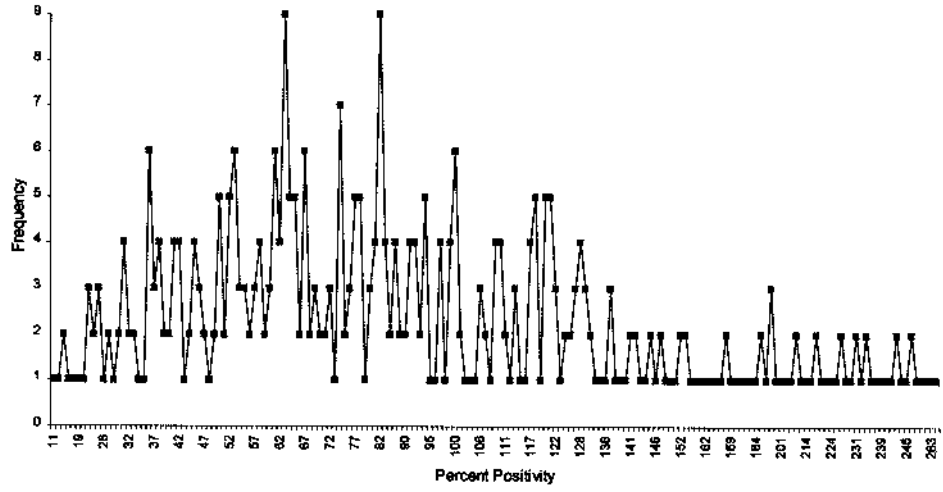

FIG. 6c. ELISA results of antibodies directed against Trypanosoma congolense *and* T. vivax *for parasitologically-positive samples, Tanga Region, Tanzania Mainland.*

4. DISCUSSION

Studies of trypanosomosis in four districts of Tanga region, using parasitological (BCT, blood smear examination and mouse subinoculation) and serological techniques (antibody-detection ELISA), showed that the prevalence of trypanosomosis varied from 0 – 18.2%. Application of antibody-detection ELISA for serological monitoring of trypanosomosis, was reported in the mid-seventies [4], but lacked species specificity and was unable to detect current infections. The development of an antigen-detection ELISA [5] was expected to overcome these shortfalls.

Unfortunately, the sensitivity and specificity of the test were subsequently discovered to be poor [6]. Hence, the need to continue studies for refining both techniques. The specificity and sensitivity of Ab-detection ELISA as reported in the present studies is encouraging. Whereas the specificity of Ab-detection ELISA (*T. congolense* and *T. vivax*) for a trypanosomosis-negative population on Mainland Tanzania is 97.2%, the sensitivity for the test from parasitologically positive samples in the coastal lowland areas (<200m above sea level) of Tanga Region is 94.5%. The specificity of the Ab-detection ELISA using pre-coated microtitre plates for samples from Pemba island was 94.75% (Fig. 5c.) at a threshold of 45 PP. Although this cut-off point is higher than the one used for Mainland samples (30.0PP), the 45 PP threshold is still thought to be appropriate for the samples, recovered from a serum bank at ADRI. Antibody-detection ELISA results for parasitologically positive samples from the mainland indicated that 5.5% of the samples assayed were below the 30 PP threshold. According to work done on *T. brucei rhodesiense, T. b. brucei, T. congolense* and *T. vivax* in the mid-seventies [4], 15-20% of BCT-positive samples showed optical density(OD) values of <0.20.

In the present studies OD values of about 0.20 to 0.30 corresponded to <30.0 PP, which is the threshold for mainland samples and constituted 2.8 to 5.5% of the trypanosomosis-negative and positive populations, respectively. Comparable results were obtained for trypanosomosis-negative-samples from Pemba, in which 5.25% of the samples were above the 45.0 PP. The 45.5PP corresponded to OD values of about 0.45. Indeed the present results are in agreement with previous reports [4], in which it was reported, that uninfected animals never gave OD values higher than 0.44 for *T. brucei. Trypanosoma brucei* antigen was not tested during the present studies, but its immunogenicity is not expected to differ much from *T. congolense* or *T. vivax* antigens. Samples parasitologically positive for *T. brucei,* reacted strongly to *T. congolense* and *T. vivax* antigens during the present studies.

Trypanosoma brucei brucei antigen was described to have been able to detect high levels of *T. vivax* antibodies in a calf, from week 2.5 to 24.0 after experimental infection in West Africa [7]. In the present studies, in which *T. congolense* and *T. vivax* antigens were used, trypanosomal antibodies were detected not only in samples parasitologically positive for *T. congolense* and *T. vivax*, but also positive for *T. brucei*, both in the case of single and mixed infections. *Trypanosoma congolense* antigen is reported to be less immunogenic, showing lower optical density values against trypanosomal antibodies as compared to antigens prepared from *T. b. brucei, T. b. rhodesiense* or *T. vivax* [4]. *Trypanosoma congolense* is considered to be poorly immunogenic on the basis of findings with immunofluorescence [8]. The present results showed that the IAEA-produced antibody-detection ELISA kit (using *T. congolense* and *T. vivax* antigens) may be superior to the antigens used by previous workers. The immunogenicity of both *T. congolense* and *T. vivax* antigens was satisfactory in the present findings (Figs 5a, 5b, 6a, 6b). Serological tests have long been reported to be less definitive than parasitological tests when used alone and not completely reliable for the diagnosis of trypanosomosis in individual animals [4, 9, 10, 11]. Experience from Mangapwani on Unguja Island of Zanzibar, showed that parasitological and serological tests could complement each other and should not be used on their own. Mangapwani was previously reported to be free from tsetse flies and trypanosomosis by parasitological techniques, but was later discovered to be antigenaemic [12] and, consequently, control operations had to be initiated. Recent work on adaptation and validation of antibody-detection ELISA in epidemiological surveys in Zambia with dried blood spots on filter paper showed a sensitivity and specificity of 93.7 and 98.1%, respectively [13], which are comparable to the present findings.

Based on investigations of samples from trypanosomosis-free and endemic areas, it is concluded, that the prevalence of trypanosomosis in Tanga region varied from 0 – 18.2%. Furthermore, antibody-detection ELISA using pre-coated *T. congolense* and *T. vivax* antigens has an acceptable specificity and sensitivity. However, additional studies are required, not only to include *T. brucei* antigen, but also to improve both specificity and sensitivity of the antibody-detection ELISA, and to refine the antigen-detection ELISA for detecting current infections.

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EVALUATION OF THE ANTIBODY-DETECTION ELISA USING PLATES PRE-COATED WITH DENATURED *T. CONGOLENSE* AND *T. VIVAX* ANTIGENS FOR MONITORING TSETSE AND TRYPANOSOMOSIS CONTROL IN ZAMBIA

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Abstract

EVALUATION OF THE ANTIBODY-DETECTION ELISA USING PLATES PRE-COATED WITH DENATURED *T. CONGOLENSE* AND *T. VIVAX* ANTIGENS FOR MONITORING TSETSE AND TRYPANOSOMOSIS CONTROL IN ZAMBIA.

An evaluation of the indirect enzyme-linked immunosorbent assay (ELISA) for the detection of anti-trypanosomal antibodies in bovine serum was conducted using ELISA plates pre-coated with denatured *T. congolense* and *T. vivax* antigen. The study was conducted on 280 samples from a parasitologically positive cattle population and 200 samples from a negative cattle population. The overwhelming majority of trypanosome infections in the parasitologically positive cattle were *T. congolense*. The Ab-ELISA was able to discriminate between the negative and positive cattle populations, at an optimum cut-off point of 40% positivity (*T. congolense* antigen), with 89% sensitivity and 86% specificity; and for the *T. vivax* antigen, the optimum cut-off point was at 30%, with 81% sensitivity and 70% specificity. However, the optical densities (OD) and percentage positivity (PP) values for sera from both the reference positive and negative cattle populations were unacceptably high particularly in the ELISA using *T. congolense* antigen. Furthermore the quality control sera used in the assay appear to have inappropriately low OD and PP values by comparison to typical sera from the reference positive and negative cattle populations. It is suggested that these ELISA's be re-titrated using more appropriate quality assurance sera. This should result in OD and PP values for sera from the reference populations falling within acceptable ranges.

1. INTRODUCTION

Zambia has benefited from a large-scale tsetse and trypanosomosis control program that covers the common tsetse fly belt of Zambia, Zimbabwe, Malawi and Mozambique. This was the EU-funded Regional Tsetse and Trypanosomosis Control Programme (RTTCP). Another EU-funded (INCO-DC) project in partnership with the Central Veterinary Research Institute (CVRI) is targeting specific areas of the same fly belt for monitoring the disease in the context of trypanocidal drug resistance.

The indirect antibody ELISA for bovine trypanosomosis has been established in Zambia since 1996, and has been shown to be useful in the sero-diagnosis of bovine trypanosomosis [1]. The method was adapted for use with either conventional serum samples, or bloodspots dried on filter paper, which reduces the need for expensive cold-chain facilities under field conditions.

The FAO/IAEA Joint Division is adopting the indirect antibody ELISA as a potentially useful method for epidemiology of bovine trypanosomosis. Preliminary studies were done on an ELISA that was provided in 1998. The results obtained were not very encouraging because of the difficulties with the transportation of reagents over long distances with the result that the reactivity of biologicals diminished. To avoid this problem the FAO/IAEA Joint Division decided to adopt the use of pre-coated ELISA plates, which do not require a cold chain for storage or transport, and therefore, avoids the problems previously associated with transportation of trypanosomal antigens to the various laboratories. The ELISA plates were coated using denatured *Trypanosoma congolense* and *Trypanosoma vivax* antigen. As a component of the validation of this new assay, studies were conducted to assess the sensitivity and specificity using samples of known provenance regarding trypanosome infection status.

Aims of the study were:

- To establish the specificity of the FAO/IAEA antibody-detection ELISA using negative reference serum samples from cattle without pathogenic trypanosome infections.
- To establish the sensitivity of the FAO/IAEA antibody-detection ELISA using positive reference serum samples from cattle with pathogenic trypanosome infections.

- To determine the optimum cut-off point of the assay for estimation of the prevalence of trypanosomosis in defined geographical areas.

2. MATERIALS AND METHODS

2.1. Reference negative cattle population

Bovine serum samples (n = 300) were collected from uninfected cattle in tsetse-free areas around Lusaka. Packed red blood cell volumes (PCV) were determined for 40 of these cattle.

2.2. Reference positive cattle population

Over 3000 cattle in tsetse-fly infested areas in Eastern Province, Zambia were sampled for trypanosome infections over three years (1996 - 1999) under the EU INCO-DC Project at CVRI, Lusaka. Parasitological diagnosis was conducted using the dark-ground/buffy-coat technique [2] and thick and thin Giemsa-stained blood smears. Serum samples were also collected from these cattle.

2.3. Trypanosomal antibody ELISA

The FAO/IAEA ELISA for detection of antibodies to trypanosome infections in bovine sera was performed on serum samples from the negative and positive reference cattle populations. The assays were conducted using 96-well ELISA plates pre-coated with either denatured *T. congolense* or *T. vivax* antigen, in accordance with the test protocol provided by FAO/IAEA. Each sample was tested in two ELISA plates simultaneously, one coated with each antigen. These ELISA's were conducted using the strong positive (C++), moderate positive (C+) and negative (C-) control sera provided by FAO/IAEA.

2.4. Comparison of FAO/IAEA antibody-ELISA control sera with control sera in routine use in the Southern African Region

The strong positive control (C++), weak positive control (C+) and negative control (C-) sera provided by FAO/IAEA (November 1997) were compared with C++, C+ and C- control sera in routine use in the RTTCP trypanosomosis Ab-ELISA in the Southern African Region. The RTTCP C++ serum was produced by repeated experimental infection and treatment of two Zambian cattle with *T. congolense*, while the RTTCP C- serum was a pool of sera from trypanosome-uninfected cattle from the tsetse-free area around Lusaka [1]. The RTTCP C+ serum was prepared by making an appropriate dilution of the C+ serum in the C–. These control sera have previously been shown to be suitable for routine testing of cattle from this region [1].

The comparison was done using the FAO/IAEA antibody-ELISA protocol provided in November 1997, on one half of the ELISA plate and the FAO/IAEA reference C++, C+ and C- sera on the other half, and the same exercise was done using the RTTCP/CVRI antibody-ELISA protocol.

3. RESULTS

3.1. PCV and parasitological findings

The average PCV for 40 cattle sampled in tsetse-free areas around Lusaka was 30.4%, with individual values ranging from 26 to 35%.

Trypanosome infections were detected in 327 cattle sampled in tsetse-infested areas of Eastern Province, Zambia. Ninety-six per cent of these infections were *T. congolense*, approximately 3% were *T. vivax*, and 1% were *T. brucei*. The average PCV for these parasitaemic cattle was 24.5%, with individual values ranging from 11 to 41%. From these 327 trypanosome-infected cattle, 280 sera were available for testing in the antibody-ELISA's.

3.2. Antibody-ELISA using denatured *Trypanosoma congolense* antigen

The OD values of sera (n = 280) from parasitologically positive cattle tested in the antibody-ELISA using plates pre-coated with denatured *T. congolense* antigen were high (mean 2.19, range

0.12 – 3.51). One hundred and eighty-seven (67%) of these sera gave OD responses above 2.0. Two hundred and twenty (78.6%) of these positive samples gave OD responses above that of C++.

For the 200 sera from negative cattle, high ODs were, observed ranging between 0.176 and 2.63. The arithmetic mean OD for negative sera was 0.537, whereas the geometric mean (0.484) and median (0.475) were somewhat lower, reflecting the skewed OD distribution of the negative population.

The 280 sera from parasitologically positive cattle generally exhibited high percentage positivity (PP) values in the antibody-ELISA using *T. congolense* antigen. More than half (n = 165) had PPs over 200%, the highest value obtained being 317% positivity.

The 200 negative samples also exhibited high PPs in the antibody-ELISA. Sixty (30%) of these sera gave PP values over 30%, and 18 sera (9%) gave PPs above 50%. All but 2 of these negative sera gave PPs higher than the overall mean PP (11.9%) for C-.

The frequency and cumulative frequency distributions for the PPs obtained for these sera are shown in Figures 1 and 2, respectively. There appeared to be good separation between the positive and negative reference populations. Percentage positivity values for approximately 89% of positive reference sera fell above 40%. Percentage positivity values for approximately 86% of negative reference sera fell below 40%.

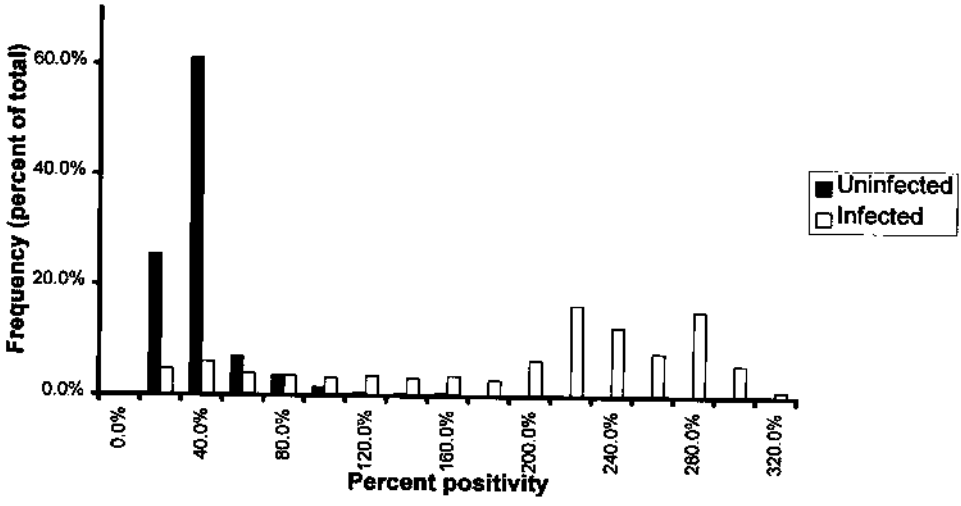

FIG. 1. Frequency distributions of PP values for reference sera from trypanosome-infected (n = 280) and uninfected (n = 200) cattle in the FAO/IAEA Trypanosomosis Antibody-ELISA using plates pre-coated with denatured T. congolense.

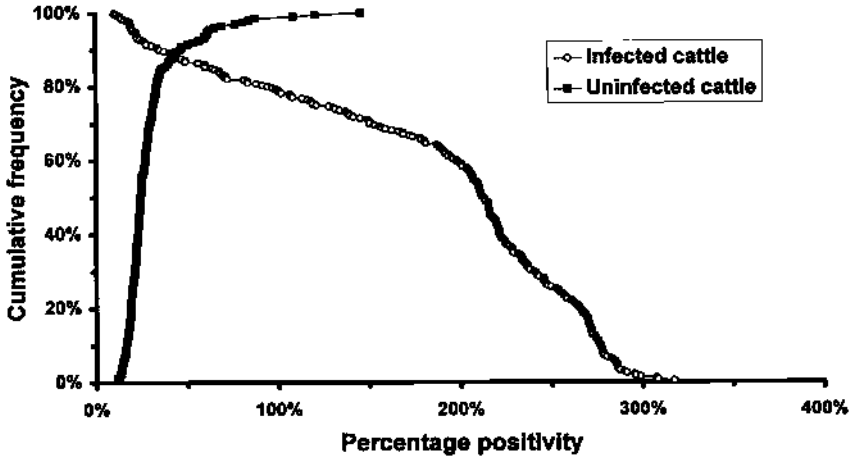

FIG 2. Cumulative frequency distributions of PP values for reference sera from trypanosome-infected (n = 280) and uninfected (n = 200) cattle in the FAO/IAEA Trypanosomosis Ab-ELISA (using plates pre-coated with denatured T. congolense *antigen).*

3.3. Antibody-ELISA using denatured *Trypanosoma vivax* antigen

When sera from 280 parasitologically positive cattle were tested in the antibody-ELISA using plates pre-coated with denatured *T. vivax* antigen, the OD values were not as high as those observed using plates pre-coated with denatured *T. congolense* antigen. The mean OD was 0.92, ranging from 0.05 to 2.96. One hundred and nine of these sera (38.9%) gave OD responses above 2.0.

For the 200 sera from negative cattle, high ODs were observed, ranging between 0.012 and 1.90. The arithmetic mean OD for negative sera was 0.443, whereas the geometric mean (0.395) and median (0.385) were somewhat lower, reflecting the skewed OD distribution of the negative population.

In the antibody-ELISA using *T. vivax* antigen, sera from parasitologically positive cattle gave lower PP values than those obtained using the *T. congolense* antigen. Of the 280 positive samples, 59 (21.1%) had PP values above 100% and 4 had PPs over 200%, with the highest value obtained being 250% positivity. The 200 samples from the negative population had rather high PPs using this antigen, with only 23 of these having PPs less than the overall mean (13.6%) for the negative control serum (C-).

The frequency and cumulative frequency distributions for these sera are shown in Figures 3 and 4, respectively. There was a reasonable separation between the positive and negative reference populations. Percentage positivity values for approximately 81% of positive reference sera fell above 30%. Percentage positivity values for approximately 70% of negative reference sera fell below 30%.

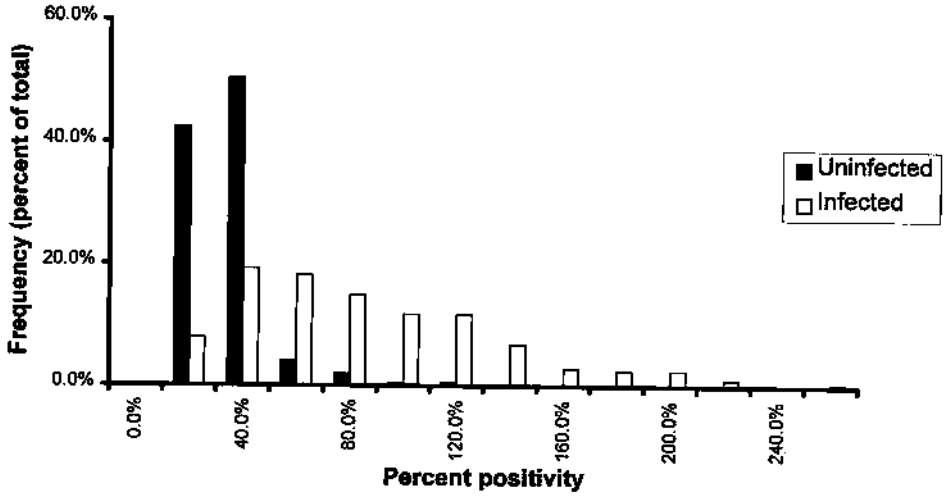

FIG 3. Frequency distributions of PP values for reference sera from trypanosome-infected (n = 280) and uninfected (n = 200) cattle in the FAO/IAEA Trypanosomosis Antibody-ELISA using plates pre-coated with denatured T.vivax.

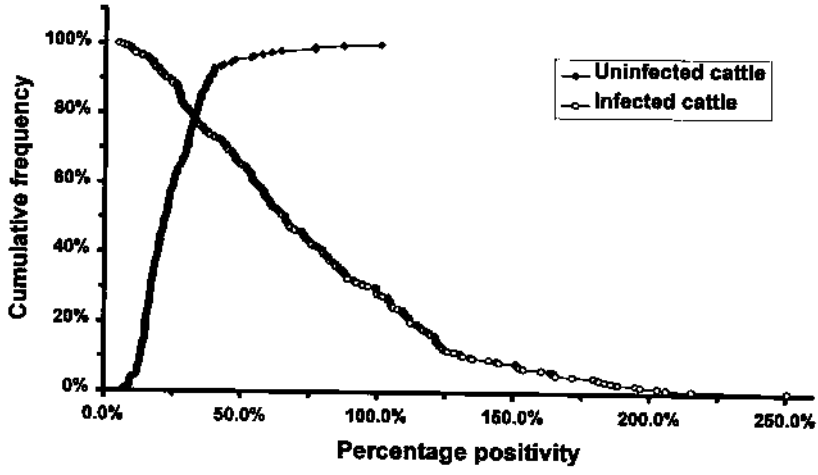

FIG 4. Cumulative frequency distributions of PP values for reference sera from trypanosome-infected (n = 280) and uninfected (n = 200) cattle in the FAO/IAEA Trypanosomosis Ab-ELISA (using plates pre-coated with denatured T.vivax *antigen).*

3.4. Comparison of FAO/IAEA antibody-ELISA control sera with control sera in routine use in the Southern African Region

The comparison of FAO/IAEA antibody-ELISA control sera with control sera in routine use in the Southern African Region when tested in the RTTCP Ab-ELISA is shown in Table 1. The FAO/IAEA C++ control serum gave an OD response (0.511) less than half that of the RTTCP C++

(1.387). Hence, the FAO/IAEA C++ had a relatively low percentage positivity of 37.4% based on a level of 100% positivity for the RTTCP C++ (Table 1). The FAO/IAEA C+ gave an OD response of 0.222, which represented only 16.2% positivity (based on a level of 100% positivity for the RTTCP C++). This would be considered negative result based on the cut-off of 25% positivity routinely used in the RTTCP Ab-ELISA.

TABLE 1: COMPARISON OF RTTCP AND FAO/IAEA AB-ELISA CONTROL SERA

Source of control sera	Level	Mean OD	Median OD	CV[2]	PP[1]
RTTCP:					
	C++	1.387	1.368	8.7%	101.4%
	C+	0.397		5.5%	29.0%
	C-	0.118		9.2%	8.7%
FAO/IAEA:					
	C++	0.511		4.2%	37.4%
	C+	0.222		5.8%	16.2%
	C-	0.069		12.6%	5.0%

[1]PP: Percentage positivity of mean OD, based on median OD of RTTCP C++ control serum
[2]CV: Coefficient of variation of OD

3.5. Discussion

From the technical point of view, the Ab-ELISA using pre-coated plates was convenient and practical, as there was no requirement to coat plates in the laboratory conducting the assay. This approach afforded a way to potentially improve standardisation of the assay among laboratories and to eliminate the problems of transportation and storage of crude trypanosome antigen in liquid form that had been encountered in an earlier version of the FAO/IAEA antibody-detection ELISA. However, transportation and storage of crude trypanosome antigen in liquid form has not resulted in any problems in the RTTCP trypanosomosis Ab-ELISA used in Southern Africa, and indeed laboratories capable of performing the other stages of the ELISA should also be capable of coating plates satisfactorily.

The results obtained in this version of the FAO/IAEA antibody-detection ELISA suggest that its analytical sensitivity is unnecessarily high. Sera from both negative and positive reference populations gave higher than expected OD and PP values, particularly when using the *T. congolense* denatured antigen pre-coated plates. Using the *T. congolense* denatured antigen pre-coated plates, most sera from parasitologically positive cattle from Zambia gave high OD responses compared to that for the strong positive control serum (C++) supplied by FAO/IAEA. This resulted in the majority of these sera giving PP values over 200%. Using the *T. vivax* denatured antigen pre-coated plates, the results OD responses for parasitologically positive cattle were also high, but not as extreme as for *T. congolense* denatured antigen.

Optical density values for the reference negative population cattle were also higher than expected, with median ODs of 0.475 and 0.385 for the ELISAs using *T. congolense* and *T. vivax* denatured antigens respectively. These high background ODs resulted in correspondingly high PP values.

Wright *et al.* [3] recommend that the strong positive control serum (C++) used for standardisation of indirect ELISA for the detection of Ab should be typical of sera from naturally-infected animals in the population under test. This was not the case for the C++ provided by FAO/IAEA, which appeared to contain significantly less Ab activity than most sera from the reference positive cattle population. If a C++ serum more typical of trypanosome-infected animals in Southern Africa were used, the assay could be re-titrated such that the C++ resulted in a response of around 1.0 OD units. Sera from the reference positive cattle population would then give ODs up to, but not

greatly exceeding, this level. The RTTCP strong positive control serum currently in routine use in the region was selected on the basis that it fulfilled these criteria.

Re-titration of the FAO/IAEA antibody-detection ELISAs as described above would most likely require an increase in the dilution of anti-bovine conjugate, and could be expected to reduce significantly the high background reactions observed for sera from the negative reference cattle population. The negative control serum (C-) supplied with the FAO/IAEA antibody-detection ELISA gave atypically low background reaction compared with those of the Zambian reference negative cattle population. However, if a negative control serum more representative of typical non-infected Zambian cattle were used, re-titration of the assay as described would be likely to reduce to acceptable levels the OD's of both negative Zambian cattle and the C- serum representative thereof. This could potentially lead to a useful increase in the specificity of the ELISAs, without any great loss in sensitivity; i.e. diagnostic performance would potentially be enhanced.

Finally, the FAO/IAEA antibody-detection ELISA using plates pre-coated with either denatured antigen gave reasonably good separation between sera from the positive and negative reference populations. On the basis of the results for these populations, using *T. congolense* antigen and giving equal weight to the importance of sensitivity and specificity, the optimum cut-off was approximately 40% positivity. This value was close to the mean PP (41.8%) of the moderate positive control serum (C+), which was classified as negative on 3 of 12 (25%) ELISA plates on the basis of this cut-off. Using this cut-off, the expected values of sensitivity and specificity would be approximately 89% and 86% respectively. Using *T. vivax* antigen and giving equal weight to the importance of sensitivity and specificity, the optimum cut-off was approximately 30% positivity. Using this cut-off, the expected values of sensitivity and specificity would be approximately 81% and 70% respectively. However, these values may have to be re-evaluated once more appropriate positive and negative control sera have been identified and the problem of excessively high analytical sensitivity has been addressed by re-titrating the assay.

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VALIDATION OF PRE-COATED ELISA TESTS TO DETECT ANTIBODIES AGAINST *T. CONGOLENSE* AND *T. VIVAX*

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Abstract

VALIDATION OF PRE-COATED ELISA TESTS TO DETECT ANTIBODIES AGAINST *T. CONGOLENSE* AND *T. VIVAX*.

The anti-trypanosomal antibody detecting enzyme linked immunosorbent assay (ELISA) was first described in 1977 and was further developed for use in large scale surveys in Zimbabwe. More recently, the IAEA initiated a programme to improve the robustness and standardisation of the assay. The IAEA supplied plates pre-coated with either a crude *T. congolense* or *T. vivax* antigen and the reagents necessary for analysing samples. Parasitologically positive and negative sera were used to validate and determine the cut-off values of the two tests. The samples were tested and results analysed using a variety of cut-off values. The tests provided similar information although the *T. congolense* pre-coated plates gave significantly higher optical density values than the plates coated with *T. vivax*. Sensitivity and specificity values were calculated using the different cut-off points. Results indicate that the test using *T. congolense* antigen had the highest specificity and sensitivity for a given cut-off value. Although the test could distinguish positive from negative sera, it was quite difficult to provide a suitable cut-off value, but the value should be dictated by the use of the test.

1. INTRODUCTION

To establish the distribution of tsetse-transmitted trypanosomosis or to evaluate the effectiveness of control measures in Africa, reliable and affordable diagnostic methods are required. The currently available techniques are either direct parasitological or indirect serological methods. Direct parasitological techniques such as wet, thick and thin stained smears identify parasites in the blood or other body fluids. These techniques are highly specific but limited in their analytical sensitivity and may lead to under-reporting of the disease [1]. This is due to the fluctuating and generally low parasitemias, which characterise the different stages of trypanosome infections. However, direct parasitological tests are simple, quick, relatively cheap and do not require sophisticated equipment or great expertise.

The diagnostic sensitivity of parasitological methods can be increased significantly by using the buffy coat technique (BCT).This method concentrates parasites in blood and has the added advantage that the packed red cell volume (PCV) can be determined being a measure of the degree of anaemia of an animal [1]. The test is reliable, simple and cost-effective, but requires an electricity source.

Indirect diagnostic assays rely on the detection of whole parasites, parts of the parasite or on immunological reactions in serum or whole blood. The techniques can be used as monitoring or survey tools. Such a test, is the enzyme linked immunosorbent assay (ELISA) first described for trypanosomosis in 1977 [2]. The test had a reasonable sensitivity and specificity, but was never developed for large scale epidemiological investigations. The test was further developed and used extensively to determine the distribution of bovine trypanosomosis in Southern Africa [3]. The Joint FAO/IAEA Division improved the indirect antibody-detection ELISA with the aim to develop a more robust and standardised assay. The test used plates pre-coated with either *T. congolense* or *T. vivax* antigens. The standardisation facilitated data interpretation, quality assurance and the exchange of data between various trypanosomosis monitoring and control programmes.

2. MATERIALS AND METHODS

2.1. Sample collection

2.1.1. Parasitologically and serologically negative samples

A total of 321 cattle were sampled at four sampling sites in Masvingo Province some 300 km south of Harare, Zimbabwe. The sites are diptanks located in tsetse-free communal and resettlement areas where trypanosomosis has not been diagnosed for the past 20 years. The cattle were randomly sampled at four different dip tanks namely Chipasha (30.69704, -20.440050), Mhungudza(30.7457, -20.243140), Mukonde (30.697480, -20.203680) and Bethel (31.730970, -19.71000) showing the longitudes and latitudes in brackets. At least sixty animals were sampled at each diptank. Animals sampled were Mashona crosses, aged between 3 and 7 years with no sex bias and care was taken not to include exotic breeds. The sampling was stratified by grazing patterns.

Blood samples were collected into heparinised capillary tubes by puncuring a peripheral ear vein using a sterile lancet. From each animal two capillary tubes were collected. One capillary tube was sealed with Cristaseal® (Hawksley) and centrifuged immediately in a microhaematocrit centrifuge for 5 min at 9000 *g*. After centrifugation, the PCV was determined using a reader (Hawksley). Buffy coat smears were prepared by cutting the capillary tube a few millimetres below the buffy coat/red blood cell interface and examined under a microscope for the presence of motile trypanosomes with a phase contrast microscope using a 40x objective lens [4]. The thick and thin blood smears were dried away from direct sunlight and stored in slide boxes for staining with 10% Giemsa. The stained thick and thin blood smears were examined under oil immersion using a 40x objective lens. Blood samples for the detection of antibodies were collected from the tail vein in plain vacutainer tubes. The blood was allowed to clot, serum was separated into 2 ml cryogenic vials and stored in an ice packed cool box before storage at –20°C at the Regional Laboratory in Harare. Cryogenic vials were labelled indicating the place and date of sampling and sample number. The crushpens or dip tanks where the animals were sampled were geo-referenced using a global positioning system (GPS).

2.1.2. Parasitologically positive samples

A number of areas were used to collect parasitologically positive samples for the validation of the test. The sample sites were situated in trypanosome endemic areas. The areas were found in Mashonaland East and Mashonaland West and the sites (with longitudes and latitudes in brackets) were called: Gunguhwe (28.8425, -17.148080), Rukomechi (29.397430, -16.135880), Makuti (29.251900, -16.323590), Vuti (29.46244, -16.477520) and Nyakadomwe. The same procedures were used to identify the trypanosomes as described for the negative samples.

The sera were stored at –20°C at the laboratory until analysis by ELISA.

2.2. Enzyme-linked immunosorbent assay (ELISA) to detect antibodies

2.2.1. Plates coated with Trypanosoma congolense *antigen*

The antigen was produced from *in vitro* cultured bloodstream forms of stock CP-81. The antigen, reagents and test procedure used for testing serum samples are described in the Bench Protocol, version iTAB (*Tc*AGd) prototype 1.0 (November, 1998) prepared by the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria.

2.2.2. Plates coated with Trypanosoma vivax *antigen*

The antigen was produced from *in vitro* cultured epimastigote forms of stock IL3905. The antigen, reagents and test procedure used for testing serum samples are described in the Bench Protocol, version iTAB (*Tv*AGd) prototype 1.0 (November, 1998) prepared by the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria.

3. RESULTS

The characteristics of the blood and serum samples are shown in Tables I and II. The PCV levels of the negative samples were clearly higher that those of the samples containing trypanosomes, while the percent positivity values of the negative serum samples were lower than the positive ones.

TABLE I. AVERAGE PERCENTAGE POSITIVITY AND PACKED RED CELL VOLUME (PCV) FOR NEGATIVE AND POSITIVE SERA

	Trypanosoma congolense antigen		*Trypanosoma vivax*	
	negative	Positive	negative	positive
Number of samples tested	193 (6 lost)	127 (mixed + serum bank samples)	160 (33 not tested)	120.0 (mixed)
Mean PP (%)	8.87	60.82	9.70	45.00
Mean PCV (%)	30.01	24.68	30.2	24.56

PP = percent positivity; PCV = packed red cell volume

TABLE II. CHARACTERISTICS OF NEGATIVE SAMPLES

Name of dip tank	Sample size	PCV <24
Bethel	47	0
Mukonde	60	0
Mhungudza	43	0
Chipasha	49	0

PCV = packed red cell volume

The prevalence figures for the three trypanosome species pathogenic for cattle are shown in Tables III and IV. *Trypanosoma congolense* was the most frequent species encountered.

TABLE III. PREVALENCE OF TRYPANOSOME SPECIES TESTED

Trypanosome species	Number of samples positive
T. congolense	95
T. vivax	20
T. brucei	12

TABLE IV. CHARACTERISTICS OF POSITIVE SAMPLES

Name of dip tank	Sample size	*T. congolense*	*T. vivax*	*T. brucei*
Nyakadomwe	36	29	2	5
Gunguhwe	21	10	7	4
Makuti	36	30	1	5
Rukomeshe	16	5	2	9
From serum bank		16	8	0

The results of the analysis of serum samples using the ELISA tests are shown in Tables V and VI. The correlation between packed red cell volume percentage and percentage positivity using the *T. congolense* coated ELISA plates is shown in Figure 2.

TABLE V. RESULTS OF SENSITIVITY AND SPECIFICITY USING VARIOUS CUT-OFF POINTS

Trypanosome antigen	Cut-off value	Specificity (%)	Sensitivity (%)
T. congolense	45	100	58
	20	98.4	80
	15	80	92
T. vivax	55	100	25
	20	97.5	67
	15	80	82

TABLE VI. AVERAGE PERCENTAGE POSITIVITY VALUES FOR DIFFERENT TRYPANOSOME SPECIES

	ELISA plate coated with *T. congolense* antigen	ELISA plate coated with *T. vivax* antigen
Trypanosoma congolense	72.54	43.44
Trypanosoma vivax	49.65	65.67
Trypanosoma brucei	53.34	63.21

ELISA = enzyme linked immunosorbent assay

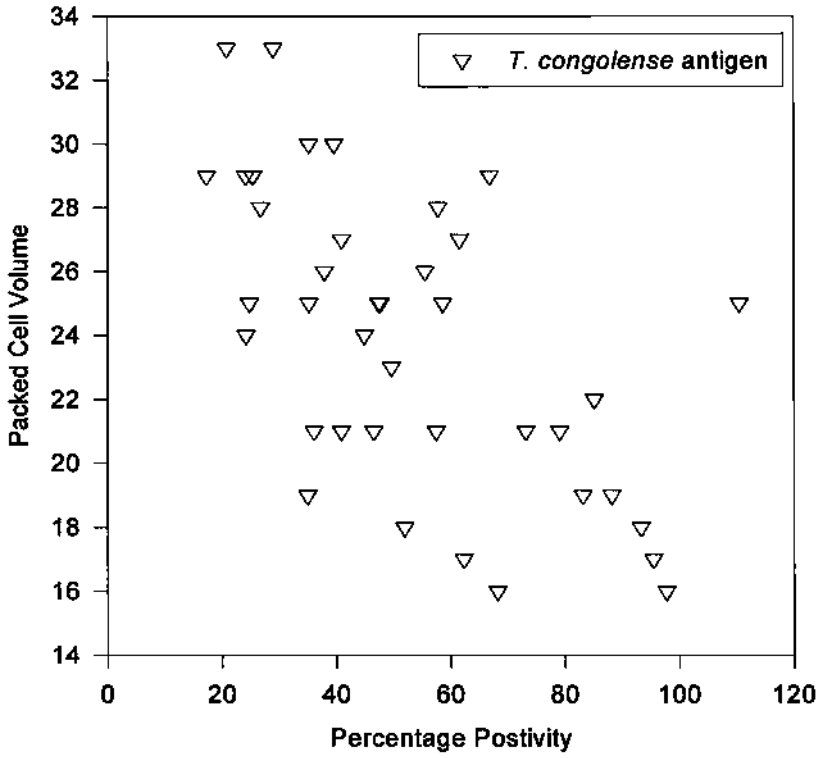

FIG. 1. Correlation between packed red cell volume and percentage positivity for T. congolense *antigen.*

The frequency distributions and the cumulative frequency distributions of the percentage positivity data for sera from trypanosome infected and uninfected cattle using *T. congolense* and *T. vivax* antigen is represented in Figs 2 and 3, respectively.

4. DISCUSSION

Trypanosomal antibodies are not detectable until 8-21 days post primary infection [5, 6, 7]. Thus, false negative results are encountered during the initial phase of infection, while false positive results can be due to the persistence of antibodies up to ten months after self cure or therapy [8, 9]. For the determination of cut-off values, samples from trypanosomosis free and trypanosomosis infested areas were used. The packed red cell volume from parasitologically positive sera was significantly lower than that from parasitologically negative sera and there was a correlation between PCV and PP values.

A significant difference between the PP values of negative and positive samples was detected. consequently, a clear discrimination between negative and positive sera could be made, although the cut–off value can be changed depending on the objective of the study (monitoring versus control). Selection of an appropriate cut-off value will affect the number of false positives and false negatives [10, 11]. For example, selection of a cut off value of 20% positivity for the test using *T. congolense* antigen, will result in a specificity of 98.4% and a sensitivity of 80%. The same cut-off value for the test using *T. vivax* antigen, will result in a specificity of 97.5% and a sensitivity of 67%. Thus, the *T.*

congolense test seems to be performing better than the *T. vivax* test both in sensitivity and specificity. However, it is advisable to test a larger number of *T. vivax* samples for a more thorough validation of the test. The sensitivity of the test using the *T. congolense* positive samples might be relatively low due to the manner in which samples were collected. Animals were sampled in the field whenever they were found to be positive.

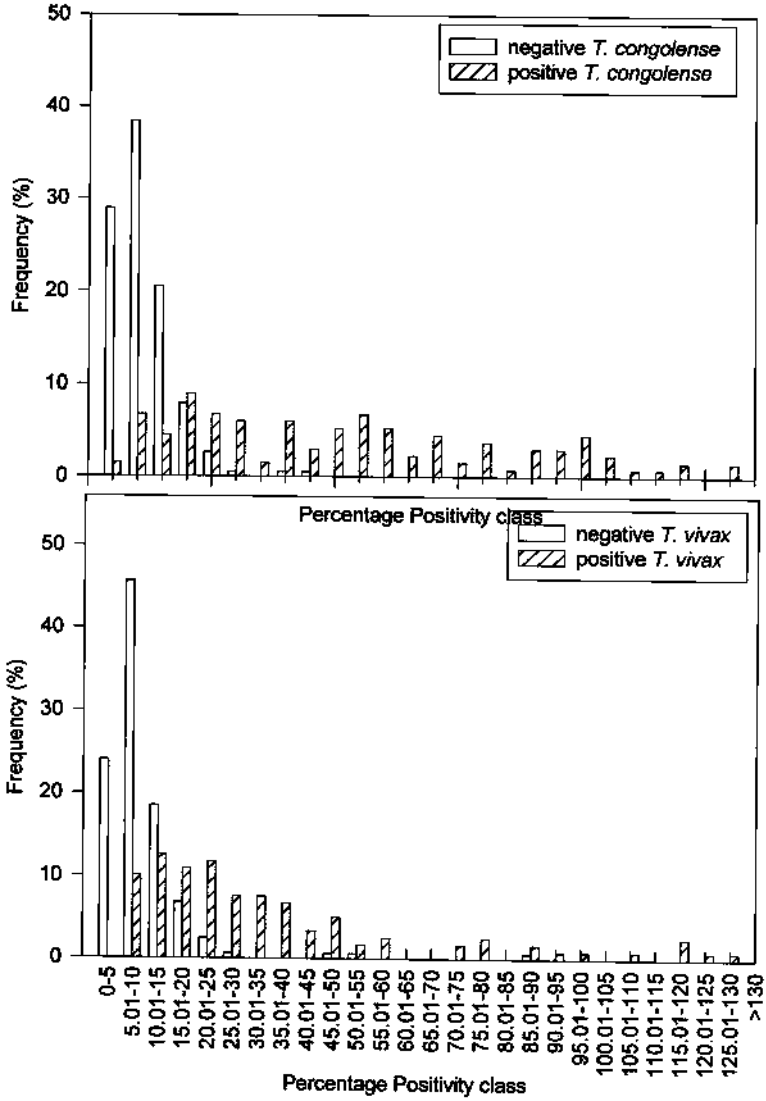

FIG. 2. Frequency distributions of the percentage positivity data for sera from trypanosome infected and uninfected cattle using T. congolense *and* T. vivax *antigen.*

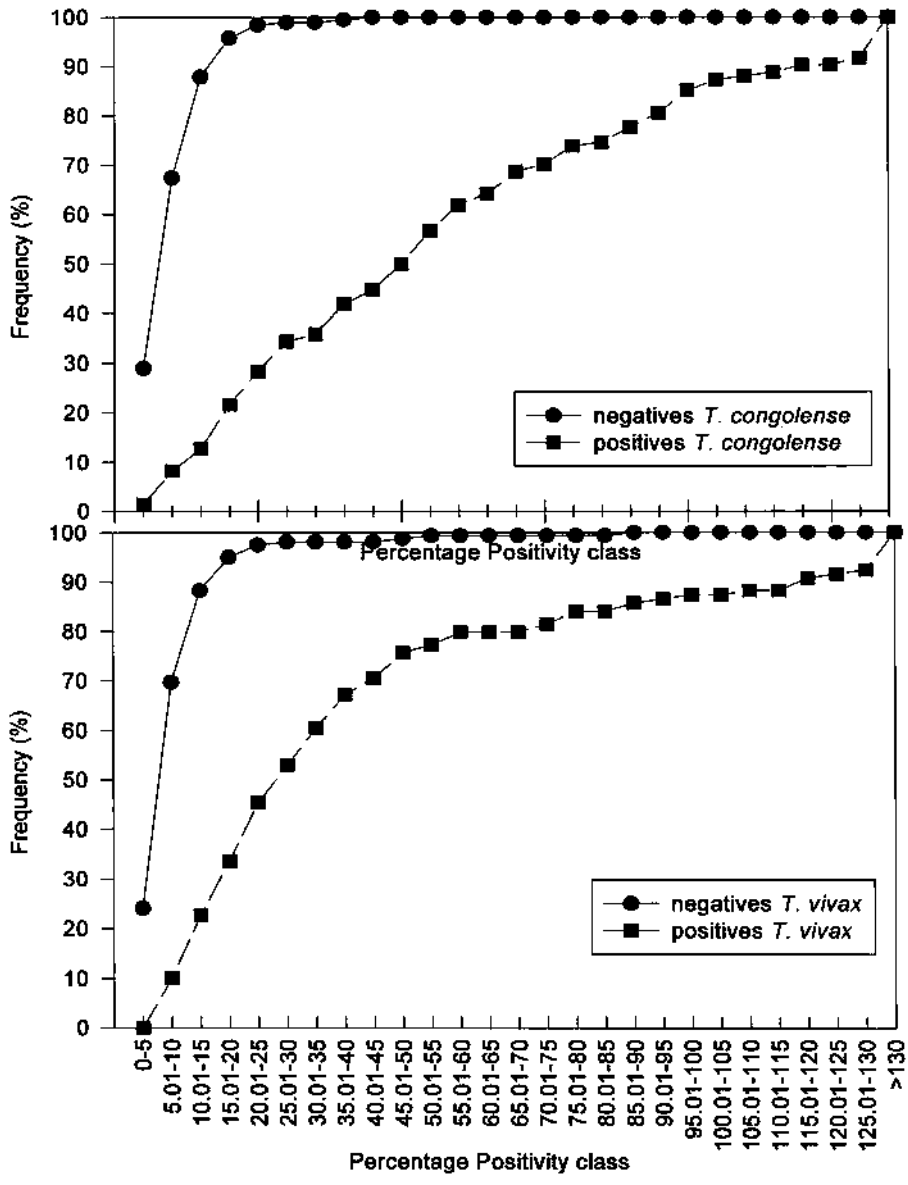

FIG. 3. Cumulative frequency distributions of percentage positivity data of sera from trypanosome-infected and uninfected cattle using T. congolense *and* T. vivax *antigen.*

However, no information was available on the duration of infection at the time of sampling. Thus, it is possible that the sample was collected during the period (8-21 days post-infection) when not enough antibodies were present to elicit a good signal, thus lowering the sensitivity of the test.

Not enough *T. vivax* positive samples (n = 20) were used for a proper validation of the *T. vivax*-antibody-detection test [12]. The sensitivity of the test using *T. vivax* antigen was quite low possibly due to the low number of samples used.

The collection of blood spots on filter paper could greatly simplify the sampling procedure. Moreover, samples could be collected from remote areas, as blood spots are not affected by the lack of a cold chain. Another improvement of the test would be to use plates coated with a single antigen, containing a mixture of all pathogenic species, since species specific diagnosis is not an important feature in large scale epidemiological surveys.

It is concluded that further validation of the assay will provide the end users with a better product and that extensive field application of the test is required before it is distributed for routine use to laboratories in Africa.

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GRAPHICAL CONTROL AND EVALUATION OF THE OPERATIONAL PERFORMANCE OF ELISA METHOD FOR DETECTION OF TRYPANOSOMAL ANTIBODIES

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Abstract

GRAPHICAL CONTROL AND EVALUATION OF THE OPERATIONAL PERFORMANCE OF ELISA METHOD FOR DETECTION OF TRYPANOSOMAL ANTIBODIES

The performance of four indirect trypanosomal antibody detection enzyme-linked immunosorbent assays (I-TAB ELISA's), exploiting native and denatured antigens of *Trypanosoma congolense* (*T.c.*AGn, *T.c.*AGd) and *T. vivax* (*T.v.*AGn, *T.v.*AGd), has been evaluated in fifteen laboratories from Africa and Europe. Standardised internal quality control samples (IQCs) were used as indicators and data plotted on charts to monitor and control the ELISA's at individual laboratories. Based on the overall data, dispersion of true values from the population data range was estimated plotting the location and deviation of raw and normalised absorbance values of the IQCs. Binding ratios were calculated to estimate the assay proficiency with respect to the accuracy of assessing that the IQC samples tested positive or negative in the test proper. The frequency distribution of coefficients of variation < 10 % of IQCs was monitored. The use of this standardised and transparent IQC data charting provides a useful quality control tool for the evaluation of the performance of the ELISA's used for trypanosomosis serology. The method provides a measure of confidence in estimating the proficiency of national laboratories with respect to reported ELISA results on disease occurrence.

Moreover, data were compiled of the inter-laboratory ELISA performance by means of summary data charts with reference to the performance criteria described. The data were also analysed using modified Youden plots. The analysis demonstrated similar laboratory proficiency for the I-TAB ELISA (*T.c.*AGn) (four of five laboratories), I-TAB ELISA (*T.c.*AGd) (eleven of fourteen laboratories), I-TAB ELISA (*T.v.*AGn) (three of five laboratories), and I-TAB ELISA (*T.v.*AGd) (eleven of fifteen laboratories). The impact of data variation between test and specified values as a sole decision criterion for acceptance or rejection of test plates is discussed.

1. INTRODUCTION

Work on serological methods has demonstrated that the enzyme-linked immunosorbent assay (ELISA) is the most suitable method for complementary use with traditional parasitological techniques to aid control and diagnosis of trypanosomosis in animal livestock caused by *Trypanosoma congolense*, *T. vivax* and *T. brucei* [1]. Indirect ELISA's have been evaluated for antibody detection in serum samples using trypanosomal crude antigen preparations or purified antigen fractions originating from rodents or cell culture conditions [2, 3, 4, 5]. For the detection of circulating trypanosomal antigens in serum samples, direct sandwich assays were developed exploiting monoclonal antibodies [6]. However, these efforts have not led to the distribution of a robust, sustainable and internationally recognized ELISA method. For international trade, methods for direct examination and parasite concentration rather than ELISA are still the prescribed tests for examination of tsetse-borne trypanosomosis by the Office International des Epizooties [7], although it is demonstrated that they provide low sensitivity with a high specificity.

Recently, four indirect ELISA's have been developed, [8, 9, 10]. In addition, a standardised and transparent control system monitoring the operational performance of the ELISA within specified limits was developed for implementation as routine application in diagnostic laboratories in the tropics.

The objective of this study was to obtain and present data on the quality control of four ELISA's for detecting antibodies against trypanosomosis through the use of charting methods. Such methods ensure the constant control and monitoring of the operational performance of ELISA's with

respect to the tentative control data ranges determined under conditions at the FAO/IAEA Agriculture and Biotechnology Laboratory, during the assay development stages. The data were processed using Shewhart-like charts [11], and used data from standardised internal quality control samples [12] referred to as ELISA performance indicator data. The methods gave immediate visual monitoring and helped in controlling the operational performance from plate to plate and day to day. The overall operational performance of the assays was compared from fifteen laboratories in Africa and Europe. The results were analysed graphically using summary data charts and modified Youden plots [13, 14].

2. MATERIALS AND METHODS

2.1. Laboratories

The operational performance of the ELISA's was monitored in fifteen laboratories in Austria and Belgium, Burkina Faso, Cameroon, Cote d'Ivoire, Ghana, Kenya, Mali, Nigeria, Sudan, Tanzania, Uganda, Zambia, Zanzibar and Zimbabwe.

2.2. ELISA reagents and shipment

Four indirect trypanosomosis antibody (I-TAB) ELISA systems were evaluated. Briefly, they exploited native (AGn) or detergent-heat treated antigen preparations (AGd): two *T. congolense* (*T.c.*) AG-based indirect ELISA's (I-TAB ELISA (*T.c.*AGn) and I-TAB ELISA (*T.c.*AGd)), as well as two *T. vivax* (*T.v.*) AG-based indirect ELISA's (I-TAB ELISA (*T.v.*AGn) and I-TAB ELISA (*T.v.*AGd)) [9]. The antigen-precoated ELISA plates were sealed, packed in plastic bags with silica gel desiccant packets (Sigma, USA), and stored at +37°C until shipment in the original cardboard boxes of the ELISA plate manufacturer by air freight without special conditions. The plates were stored at room temperature in counterpart laboratories until used. The frozen biological reagents (control sera and conjugated antibody) were dispatched in vacuum flasks and kept at -20°C until used.

2.3. ELISA procedure

The ELISA's were performed according to the corresponding standardized FAO/IAEA bench protocols (prototype version 1.0, November 1998). The assay procedure included the testing of four internal quality control (IQC) samples in four replicates: a defined strong positive (C++), a moderate positive (C+), a negative serum sample (C-), and serum diluent buffer as a conjugate control (Cc) as described elsewhere [12]. The IQCs were used as operational performance indicators of the ELISA method.

2.4. Tentative internal quality control limits

As part of the assay standardisation procedure at the FAO/IAEA Agriculture and Biotechnology Laboratory, preliminary internal quality control limits were established based on theconsensus from a Joint FAO/IAEA Meeting of Consultants convened in Vienna in January 1992. Replicates of each IQC (n=24) were repeatedly tested in six quadruplicate wells/plate on fifteen occasions. For each plate, the optical density (OD) value of each IQC replicate was expressed as a percentage of the median of four replicates of the C++ OD according to the ELISA data interchange software programme (EDI version 2.3.1, 1999) supplied by the FAO/IAEA. For each IQC, the preliminary upper and lower control limits of the raw absorbance signal was determined from the overall mean OD value ± 3 standard deviations (*SD*) of 90 mean OD values from 90 quadruplicates. Similarly, the tentative upper and lower control limits of the percent positivity (PP) values of each IQC was determined from the overall mean PP value ± 3 *SD* of 90 mean PP values from 90 quadruplicates.

2.5. ELISA charting methods

For the generation of Shewhart-like ELISA control data charts [11] and data processing, the spreadsheet software program Microsoft Excel for Windows '95, version 7.0, was used. Figure 1 illustrates the use of ELISA IQC data charting methods at the operator's level, which were then subjected to inter-laboratory explorative analysis. The charts were meant to visualize the agreement of the true operational performance observed under local conditions with the expected performance determined at the FAO/IAEA Agriculture and Biotechnology Laboratory to control and monitor the plate to plate, day to day and trend performance.

Internal explorative assay analysis		External explorative assay analysis
The ELISA operator generates...		The FAO/IAEA laboratory generates...
1) detailed & summary daily data charts	→	A) summary laboratory data chart and B) modified Youden plot analysis chart
2) detailed daily precision chart	→	C) summary laboratory precision chart
3) detailed daily proficiency chart	→	D) summary laboratory proficiency chart

FIG 1. Data charts generated for monitoring and evaluation of the operational ELISA performance analysing internal quality control data.

2.5.1. Detailed and summary daily data (D&SDD) chart

For each laboratory, Shewhart-like control detailed (D) & summary daily data (SDD) charts were generated to plot the daily distribution of the C++ OD values. Similar charts plotted the percentage positivity (PP) of each IQC from individual plates expressing the raw OD value as PP relative to the mean of the intermediate OD value (median OD value) of the strong positive control. Shewhart-like control charts plotted the number of plates along the x-axis against the actual absorbance values or the percent positivity values (y-axis), respectively. The upper and lower control limits (UCL-LCL) representing OD and PP mean values ± 3 *SD* were determined at the FAO/IAEA Agriculture and Biotechnology Laboratory as described under paragraph 2.4. The daily IQC results from single plates (OD and PP mean values ± 2 *SD*) and the overall mean ± 2 *SD* derived from all plates on one occasion are plotted. Some OD or PP values have been highlighted to illustrate what are extremes for the *SD*.

2.5.2. Detailed daily precision (DDPre) chart

The intra-laboratory analysis of the variation of the IQC replicates within and between plates is referred to as assay repeatability. Detailed daily precision (DDPre) charts plot the percent coefficient of variation (CV %), which is a measure of relative dispersion of IQC replicates based on the*SD*. The CV % was calculated by the *SD* of four PP replicates divided by the corresponding mean for single plates. For this study, the upper control limit was set as CV =10 %, which was empirically determined and recommended for evaluation of standardised ELISA's [15]. In addition to the CVs of C++ and C+, the DDPre chart plotted the CVs of C- and Cc. These were considered useful for monitoring but considered less meaningful for final judgement of the assay precision because their mean values approached zero.

2.5.3. Detailed daily proficiency (DDPro) chart

The detailed daily proficiency (DDPro) charts plotted the intra-laboratory assay proficiency computing the ratios of antibody binding to antibody non-binding (B/B0) of the median PPs of C+/C- from each plate. For calculation, the median of four IQC replicates rather than the mean was chosen to approach the true value rather than the value more biased by dispersion of four replicates. Also, the small difference of antibody activity of C+ compared to C- was considered more indicative to alert to reduced assay proficiency than the higher ratio of C++/C-. The tentative UCL-LCL range was determined from the overall mean value of C+/C- binding ratios ± 3 *SD* at the FAO/IAEA Agriculture and Biotechnology Laboratory (see 2.4.).

2.6. Interlaboratory explorative analysis

For each ELISA system, data of the performance indicators were generated under local conditions in laboratories in Europe and Africa. The data were reported to the FAO/IAEA Agriculture and Biotechnology Laboratory and plotted on summary data charts for explorative analysis of the ELISA performance within control limits.

2.6.1. Summary laboratory data charts

The overall IQC mean values of raw (OD) and normalised (PP) absorbance values from each laboratory representing the true data range were compared with the tentative UCL-LCL range (OD and PP mean values ± 3 SD) determined at the FAO/IAEA Agriculture and Biotechnology Laboratory.

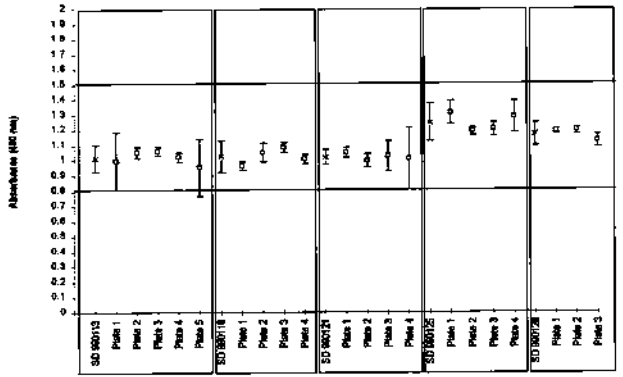

FIG. 2a. I-TAB ELISA (T.c.AGd): illustration of daily single and summary plate absorbance values plotted on a detailed and summary daily data chart at a laboratory in Africa (C++ AVG ODs ± 2 SD). Lines represent tentative upper and lower control limits (AVG ODs ± 3 SD).

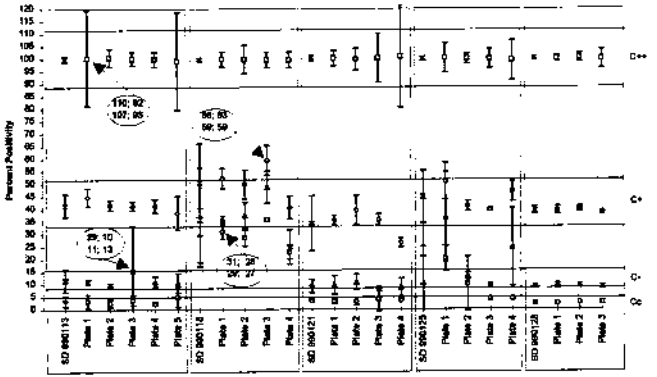

FIG. 2b. I-TAB ELISA (T.c.AGd): illustration of daily single and summary plate percent positivity (PP) values plotted on a detailed and summary daily data chart at a laboratory in Africa (C++, C+, C- and Cc AVG PPs ± 2 SD). Lines represent tentative upper and lower control limits (AVG PPs ± 3 SD). Numbers in circles represent examples of alarming PP values.

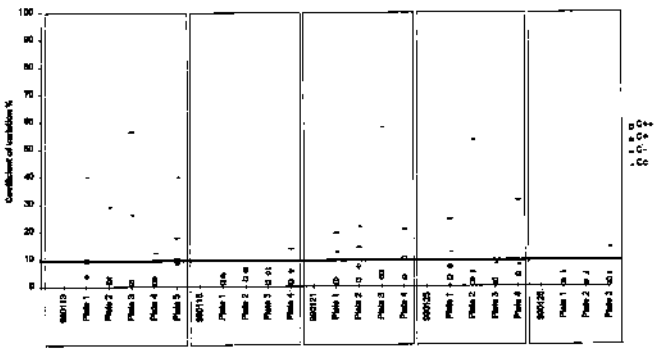

FIG. 2c. I-TAB ELISA (T.c.AGd): illustration of CV % of PP values plotted on detailed daily precision chart at a laboratory in Africa. Bold line represents tentative 10 % upper control limit.

FIG. 2d. I-TAB ELISA (T.c.AGd): illustration of C+/C- binding ratios plotted from median PP values of 4 replicates each on daily detailed proficiency chart at a laboratory in Africa. --- tentative upper and lower control limit (AVG ± 3 SD).

2.6.2. Summary laboratory precision chart

For each IQC on single plates and individual laboratory, the frequency distribution of the CVs < 10 % was plotted and compared between laboratories (reproducibility). For each IQC, a box represented the true frequency range based on the overall mean of the frequency ± 1 *SD* of CVs < 10 % obtained from all laboratories.

2.6.3. Summary laboratory proficiency chart

For each laboratory, the overall mean ± 2 *SD* of binding ratios was plotted to demonstrate the intra-laboratory variation of the assay proficiency within the tentative UCL-LCL range (overall mean ± 3 *SD*) obtained from data described under paragraph 2.4. In addition, computing the overall mean ± 1 *SD* of pooled B/B0 ratios from single plates of all laboratories a range was defined to evaluate the inter-laboratory variation of the assay proficiency.

2.6.4. Modified Youden plot analysis

The modified Youden plot analysis identified systematic and random errors between laboratories [13, 14]. Briefly, a result obtained by a laboratory on one sample was plotted with respect to the result it obtained on a similar sample. Depending on the relation of the plotted point to the true value, it can be decided whether discrepant results are due to bias, imprecision, or both.

For each laboratory, the overall mean PP values of C+ (y-axis) were plotted against those of C- (x-axis). A rectangular was formed by the overall laboratory mean PP values ± 1 *SD* of C+ and C-. Laboratories reporting both IQCs outside the mean ± 1 *SD* defined quadrant indicated systematic errors (upper right or lower left region). Laboratories revealing random errors for both IQCs were visualised in the upper left or lower right region outside the mean ± 1 *SD* defined quadrant. Laboratories falling within the vertical or horizontal medium region outside the mean ± 1*SD* defined quadrant indicated a random error for 1 IQC sample.

3. RESULTS

3.1. Routine Shewhart-like charting methods of IQC data

Figures 2a to 2d illustrate Shewhart-like data charts for monitoring the ELISA under conditions in Africa. Plots of the IQC data show, at a glance, the unprocessed absorbance values of C++; the IQC PP; the CV % and B/B0 values.

Each plot shown in Figs. 2a and 2b reflects the mean value and its variation in all plates on various occasions. The bar shows the absolute range of variation of the four replicates to the mean within the measured probability on each plate and occasion. Different situations were encountered which allowed immediate interpretation of the assay performance. These were: 1) Plots showing means to be within limits and the error bars to be short and also within limits, which was ideal with reference to the IQC sample tested. 2) Plots showing the mean to be within limits, but one tail of the error bar to be out of limits. The error here was probably higher than acceptable. Reference to the individual data is recommended e.g., a single replicate could have been missed out or gave an out of limit result, which both reduced the overall mean plot and increased error. 3) Plots showingthe mean, and most or all of the error bar to be outside limits. The data must be examined with reference to other IQCs, which may indicate a systematic or random error. 4) Plots showing the mean valueto be below or above limits, but the error to be small, indicating little variation in results for all plates used. Similarly, occurrence of systematic or random errors needed to be carefully addressed.

Disparate situations were observed for the assay performance over time: A) All means and error bars were within limits. This indicated that the IQC values were constant and it wouldn't be expected that the test had altered in sensitivity. B) The plotted curveconnecting results temporally was irregular with large "swings" in mean OD and PP values throughout time. Differences in assay variability due to frequent changes of operators might be considered. C) The plotted time curve was irregular with areas of similar means, which could have been a result of notable changes in personnel performing the assay and/or reagents. D) The curve demonstrated a fairly constant downward or upward trend irrespective of operators. This could signify altering of reagents over time.

The DDPre chart (Fig. 2c) showed estimates of the relative variation of the plotted means of IQCs from plate to plate. In the example given, the ELISA revealed CVs < 10 % for C++ and C+ indicating excellent precision. Higher CVs % were generally observed for C- and Cc as expected, even though CVs < 10 % were occasionally found.

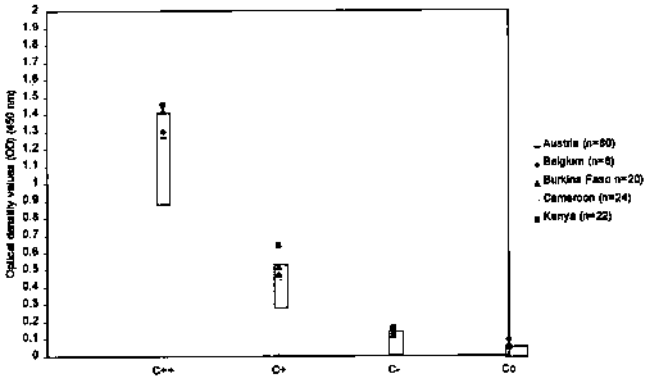

FIG. 3a. I-TAB ELISA (T.c.AGn): summary laboratory data chart plotting IQC values expressed as overall mean absorbance values. Boxes represent tentative range of upper and lower control limits (UCL-LCL) (AVG OD ± 3 SD) as determined at the FAO/IAEA Laboratory.

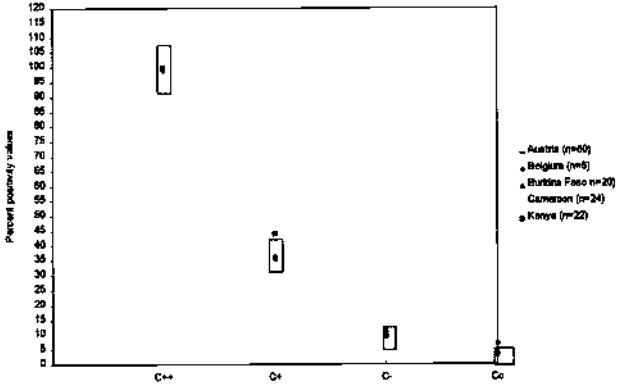

FIG. 3b. I-TAB ELISA (T.c.AGn): summary laboratory data chart plotting IQC values expressed as overall mean percent positivity values. Boxes represent tentative UCL-LCL range (AVG OD ± 3 SD) as determined at the FAO/IAEA Laboratory.

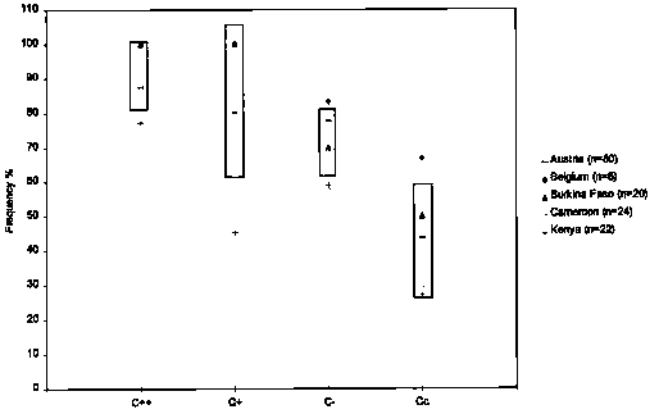

FIG. 3c. I-TAB ELISA (T.c.AGn): summary laboratory precision chart illustrating the frequency distribution of CVs < 10 %. Boxes represent the true UCL-LCL range (overall AVG ± 1 STD) obtained from all laboratories.

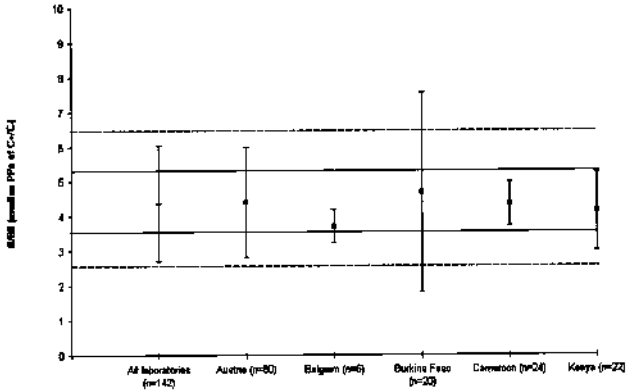

FIG. 3d. I-TAB ELISA (T.c.AGn): summary laboratory proficiency chart. - - - tentative UCL-LCL range (AVG ± 3 SD) determined at the FAO/IAEA Laboratory; — true UCL-LCL range (overall AVG ± 1 STD) obtained from all laboratories.

The DDPro chart (Fig. 2d) gave an example of the effect of systematic or random errors described above on the assay performance. The plates tested on day 990118 and two plates tested on day 990125 showed binding ratios below expected limits.

3.2. Explorative analysis of interlaboratory ELISA performance

For assessment of the ELISA performance in fifteen laboratories, the true dispersion of raw and relative absorbance, and binding ratios of IQC data were compared with tentative limits (see 2.4.). With respect to the laboratory proficiency testing, the absorbance range expressed as PP values was also explored with reference to the true data range as computed by the modified Youden plot analysis. The frequency distribution of CVs < 10 % was analysed to estimate the expected assay precision under various laboratory conditions.

3.3. I-TAB ELISA (*T.c.*AGn)

The I-TAB ELISA (*T.c.*AGn) was evaluated in three laboratories in Africa and two laboratories in Europe. In Kenya, higher absolute absorbance values for the C++, C+ and C- were observed (Fig. 3a). Similar absorbance of C++, but to a lesser extent, was observed in Burkina Faso. In Belgium and Burkina Faso, slightly more background absorbance (Cc) occurred (Fig. 3a), which did not change when PPs were plotted in the Belgium laboratory (Fig. 3b). The best assay precision was observed in Belgium and Burkina Faso, where all plates demonstrated CVs < 10 % for the C++ and C+ (Fig. 3c). A high variation of IQC replicates was observed in the Kenyan laboratory. At all laboratories, the assay proficiency was within the expected limits (Fig. 3d). The modified Youden plot analysis demonstrated that the ELISA performance under the local circumstances in Kenya was affected by systematic errors and, therefore, different to the other laboratories (Fig. 7).

In summary, for each of the four IQCs, expected absorbances were observed in two of the five laboratories. Computing PP values, three of five laboratories demonstrated controlled ELISA performance inside the established tentative limits. Comparing the assay precision between laboratories (reproducibility), four of the five laboratories demonstrated similar frequency distribution of CV < 10 % of C++ and C+ within the overall mean frequency distribution ±1 *SD*, namely 82.38 % – 101.86 % and 61.05 % - 107.46 %, respectively. The assay proficiency with respect to assay accuracy was demonstrated in all laboratories as expected. Among five laboratories, four laboratories showed similar laboratory proficiency.

3.4. I-TAB ELISA (*T.c.*AGd)

The I-TAB ELISA (*T.c.*AGd) was evaluated in twelve laboratories in Africa and two laboratories in Europe. Special attention was given to the ten laboratories where unexpected data alerted a poor ELISA performance

Ghana and Zambia consistently demonstrated higher absorbance values than expected (Fig. 4a). For Zambia, the assay performance was within expected limits on analysis of PP values (Fig. 4b). For Ghana, the high signal for C- was maintained. It was later shown that a working dilution of anti-species enzyme-conjugate of 1/14000 instead of 1/20000 was used [16]. In Mali, the C+ OD values were similar to C++ (Figs. 4a and 4b). The C- was extremely low resulting in tremendous increase of the assay proficiency (Fig. 4c). The Youden plot analysis identified that the Mali counterparts were performing the ELISA differently compared to other laboratories because of systematic errors for both the C+ and C- (Fig. 7). It was later shown that the C++ was replaced by a locally collected serum sample [17].

In Belgium and Burkina Faso a slightly higher background signal, just above the upper control limit, had to be accepted during the testing period (Figs. 4a and 4b). The latter laboratory also reported PPs of C- just above the expected limits, which was also observed in Cote d'Ivoire and Kenya.

In Uganda, the C+ was high relative to the expected C- signal (Figs. 4a and 4b) and this affected the binding ratio, which fell outside the pre-established range and approached the upper control limit based on the overall mean binding ratios ± 1 *SD* from all laboratories. The frequency of CVs < 10 % of C++ and C+ < 10 % was 50 % and 60 %, respectively, which was inferior to the frequency distribution observed in other laboratories (Fig. 4c). On Zanzibar, high overall mean absorbances outside range were observed for the weak positive and negative controls (Fig. 4a). Computing relative values, the overall mean PP of C+ was within limits, but the PP of the C- remained above the upper

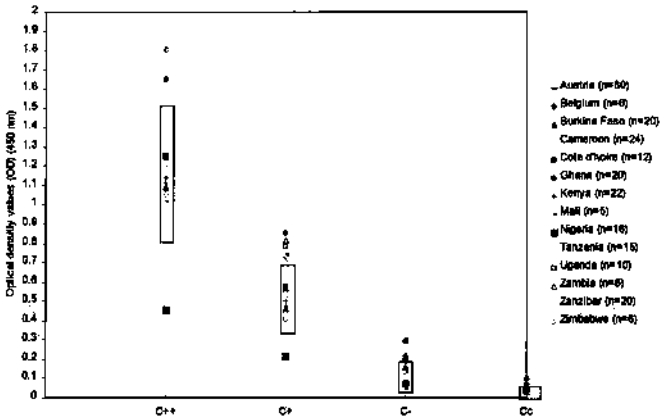

FIG. 4a. I-TAB ELISA (T.c.AGd): summary laboratory data chart plotting IQC values expressed as overall mean absorbance values. Boxes represent tentative range of upper and lower control limits (UCL-LCL) (AVG OD ± 3 SD) as determined at the FAO/IAEA Laboratory.

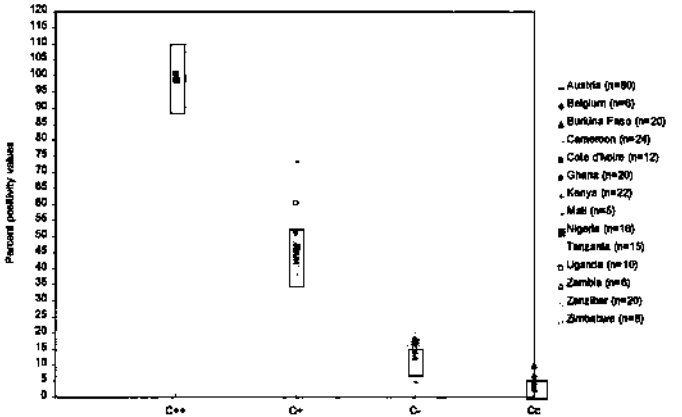

FIG. 4b. I-TAB ELISA (T.c.AGd): summary laboratory data chart plotting IQC values expressed as overall mean percent positivity values. Boxes represent tentative UCL-LCL range (AVG OD ± 3 SD) as determined at the FAO/IAEA Laboratory.

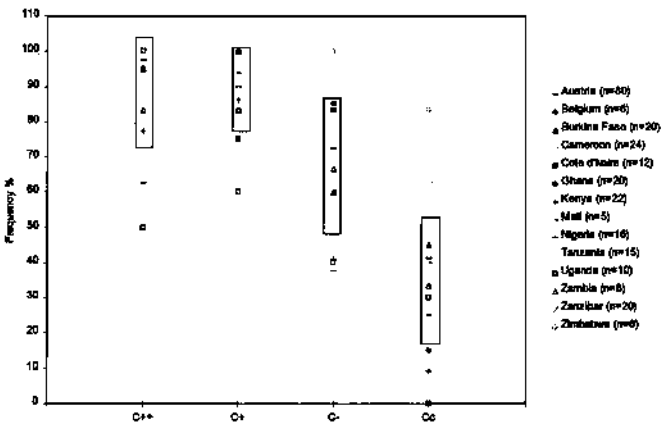

FIG. 4c. I-TAB ELISA (T.c.AGd): summary laboratory precision chart illustrating the frequency distribution of CVs < 10 %. Boxes represent the true UCL-LCL range (overall AVG ± 1 SD) obtained from all laboratories.

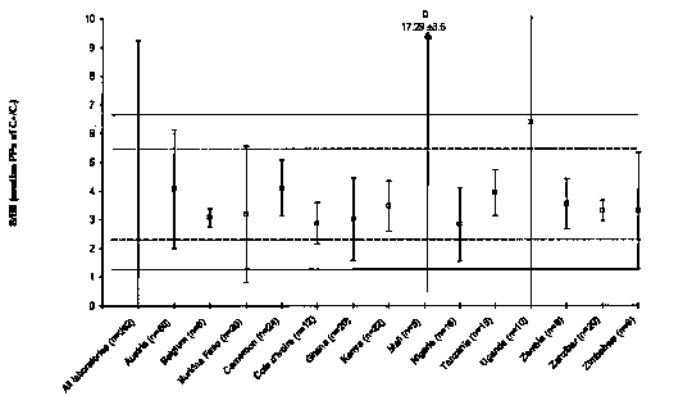

FIG. 4d. I-TAB ELISA (T.c.AGd): summary laboratory proficiency chart. - - - tentative UCL-LCL range (AVG ± 3 SD) determined at the FAO/IAEA Laboratory; — true UCL-LCL range (overall AVG ± 1 SD) obtained from all laboratories.

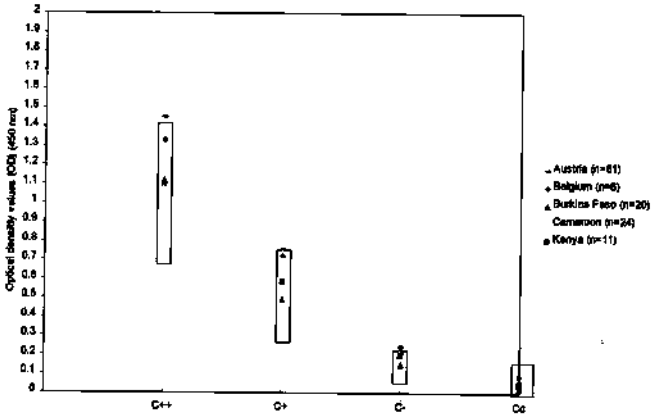

FIG. 5a. I-TAB ELISA (T.v.AGn): summary laboratory data chart plotting IQC values expressed as overall mean absorbance values. Boxes represent tentative range of upper and lower control limits (UCL-LCL) (AVG OD ± 3 SD) as determined at the FAO/IAEA Laboratory.

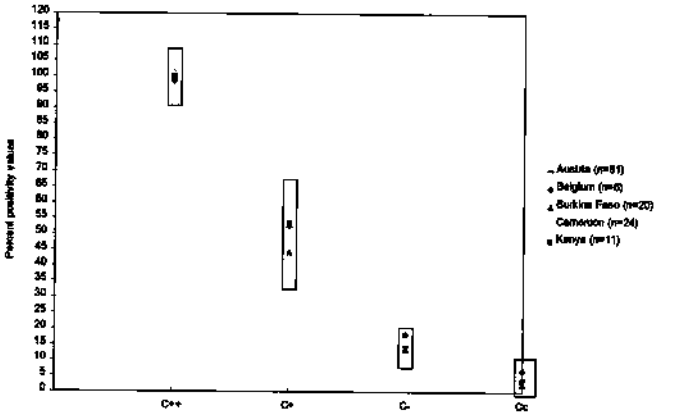

FIG. 5b. I-TAB ELISA (T.v.AGn): summary laboratory data chart plotting IQC values expressed as overall mean percent positivity values. Boxes represent tentative UCL-LCL range (AVG OD ± 3 SD) as determined at the FAO/IAEA Laboratory.

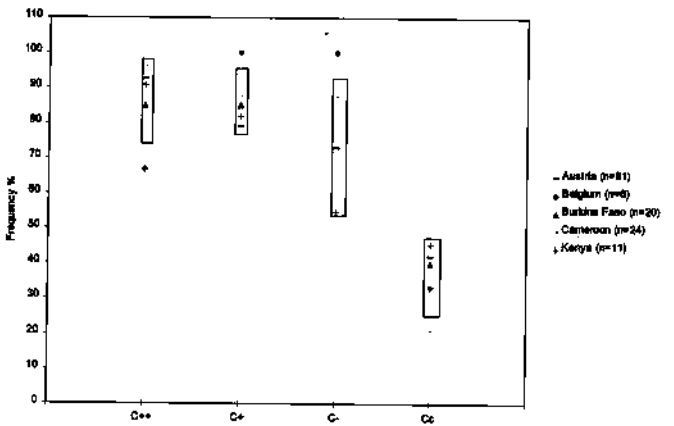

FIG. 5c. I-TAB ELISA (T.v.AGn): summary laboratory precision chart illustrating the frequency distribution of CVs < 10 %. Boxes represent the true UCL-LCL range (overall AVG ± 1 STD) obtained from all laboratories.

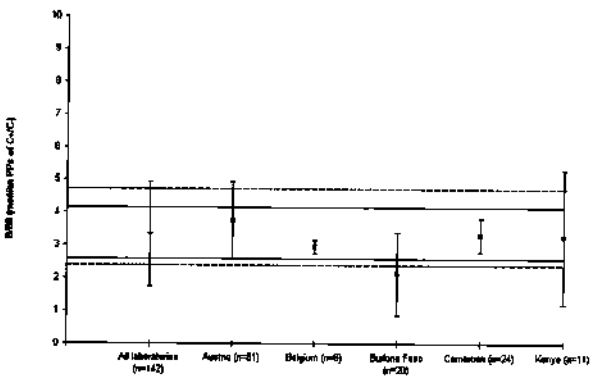

FIG. 5d. I-TAB ELISA (T.v.AGn): summary laboratory proficiency chart. - - - tentative UCL-LCL range (AVG ± 3 SD) determined at the FAO/IAEA Laboratory; — true UCL-LCL range (overall AVG ± 1 STD) obtained from all laboratories.

limit, which did not seriously affect the binding ratio (Figs. 4b and 4d). For the C+ (Uganda) and C- (Zanzibar) random errors were found (Fig. 7).

In Nigeria, the ELISA showed very low colour of C++ and C+. Expressed in PP values, the C- was unexpectedly high (Figs. 4a and 4b), without affecting the assay proficiency with respect to the assay accuracy (Fig. 4d). As observed in Uganda, the assay precision was not as good as shown in other laboratories (Fig. 4c).

In summary, for each of the four IQCs, expected absorbance and PP values was observed in five of fourteen laboratories. Similar expected assay precision was found in twelve laboratories as demonstrated by the frequency distribution of CVs < 10 % of C++ and C+ within the overall mean frequency distribution ±1 *SD* namely 73,77 % – 105.60 % and 76.87 – 100.52 %, respectively. Expected assay accuracy was obtained in twelve laboratories. Eleven of the fourteen laboratories, showed similar laboratory proficiency.

3.5. I-TAB ELISA (*T.v.*AGn)

The I-TAB ELISA (*T.v.*AGn) was evaluated in three laboratories in Africa and two laboratories in Europe. For this ELISA, the tentative limits of C++ and C+ overlapped, indicating high variation of absorbance for the antibody positive controls (C++, C+) which did not interfere with clear discrimination from C- (Fig. 5a). High absorbance of C++ was also found when the ELISA was used at the FAO/IAEA Agriculture and Biotechnology Laboratory (Fig. 5a).

Cameroon consistently gave high readings for the C- and Cc (Figs. 5a and 5b), although the C++ and C+ fell within expected limits and the diagnostic proficiency was similar to other laboratories (Fig. 5d). However, from all the five laboratories, Cameroon revealed the highest signal for C+ and C- (Fig. 7). In Belgium, the absorbance of C- was just above the upper control limit (Fig. 5a).

In Burkina Faso, the overall mean absorbance and PPs were within the expected range. However, the C+/C- binding ratio dropped just below the expected lower control limit indicating a loss of assay proficiency. This occurred due to a random error in the ELISA performance leading to reduced C+ PPs (Fig. 7). Among the five laboratories, Cameroon and Burkina Faso demonstrated an altered ELISA performance. Analysing the assay reproducibility, Belgium demonstrated 66.67 % of C++ and 100 % of C+ quadruplicates revealing CVs < 10 %.

In summary, for each of the four IQCs, expected absorbance was observed in two of the five laboratories. Absorbance expressed as PP values demonstrated controlled ELISA performance within tentative limits in four laboratories. Similar expected assay accuracy and assay precision was found in four of the five laboratories. They demonstrated a frequency distribution of CV < 10 % of C++ and C+ within the overall mean frequency distribution ±1 *SD*, namely 74,59 % – 97.81% and 78.55 % – 94.78 % respectively. Among five laboratories, three laboratories showed similar laboratory proficiency.

3.6. I-TAB ELISA (*T.v.*AGd)

The I-TAB ELISA (*T.v.*AGd) was evaluated in thirteen laboratories in Africa and two laboratories in Europe.

Nine of the total of fifteen laboratories showed unexpected assay performance. Two laboratories reported very low colour development. In Nigeria, absorbance of C++ and C+ was reduced. Data normalisation revealed PPs of C- and Cc, which were too high relative to the C+ (Figs. 6a and 6b). The binding ratio approached the lower limit of the expected range (Fig. 6d) due to a random error (Fig. 7). In Ghana, absorbances for the C++ were too low leading to unexpected high PPs of C+, C- and Cc (Figs. 6a and 6b). The binding ratio remained inside range (Fig. 6d).

Burkina Faso showed reduced overall mean PP of C+ relative to the expected overall mean absorbance of C++ dropping the assay proficiency below the lower control limit outside limits (Fig. 7). In Burkina Faso and Belgium higher backgrounds were observed. Mali and Zimbabwe reported expected overall mean absorbance of the four IQC samples. However, the overall mean PP of C+ was lower than expected indicating that the intensity of colour development varied from plate to plate.

Comparing the laboratory proficiency, among the fifteen laboratories, a discrepancy was observed in Mali and Burkina Faso due to random errors for C+; in Nigeria, a random error encountered for C-, and in Ghana a systematic error affected C+ and C- (Fig. 7).

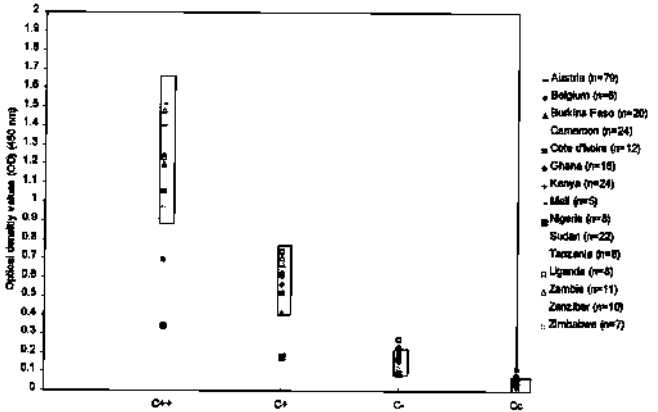

FIG. 6a. I-TAB ELISA (T.v.AGd): summary laboratory data chart plotting IQC values expressed as overall mean absorbance values. Boxes represent tentative range of upper and lower control limits (UCL-LCL) (AVG OD ± 3 SD) as determined at the FAO/IAEA Laboratory.

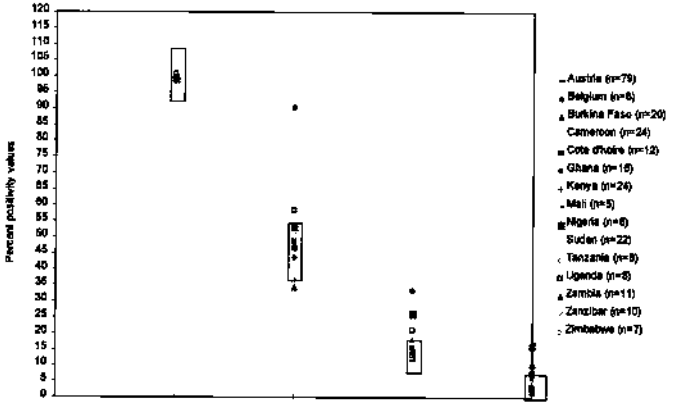

FIG. 6b. I-TAB ELISA (T.v.AGd): summary laboratory data chart plotting IQC values expressed as overall mean percent positivity values. Boxes represent tentative UCL-LCL range (AVG OD ± 3 SD) as determined at the FAO/IAEA Laboratory.

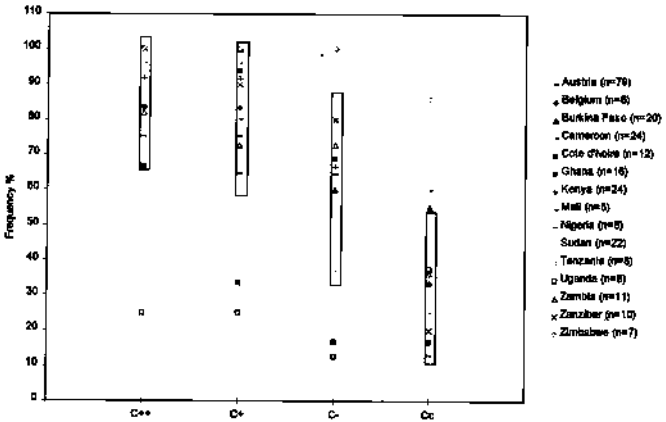

FIG. 6c. I-TAB ELISA (T.v.AGd): summary laboratory precision chart illustrating the frequency distribution of CVs < 10 %. Boxes represent the true UCL-LCL range (overall AVG ± 1 STD) obtained from all laboratories.

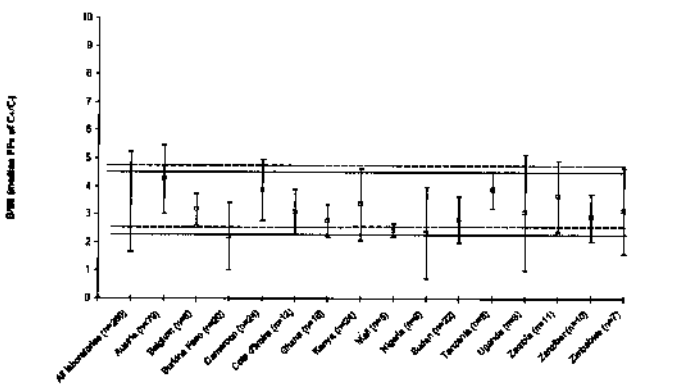

FIG. 6d. I-TAB ELISA (T.v.AGd): summary laboratory proficiency chart. - - - tentative UCL-LCL range (AVG ± 3 SD) determined at the FAO/IAEA Laboratory; — true UCL-LCL range (overall AVG ± 1 STD) obtained from all laboratories.

In Uganda unexpected high overall mean PPs of C+ and C- were found without effecting the binding ratios. Estimating the assay repeatability, CVs < 10 % of C++ and C+ quadruplicates were only rarely found (25 %). The assay performance on Zanzibar was characterised by an overall mean absorbance and PP value of C- above the tentative upper limit, which did not affect the assay proficiency. In Sudan, the assay revealed marginally less absorbance, which did not affect the assay proficiency.

In summary, for each the four IQCs, expected absorbance was observed in eight of fifteen laboratories and expected PP values were observed in seven of fifteen laboratories. Analysing the interlaboratory assay precision, fourteen and thirteen laboratories demonstrated similar frequency distributions of CV < 10 % of C++ and C+, respectively, within the overall mean frequency distribution ±1 *SD*, namely 66.30 % – 106.21 % and 56.74 % - 103.96 %, respectively. Expected assay proficiency was observed in twelve laboratories. Among fifteen laboratories, eleven laboratories showed similar laboratory proficiency.

4. DISCUSSION

The use of charting methods to plot standardised IQC data on Shewhart-like data control charts proved useful to monitor and evaluate the operational performance of indirect ELISA methods for the detection of trypanosomal antibodies. The charting methods: i) kept a constant record of all data, ii) monitored the ELISA's from day to day, and week to week, iii) rapidly identified unacceptable results, iv) helped to determine problems with specific reagents, v) noted trends in results, e.g., a decrease in performance, vii) identified bias of ELISA performance due to different operators, and vii) fulfilled various criteria for good laboratory practice (GLP). In addition, the establishment of standardised and transparent IQC data charting methods for quality control of the ELISA performance should provide a measure of confidence to national laboratory proficiency with respect to reports on disease occurrence.

The evaluation of the operational performance of four indirect ELISA systems in fifteen laboratories demonstrated that the majority reported similar laboratory proficiency for the I-TAB ELISA (*T.c.*AGn) (four of five laboratories), I-TAB ELISA (*T.c.*AGd) (eleven of fourteen laboratories), I-TAB ELISA (*T.v.*AGn) (three of five laboratories), I-TAB ELISA (*T.v.*AGd) (eleven of fifteen laboratories. These findings were in agreement with those obtained from a previous pilot study [18]. The data reported here and those with reference to the diagnostic sensitivity and robustness of ELISA method [9, 10, 19] provided evidence that the ELISA's exploiting heat-denatured antigens of *T. congolense* and *T. vivax* can be successfully used for trypanosomosis control following transfer to diagnostic laboratories in the tropics.

The detailed plate to plate analysis of the replicates of C++, C+, C- and Cc allowed comparison with the defined population data determined at the FAO/IAEA Agriculture and Biotechnology Laboratory, so that unexpected absolute (OD) and relative absorbance (PP) values alerted operators to problems in good time. The discrepancies between the test absorbance of IQCs result and specified absorbance was attributed to unavoidable random errors inherent in every measurement procedure. The factors that influence the outcome of a measurement can not all be completely controlled. In the practical interpretation of ELISA measurement data, this variability has to be taken into account and requires additional explorative analysis. Therefore, the method of using binding ratios for C+/C- was analysed to allow comparison of the analytical sensitivity with respect to the accuracy of assessing that a sample was positive both within and between tests in laboratories. The frequency distribution of CVs < 10 % of C++ and C+ PP values, indicative of the assay precision, was also examined. It was demonstrated that the assays performed reasonably well within the true range represented by overall mean value ± 1 *SD* of all laboratories. This suggested that the ELISA systems were similarly affected by uncertainties occurring at individual laboratories which can not be controlled by the production site of the ELISA's.

It should be noted that the results of IQCs performance indicators such as reduced binding ratios do not automatically control the assay proficiency with reference to the diagnostic sensitivity and specificity. Here, re-testing of test serum samples would be recommended or even better the consistent plate to plate analysis of defined positive and negative reference sera representing the studied population.

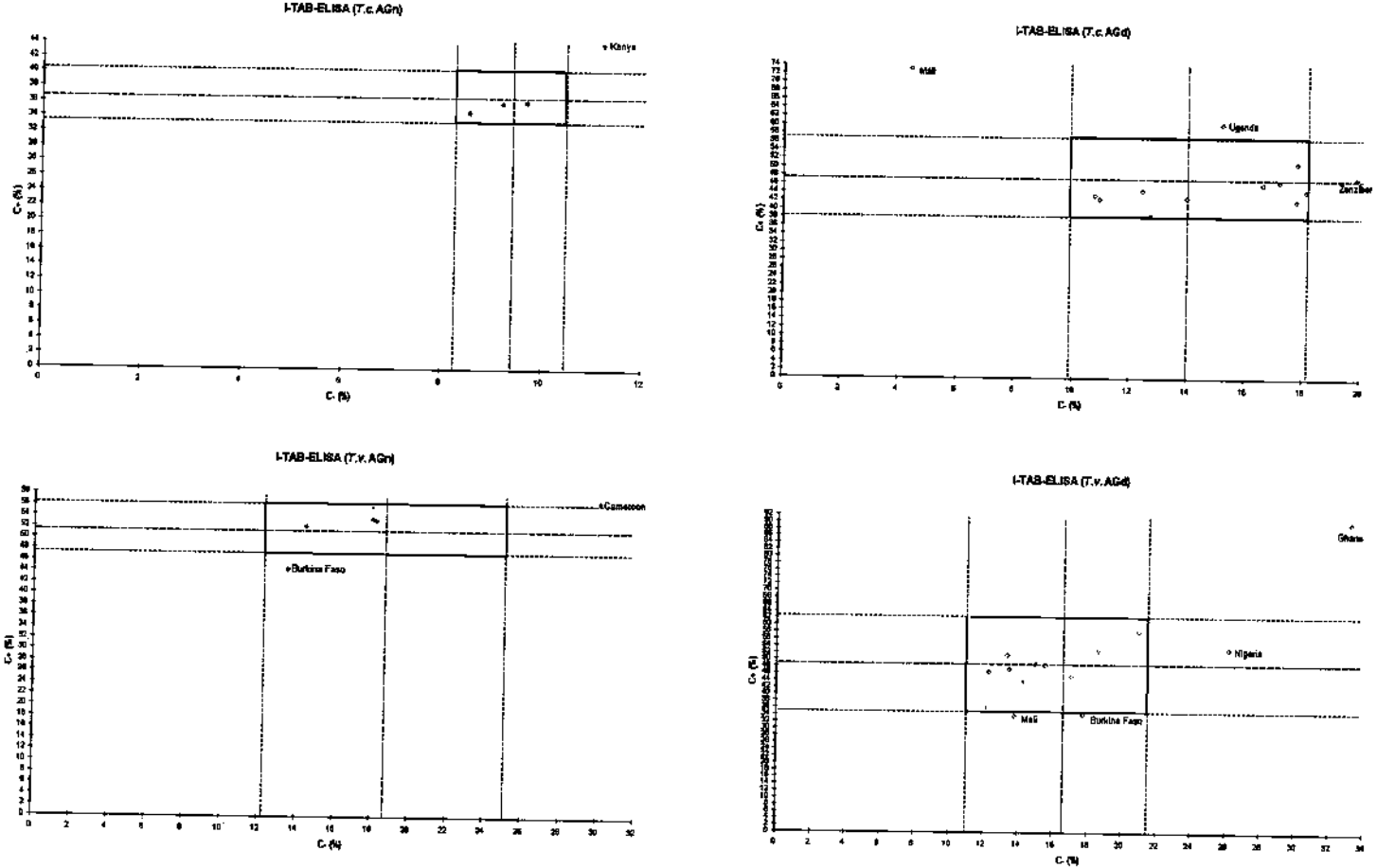

FIG. 7. Results of modified Youden plot analysis used for laboratory proficiency testing. Using indirect antibody detection ELISAs for trypanosomosis random and systematic errors in laboratories were identified. For I-TAB ELISA (T.c.AGn), I-TAB ELISA (T.c.AGd) and TAB I-ELISA (T.v.AGd) note that data within range overlapped with results from one additional laboratory. For further explanation see under MATERIALS AND METHOD.

From the data it became evident that the sole use of the IQC absorbance range did not truly reflect the potential ELISA performance and should, therefore, not be used as the only decision criteria for plate acceptance or rejection. It is proposed to refer also to the assay precision and binding ratios, which can be easily recorded on Shewhart-like data charts at individual laboratories. For interlaboratory evaluation of the ELISA performance, data should then be reported to the ELISA production site, responsible for continuous control and monitoring of the performance of ELISA systems.

In conclusion, a control quality procedure was established for evaluation of the operational performance of indirect trypanosomosis ELISA method within and between laboratories. The results provided a measure of confidence in the reliable use of trypanosomosis antibody ELISA's with reference to controlled assay performance in diagnostic laboratories in Africa and Europe. The trypanosomosis ELISA method was therefore considered "fit for purpose".

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VALIDATION OF AN INDIRECT ELISA[1] FOR THE DETECTION OF *TRYPANOSOMA CONGOLENSE* ANTIBODIES IN ETHIOPIAN CATTLE

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Abstract

VALIDATION OF AN INDIRECT ELISA FOR THE DETECTION OF *TRYPANOSOMA CONGOLENSE* ANTIBODIES IN ETHIOPIAN CATTLE.

Control and eradication of African Animal Trypanosomosis can be achieved if the reliability of the methods to diagnose the disease could be improved. The techniques currently used in the diagnosis of trypanosomosis in Ethiopia are not sufficiently sensitive to detect all infected animals. In order to improve disease diagnosis the indirect antibody detection ELISA, developed by FAO/IAEA, was evaluated under field conditions. Accordingly, reference serum samples were collected from trypanosomosis free and endemic areas. Serum samples negative for trypanosomes were collected from the Central highlands of Ethiopia where there is no previous record of trypanosomosis. The samples were used to establish the threshold and the specificity of the test. *Trypanosoma congolense* positive sera (based on thin and thick smears and BCT) were collected from endemic areas in the Southwestern part of the country to estimate the sensitivity of the test. Out of 701 negative serum samples, 690 were identified as negative with the indirect antibody ELISA, whereas the remaining 11 were detected as positive. Moreover, of the 282 infected samples the ELISA detected 155 sera as positive, but the remaining 127 cases fell in the negative range. The positive/negative threshold established from negative reference sera was found to be 81.38%. Based on this threshold the specificity of the test was 98.43%, whilst the sensitivity was calculated as 54.96%. Thus, the complementary use of both the ELISA and parasitological methods is encouraged.

Since the internal quality controls (IQC) did not fall in the ranges prescribed in the protocol provided by FAO/IAEA precision was achieved by comparing the plate to plate variation of the IQC based on the means plot. Accordingly the assay process indicated that there was no significant difference between individual mean of each plate regarding the strong positive (C++) and moderate positive (C+) controls. Nevertheless, considerable discrepancies were encountered between plates when each individual mean of negative (C-) and conjugate (Cc) controls were compared. This variation indicated an instability of the antigens requiring further refinement to achieve a standard quality.

1. INTRODUCTION

A significant proportion of Ethiopia is infested with tsetse flies and some 6 million head of cattle are exposed to trypanosomosis, the disease tsetse transmit. This situation greatly impedes sound agricultural development in the Western and Southwestern parts of the country. During the past years the disease has encroached previously non-infested areas reaching altitudes of up to 2000 meters above sea level [1]. Consequently, the distribution of tsetse flies, which had been covering 98.000 km^2 two and half decades ago [2] is estimated to have almost doubled recently [3]. It is even possible that a larger area is affected if more sensitive diagnostic tools were available.

In an attempt to investigate the exact foci and boundaries of the disease problem and to monitor control activities in the country, it is essential to use sufficiently reliable diagnostic techniques. The diagnostic methods currently in use are primarily observation of clinical signs followed by the detection of parasites in the peripheral blood using parasitological techniques such as thin and thick blood smears and the buffy coat technique (BCT) [4, 5]. These techniques are tedious when applied in the field and do not detect a low parasitemia, which is common in chronic disease situations.

With a view to solving these drawbacks and to develop improved diagnostic techniques monoclonal antibodies (Mabs) were produced that recognize species specific antigens and can be used to develop immunodiagnostic assays [6, 7]. These assays can also be used in drug efficacy tests and for the study of trypanotolerance [8]. However, the antigen-detection ELISA proved to be not sufficiently reliable when applied independently [9, 10] and even identified less positive cases of *T. congolense* and *T. vivax* as positive under experimental conditions than the standard parasitological techniques [11, 12].

On the other hand, the application of an antibody-detection ELISA although showing a good diagnostic sensitivity, requiring simple equipment and permitting wide scale use as a routine diagnostic test, is limited because of poorly defined antigens and the persistence of antibodies for

[1] It should be noted that unlike in the other reports, this study did not use plates pre-coated with antigen, a procedure which greatly improved antigen stability.

several months in treated or recovered animals [13]. Consequently, a standardized procedure for antigen production was introduced and an improved version of the indirect antibody ELISA to detect *T. congolense* was developed by the FAO/IAEA Agriculture and Biotechnology laboratory. We report the cut-off point of the test, its sensitivity and specificity under field conditions in Ethiopia.

2. MATERIALS AND METHODS

2.1. Sampling methods

Privately owned local Zebu cattle were used for collecting sera. Blood was aspirated from the jugular vein using 10 ml plain vacutainer tubes. Following clotting of the blood, serum was extracted and stored at -20°C until used.

2.2. Antibody negative serum samples

In order to collect samples negative for antibodies against *T. congolense*, areas were selected from the Central highlands of Ethiopia, with an altitude higher than 2500 masl and with no history of trypanosomosis. During the sampling exercise the history of each individual cow with regard to its origin was investigated. In addition, every animal was checked for the presence of parasites using thin and thick blood smears and the BCT prior to serum collection. The negative samples was used to establish the positive/negative threshold and to estimate the specificity of the assay.

2.3. Antibody positive serum samples

Sera of cattle actively infected with *T. congolense* from the Western lowlands of the country, particularly Gambella and Eastern Wellega, were used as an antibody-positive reference population. In order to identify positive infections, the conventional parasitological diagnostic methods were used: thin and thick blood smears and BCT. The positive serum samples were used to estimate the sensitivity of the assay.

2.4. Antigen and other reagents

Crude antigen preparations following ultrasonic disruption of *in vitro* propagated *T. congolense* (strain CP81), standardized controls and other consumables were prepared and provided by the Joint FAO/IAEA Division and the assay was conducted following the bench protocol version iTCA 1.0, November 1997.

3. RESULTS

3.1. Comparison of plate to plate variation

Despite the fact that the assay was conducted according to the bench protocol, the results of internal quality controls (IQC) failed to meet the prescribed acceptance criteria. But in an attempt to analyze the specificity and sensitivity of the test, precision was achieved by comparing plate to plate variation based on values of IQC. In doing so, the variation of individual mean percent positivity (PP) values of IQC from various plates relative to the overall mean +/-one standard deviation was assessed and displayed by plotting the data against the plate number. The results indicated that there is no statistically significant difference between the individual means of C++ and C+ from the overall mean of 98.18% (with standard error 1.41) and 55.87% (with standard error 0.62), respectively. Nevertheless, a significant difference existed between the individual means of the negative control (C-) and conjugate control (Cc) and their overall means of 28.14% (standard error of 0.5) and 23.18% (standard error of 0.068), respectively.

3.2. Diagnostic results of serum samples

Out of 701 serum samples collected from negative animals, the indirect antibody-detection ELISA revealed the absence of detectable antibodies to *T. congolense* in 690 animals, whilst the remaining 11 samples were detected as positive. On the other hand, 155 animals from 282 infected animals were confirmed positive with Ab-ELISA and the remaining 127 were testing negative. The threshold value of the test was established by doubling the mean PP from the

negative or disease free reference samples and was set at 81.38%. Therefore, the diagnostic sensitivity of the indirect Ab-ELISA was found to be 54.96% and the diagnostic specificity 98.43% (Table I).

TABLE I. SENSITIVITY AND SPECIFICITY OF INDIRECT AB-ELISA TO DETECT T. CONGOLENSE

		Infected*	Uninfected*
Antibody-ELISA	Positive	155	11
	Negative	127	690
	Total	282	701
		Diagnostic sensitivity: 54.96%	Diagnostic specificity: 98.43%

* samples collected from animals of known infection status (using parasitological techniques)

4. DISCUSSION

The validation of the indirect ELISA for the detection of antibodies against *T. congolense* infections under field conditions was done using two sets of sera, one from a population of parasite free animals and the other from a cattle population infected with trypanosomes. Although there is no test that can serve as a "gold standard", a set of subjective and objective criteria was used to identify the two populations.

The internal quality controls (IQC) prepared by the Joint FAO/IAEA Division were used for the analysis of the test samples. The results indicated that the OD values of the IQCs failed to fall within the range prescribed in the bench protocol. This could be due to poor antigen quality giving rise to reduced OD readings of the standardized controls.

Reduced absorbance values of IQC particularly that of C++ appeared to affect the validation process of the test. If the antigen was stable enough to produce a lower threshold falling within the normal range, the diagnostic specificity and sensitivity of the analysis would be increased. Nevertheless, despite the fact that the antigen used was unstable and produced reduced absorbance values for IQC, an acceptable test proficiency was achieved by comparing plate to plate variation of internal quality controls. Individual mean percent positivity (PP) values of strong positive control (C++) and moderate positive control (C+) of each plate agreed with each other 95% of the time indicating a good test performance. But absorbance values of the moderate control still remained in the negative range of reading, i.e. below the threshold of the test but yet at higher values in most cases than the true negative samples. However, considerable variation appeared to occur between individual means of negative (C-) and conjugate (Cc) controls of each plate. Refinements to the quality and maintenance of the antigen are indicated to improve the antibody-detection ELISA [14].

With regard to the test samples the threshold value was established by doubling the mean PP values of negative reference serum samples. At the threshold value of 81.38% the sensitivity of the indirect antibody ELISA for *T. congolense* is relatively low (54.96%), but encouraging provided optimum quality of the antigen is maintained. The diagnostic specificity of the test is still very high (98.43%) regardless of the absorbance value of C++ which was below the expected reading.

The indirect antibody ELISA showed great potential in the detection of low parasitaemias. Generally, most false negative serological results are of animals with a high level of parasitaemia as detected by BCT. A possible explanation for such a discrepancy would be that serum samples were collected during the earliest peak of parasitaemia at which time internal antigens were not present in sufficient quantities to produce detectable antibodies. Thus, the complimentary use of both parasitological techniques and indirect Ab-ELISA in the diagnosis of trypanosomosis is encouraged.

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SELECTION OF PRIORITY AREAS FOR TSETSE CONTROL IN AFRICA; A DECISION TOOL USING GIS IN DIDESSA VALLEY, ETHIOPIA, AS A PILOT STUDY

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Abstract

SELECTION OF PRIORITY AREAS FOR TSETSE CONTROL IN AFRICA; A DECISION TOOL USING GIS IN DIDESSA VALLEY, ETHIOPIA, AS A PILOT STUDY.

Trypanosomosis is one of the main constraints to livestock production, particularly in the sub-humid and the semi-arid zones in Africa. To study the impact of the disease and the people economically affected by the disease GIS has proven to be a valuable decision tool to prioritise intervention areas and to select control or eradication methods.

In this study a GIS and remote sensing based model have been used in the Didessa Valley and South-Western Ethiopia to study valley specific relationships of all factors (host, vector, disease, human population/activity) with geography, environment and farming systems to answer the following questions:

◊ Where does trypanosomosis have a negative effect on (agricultural) development?

◊ In which areas will control measures have the highest impact/economical benefit?

These findings have been extrapolated to set priority areas for tsetse control for Ethiopia as a whole, using a multi-criterion evaluation technique and a Boolean disease data-set to create signatures (training sets) to predict the probability of agricultural suitability and disease suitability based on a set of environmental predictors. Data sets have been validated through ground-truthing using a random data-set for the specified window of South-western Ethiopia. A logical expression has been used to combine the factors (vector distribution, agricultural suitability, climatically disease suitability, control area suitability) to select the priority areas for tsetse control. Areas permanently infested by malaria, one of the life-threatening human diseases in Africa, have been considered as not suitable for intervention. The result, a Boolean representation of the priority areas for South-western Ethiopia can be prioritised further by the decision maker (government, donor, specialist) using criteria such as cost/benefit, administrative organisation and control method.

The unique altitude related dynamic tsetse situation in Ethiopia, makes wider extrapolation using the same developed training-set, unpredictable. The dynamics of tsetse should be studied in more detail to prevent re-invasion of the control areas.

1. INTRODUCTION

1.1. General background

1.1.1. Trypanosomosis

Trypanosomosis is a haemoprotozoan disease of humans and animals (plants, fishes) caused by several species of trypanosomes. In Africa, the most important trypanosome species affecting domestic ruminants are those transmitted by tsetse flies [1].

Trypanosomosis is one of the main constraints to livestock production, particularly in the sub-humid and to a lesser extent in the wetter parts of the semi-arid zones in Africa [2]. Domestic livestock in Africa are important as a source of protein (milk & meat) to humans, as a source of animal traction, as a source of income (e.g. hides) and investment (social security) and as a source of manure for enhancing agricultural (crop) production. Tsetse transmitted trypanosomosis affects 37 sub-Saharan countries; an estimate of 160 million cattle and 260 million sheep & goats are kept in this area of risk extending over 10 million km^2 of land [1].

1.1.2. Impact of the disease

The impact of trypanosomosis on African agriculture is most obviously seen in the birth and mortality rates of young animals. In susceptible cattle breeds, the disease reduces calving by up to 20% and causes the deaths of another 20% of young stock. Even the so-called "trypanotolerant" animals, such as N'Dama cattle, are affected. It strongly reduces milk off-take (reduction of 26%, The

Gambia) and lambing and kidding rates (reduction of 37%, The Gambia). Trypanosomosis reduces the availability and efficiency of draught animals used for preparing land for crop-production (reduction of 33%, Ethiopia)[3]. In mixed farming systems where trypanosomosis is so severe that it constrains the number of oxen that farmers own, it can reduce the average area planted per household by as much as 50% [4].

1.1.3. Classification of the vector

The tsetse fly can be classified in the order of *Diptera* (2-wings), family *Glossinidae* and within the genus: *Glossina.* Within the genus, 23 species and 8 subspecies of Glossina have been identified [5].

Three sub-genera can be differentiated using ecological characteristics:

- Morsitans group (savannahs of Africa)
- Palpalis group (riverine forest vegetation)
- Fusca group (rain-forest)

The most important vectors of the Morsitans group transmitting disease to domestic ruminants are *Glossina morsitans* (Southern and Eastern Africa), *G. pallidipes* (thicketed areas of savannahs), *G. m.submorsitans* (savannahs of West-Africa extending eastwards to Ethiopia), whilst *G. fuscipes* of the Palpalis group can be an important vector of human trypanosomosis in West Africa. The Fusca group is less important for the transmission of trypanosomosis to domestic livestock because the habitat of livestock and the habitat of the vectors hardly overlap, except for two species which occur outside rain-forest areas: *G. brevipalpis and G. longipennis* [1].

1.1.4. Interactions of vector and host

Habitats are composed of different mixtures of soil, water, air and vegetation, each with different thermal characteristics and often with different temperatures [6]. These characteristics of the earth surface are used in remote sensing (RS) applications to study the environment and its changes. A number of factors and environmental characteristics influence the spatial distribution of all species occurrences in nature. In today's world, the geographical location of species occurrences is primarily a composite result of the impact of human civilisation on environmental features [6]. In Nigeria, agricultural activity showed a reduction in number of tsetse flies because of disturbance of the natural habitat of the vector [7], whilst Read *et al* [8] showed historical evidence suggesting that an advance of tsetse was responsible for a decrease in the area cultivated.

Many arthropod vectors of parasites are sensitive to climate, which is generally expressed as temperature, humidity and rainfall [10]. The general distribution of tsetse flies is determined principally by climate and influenced by altitude, vegetation and the presence of suitable host animals. Each of these factors may directly affect the birth, death or migration rates of the vector and thus the population size [10]. For example, the most favourable temperature for *Glossina* is between 21 and 24°C for the adult stage while too high (>35°C) or too low temperatures (<14°C) hinder puparia from completing their development [11]. The influence of humidity and rainfall can be partly seen in the relatively high mortality rates of the end of the dry season and the low mortality rates during the rainy season. However, during the onset of the dry season changes in host distribution, amount of available shade, number and kind of predators, changes in temperature (°C) and change in hours of sunshine must also be taken into account [12]. Generally, the limit of tsetse distribution is closely correlated with the tropical savannah climate, which follows the 508mm annual isohyets [2].

Vegetation is important in providing shade and maintaining a suitable microclimate for the vector as well as a habitat for their vertebrate hosts [1]. Vegetation types vary with climate and altitude. Vegetation profiles for tsetse habitats are directly related to the ecological zones where tsetse species are present and can be roughly described as woodlands, thicket woodlands, thicket or Savannah [13, 2].

Generally, the habitat of livestock is directly related to the presence of human settlements. The most important factor to develop an optimum habitat for the disease is the (direct) interaction between host, vector and parasite each with their own optimum habitat. Insect disease vectors seek the optimum microclimate they can find (the coolest available place [11]) and respond to changes in that micro-climate [14].

1.2. Role of GIS in trypanosomosis control

A geographical Information Systems (GIS) allows analysis, composition and visualisation of digital and analogue (geo-referenced) data and can be used to model spatial problems *in time and space*. It is therefore, in combination with remote sensing (RS) techniques, a unique tool for assisting with decision-making processes [15].

Various studies and applications of GIS techniques defining the epidemiological and related aspects of vector-borne and arthropod diseases have been published. Rogers *et al.* described the use of remote sensing techniques to better understand the natural habitat and epidemiology of tsetse and to predict tsetse and disease distribution in (Eastern) Africa [16,17,18,19,20,21]. Reid *et al* used GIS & RS to study the effects of tsetse presence and control methods on land-use, environment & bio-diversity [8,23,24,25,26] focusing on smaller scale areas in Eastern Africa (e.g. Ghibe Valley, Ethiopia). For West-Africa Hendrickx recently published a number of articles describing the use of GIS & RS as a decision and management tool for trypanosomosis control in West Africa using a 100% training set for Togo [27]. De la Rocque studied an area at a much smaller scale in West Africa; a 1000 km^2 area in Burkina Faso with SPOT images (30*30m) and field studies using GIS combining the different layers of information to identify factors affecting distribution and abundance of tsetse flies [28]. A number of consultancy reports produced by ERGO and TALA Research Group for FAO have used GIS to understand and predict land-use patterns, farming systems, agro-ecological zones, livestock geography and cattle densities in support of epidemiological studies [29,30,31,32]. The report "*towards identifying priority areas for tsetse control in East Africa*" presented a theoretical predictive method to identify priority areas based on low scale (7.6*7.6 km) imagery data [32]. At a higher resolution, Robinson describes a method to prioritise areas for the control of the Zambian tsetse belt [33]. He reported the participatory methods used in setting criteria for the selection of priority areas for tsetse control within a GIS environment [34].

However, lack of qualitative field-data, historical data and high-resolution data reduces the accuracy of any prioritisation exercise. For the present study a pilot area was selected in Ethiopia. Within the total tsetse belt of Ethiopia an extensive data set was available as part of a study to quantify the impact of tsetse control on farming [35]. These data sets have been used among others for the present study and as training set for the larger area selected in Ethiopia (Fig.1).

We decided to apply GIS and RS to study the valley specific relationships of all factors (host, vector, disease, human population/activity) as well as the valley specific geography and its environment and farming systems to answer the following questions:

- Where does trypanosomosis have a negative effect on (agricultural) development?
- In which areas will control measures have the highest impact/economical benefit?

These findings will be extrapolated to a larger geographical region to select priority areas for tsetse control in Ethiopia as a whole.

1.3. Pilot study in Ethiopia

For this study, Ethiopia has been selected as a pilot country. The main reasons to select Ethiopia were the availability and accessibility of quantitative and qualitative data and the enormous impact of the tsetse belts on the livelihood of the Ethiopian population and economy. It is estimated that the present tsetse fly distribution in Ethiopia threatens 92000 km^2 of productive farmland, denies Ethiopia several hundreds of thousands of hectares of agricultural production, threatens the livelihood of 5 million people and moreover places 4.5 million head of cattle at risk [36]. Secondly, as part of the regional programme "SIT (Sterile Insect Technique) for tsetse and trypanosomosis management in Africa" the IAEA is supporting the national Southern Rift Valley tsetse fly control & eradication project, located at the southern boundary of "*the window*" (Fig. 1).

The spread of tsetse in Ethiopia is not only climate dependent but also strongly related to the geomorphology and geography of the country and is characterised by the lower valley floors providing a favourable all year round breeding area for the tsetse flies with seasonal dispersal into the intensively cultivated and densely stocked and populated highlands in (South)-western Ethiopia. Low temperatures dictate the limit of the tsetse distribution along the western escarpment whilst other environmental constraints (e.g. humidity, vegetation) define barriers preventing the expansion of the

tsetse belt into Northern, Southern or Eastern parts of the country; these limits were used to identify "*the window*" definitions for our study (Fig. 1).

The average altitude limit for the tsetse species present in "*the window*", *G. pallidipes*, *G. fuscipes*, *G. tachinoides* and *G. morsitans submorsitans*, ranges from 1550-1650 m.a.s.l. (meters above sea level) depending on the species and it is unusual to find tsetse above this limit (as reported in 1976 [37]). Areas above 1900 m.a.s.l. are reported to be free of tsetse, decreasing with the distance away from the Equator [37]. Nevertheless recent reports showed an increased number of flies in the so-called intermediate zone (1600-1900 m.a.s.l.)[38]. In between 1700-2000 m.a.s.l. fly catches decrease by two to three orders of magnitude (10-100 flies/trap/day at valley floor) and at 2000 m.a.s.l. flies are rarely encountered. Flies dispersing into even higher altitudes are local exceptions but have been recorded [39].

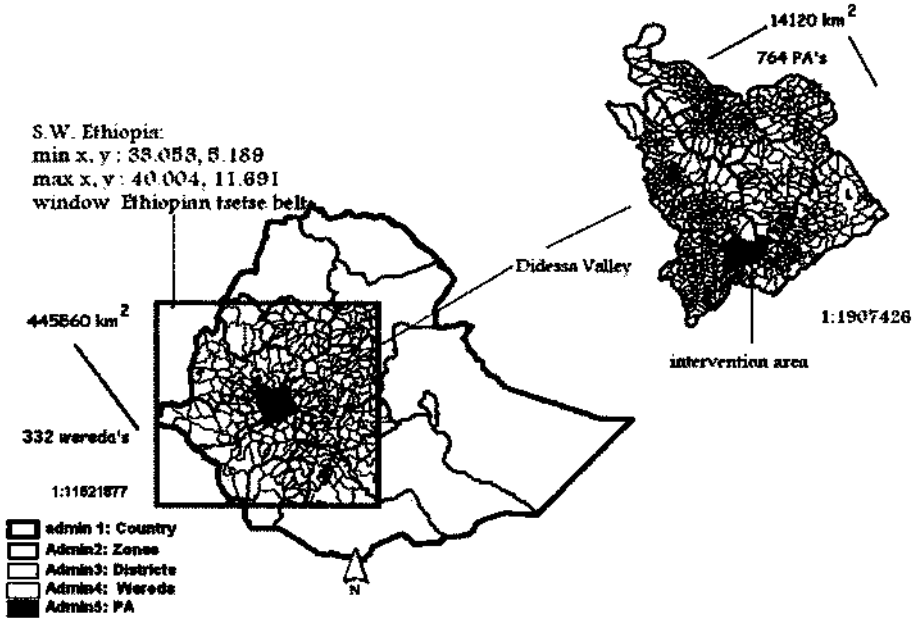


FIG.1.Window ("the window") defining the limits of the Ethiopian tsetse belt and the administrative organisation used for data collection, including the study area: the Didessa Valley.

2. MATERIALS AND METHODS

2.1. Study area

A study area has been selected where extensive data at high resolution were available, the Didessa valley. The tsetse invasion of the Upper Didessa valley was recorded during the early 70's [36]. Prior to the tsetse invasion habitats extended down to the valley floor at 1400 m.a.s.l., land-use was related to topography and livestock provided draught power, milk, skins and manure for the kitchen gardens [40]. The main impact of the tsetse invasion in the Didessa valley was the almost complete loss of all livestock types (80%), the abandonment of homesteads, a re-invasion of scrub-woodlands into the previously cultivated areas and agriculture based on hand cultivation in stead of oxen, resulting in less farm income and a reduction in nutritional status of the human population [36].

Two species of Glossina occur in the Didessa Valley, *Glossina morsitans submorsitans* and *Glossina tachinoides*. The more widespread *G. m. submorsitans* is found in the woodlands on the valley floor and to a lesser extent, along the sides of the valley. However during the dry season (December, January) the fly seeks its shade amongst the vegetation on the drainage-lines and,

therefore, moves into higher altitudes. *G. tachinoides* is less dynamic and can be found in the more dense vegetation along the main river, the Didessa (Fig.2).

Information gathered from farmers (during a field-trip in September 1999) indicated that flies were spreading across the watershed into the adjacent drainage systems and spreading into higher altitude areas. As a consequence farming areas in up- and highlands are progressively being infested by tsetse. At the same time there is pressure due to overpopulation in the tsetse free highlands to extend farming into lower altitudes thus encroaching upon the seasonal and occasional tsetse infested areas. Due to the dynamic tsetse distribution and human population pressures, livestock is repeatedly forced into contact with tsetse.

Reports and articles on the control programmes starting in 1986 (see Fig.1 for the control area, the darker area within the Didessa valley) and data collected for the FAO consultancy report [35] provided information at the lowest administrative level of Ethiopia-, that of Peasant Associations (PA). A complicating factor were changes in names and boundaries of the PA's that took place since 1986 and the lack of geo-referenced data. All data have been transferred into the latest administrative system and nomenclature. The current 764 PA's of the Didessa valley will be used as a training set for Ethiopia and be compared with available data sets at wereda (332) level for Ethiopia, representing the tsetse infested areas (Fig.1). In combination with 1 km resolution satellite imagery data [TALA Research Group, via ERGO, Oxford] areas for intervention will be identified.

2.2. Data

2.2.1. Multi-level approach of tabular data

All geo-referenced data have been transformed into geodetic co-ordinates (longitude/latitude) with reference units in decimal degrees. Data have been collected and transformed into the latest administrative level then analysed at three different levels (Fig.1):

(a) South Western Ethiopia ("*the window*"), the window specifying the Ethiopian tsetse belt. Data have been collected [41,42,43,44] at *wereda* level for 1986 and 1994. The database consists of 332 data-sets for the layers human population & livestock distribution, specifically of cattle, small ruminants and equines. Data layers of soil, climatic climax vegetation (the vegetation that would develop in absence of human influence and reflects the optimal vegetation of an area as determined by environmental conditions only), and land use have been digitised from the Ethiopian Atlas [45]. The FAO/UNESCO Soil Map (resolution 5'*5')[46] and data of FAO's Crop Production System Zone Database (CPSZ) Viewer [47] have been used as comparison and a 25 rain-station data-base have been compiled from data of the *African Data Dissemination Service* [48] using RAINMAN and WINDISP [49] to support the satellite imagery.

(b) Didessa Valley, study area for which high resolution data were available. Data have been collected at *Peasant Association* (PA) level (Admin. 5; the smallest administrative unit in Ethiopia). The database consists of 764 data-sets for the layers human population, household size, livestock distribution (sub-divided in cattle (oxen, calves), small ruminants (goats and sheep) and equines (horses, mules and donkeys) and poultry) crop production & cultivated area. The digitised data layers of the Ethiopian Atlas have been windowed from "*the window*" and rain data have been collected from nine rainfall stations in the Didessa Valley. A library of 36 geo-referenced valley vegetation photographs has been created to support the NDVI imagery interpretations. Secondly, a raster data layer of land-use [35] has been used.

(c) Intervention area in the Didessa Valley. Data have been collected at PA level (disease database) and sub-divided into pre-intervention period (<<1988), intervention period (1988-1994) and post intervention period (>>1994). The disease database consists of 29 data-sets only (number of PA's in the intervention area) [35].

2.2.2. Satellite Imagery

Satellite images [produced by TALA Research Group Oxford] have been supplied by ERGO, Oxford to support the environmental analysis. Decadal (10 days intervals) and monthly images of 1992,1993-1995 for band 6 (Normalised Difference Vegetation Index, NDVI), band 4 (temperature,

°K), monthly channel 3 (Cold Cloud Duration, CCD) and a digital elevation model (DEM) have been re-sampled and converted for "*the window*" at 1 km resolution in geodetic co-ordinates in decimal degrees, with columns/rows specifications for "*the window*" and the Didessa Valley of 770/723 and 168/184 respectively. Fourier processed [50](- a technique to achieve data reduction of time series-produced by TALA Research Group, Oxford) images have been used to study the typical vegetation, temperature & rain patterns, seasonality and growing seasons. Secondly, the environmental data-sets have been used as prediction variables and to assist in identifying areas climatically suitable for tsetse distribution (Figs 4 and 5) and for agriculture.

2.3. Developing the decision rule & data analysis

Three analysis methods have been used to develop the decision rule. Valley specific interactions have been studied with a special attention to the altitude factor, using linear regression techniques and overlay functions. The second analysis has been a multi criteria evaluation technique (MCE) using a weighted linear combination based on a set of relative weights for a group of factors [51] to study areas of agricultural suitability of the Didessa valley to define a training set for "*the window*". The third method has been developed to study the disease component and analyses the climatically suitability for trypanosomosis. A logical expression has been used to combine the results of the three analysis methods and a constraint map to develop the decision rule for the selection of priority areas for tsetse control whereby agricultural development and impact have been taken into account.

2.3.1. Valley specific interactions

The first stipulation to select areas for intervention has been identifying those areas where tsetse occurs. To identify areas at risk a Boolean tsetse distribution map (produced by ERGO & TALA Research Group, Oxford, updated [37]) has been produced as an overlay on the 1 km resolution DEM to identify the lower boundary for altitude classification where after the DEM was classified into four major tsetse distribution zones (Slingenbergh) (Figs 4 and 5):

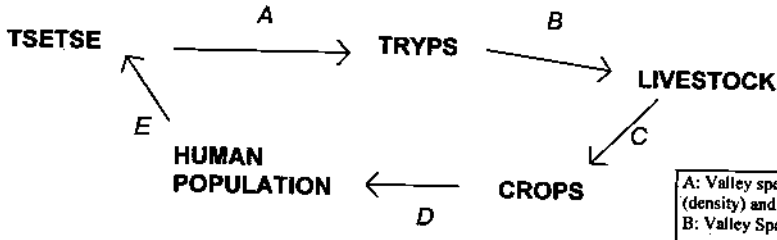
1. Tsetse infested area or tsetse breeding area; this is the valley floor, (continuously infested) and ranges up to 1600 m.a.s.l.
2. Seasonal infested area; uplands where only seasonally tsetse flies are caught. This area ranges from 1600-1800 m.a.s.l.
3. Occasionally infested areas, in these up- and highland areas occasionally a tsetse fly has been recorded. The altitude class for this category ranges from 1800-2000 m.a.s.l.
4. Tsetse free area, highlands above 2000 m.a.s.l.

The DEM has been ground-truthed with a random 47 point data-set for the Didessa Valley and corrected. The corrections have been extrapolated to "*the window*".

The areas which have been identified to support a first priority selection are those areas where control measures will have an immediate economic impact; the areas where control measures can prevent tsetse spreading upwards and areas where tsetse can be restricted to a minimum altitude level (54% of the total valley, 20 % of "*the window*") The permanently infested areas (13% of the total valley, 33% of "*the window*"), have been identified as areas where control measures will not have an immediate economic impact and set to 0, and is therefore excluded from the decision rule to identify control areas.

The valley specific interactions of the factors (Fig.6) have been studied with a linear regression analysis technique using the value-files of the Didessa valley and the intervention area (disease data) for the three different time periods (where data were available):

- pre-intervention period
- intervention period
- post-intervention period



A: Valley specific relation between tsetse presence (density) and trypanosomosis (PCV)
B: Valley Specific relation between trypanosomosis (PCV) and livestock (no/km^2)
C: Valley Specific relation between livestock (no/km^2) and cultivated land (ha)
D: Valley Specific relation between cultivated land (ha0 and human population (no/km^2)
E: Valley specific relation between human population (no/km^2) and tsetse presence (density)

FIG.6. Graphic representation of the valley specific interactions and relational flow of important factors in disease distribution.

Hypotheses

It is assumed that as the number of tsetse flies increases, trypanosomosis presence will increase. As a result of an increase of trypanosomosis presence the density of livestock (and thus oxen) will. Oxen are the main source for land cultivation, resulting in a decreased cultivated area. Less cultivation opportunities will result in migration of people. At this stage tsetse flies have an opportunity to expand their habitat and numbers of flies will increase.

3. RESULTS

3.1. Valley specific interactions

Present farming systems for the Didessa Valley and "*the window*" have been studied based on the 764 data-sets of the Didessa Valley and the 332 data-sets (1986 and 1994) of "*the window*". Data-layers of human population (no/km^2), households (no./km^2), livestock distribution/species (no./km^2) (Fig.7 and Fig.8), land-use pattern (ha. under cultivation) and land suitability (suitability classes) have been combined and, in relation with the DEM, four main Land Utilisation Patterns (L.U.P.) have been identified (Slingenbergh):

1. Lowland areas largely devoid of man and livestock << 1600 m.a.s.l.
2. Uplands with medium intensity mixed farming interspersed with under utilised land areas.1600-1800 m.a.s.l.
3. Uplands with intensive mixed farming 1800-2000 m.a.s.l.
4. Densely occupied Highland areas with highly exploitative farming systems > 2000 m.a.s.l.

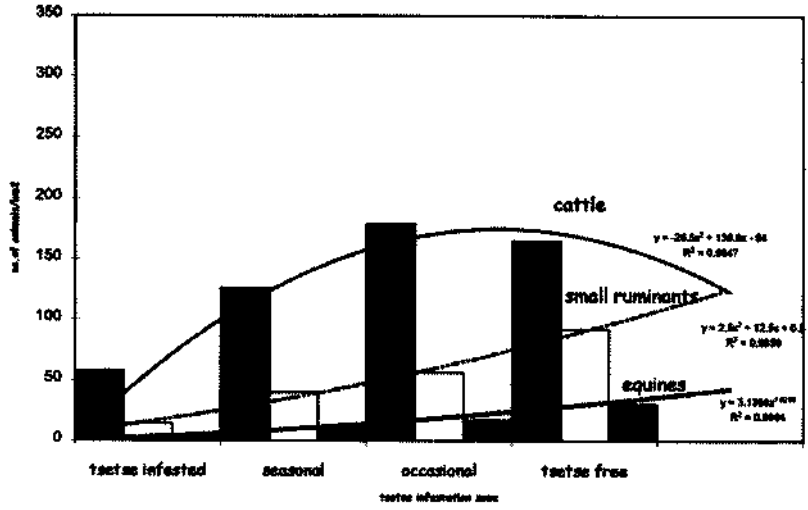


FIG.7. Livestock distribution per altitude related tsetse infestation zone in 1984

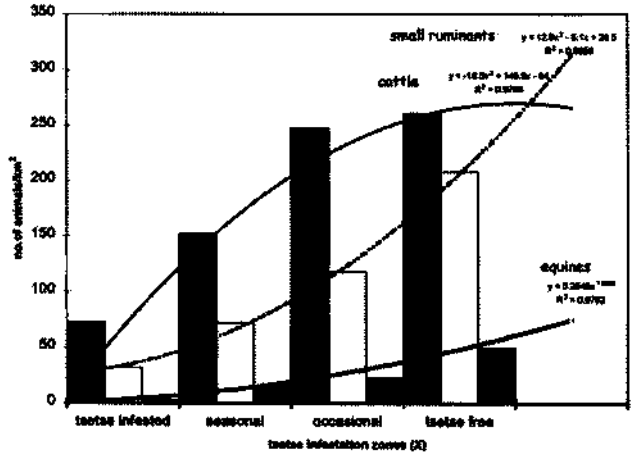


FIG.8. Livestock distribution per altitude related tsetse infestation zone in 1996

Tsetse distribution in Ethiopia is strongly related to altitude. Population and livestock distribution (Figs 7 and 8) are strongly related to the tsetse distributions. As altitude increases, the tsetse density decreases (and thus the risk of trypanosomosis), and livestock population increases. Only cattle densities are levelling off at higher altitudes. This can be an indication of lack of grazing land and/or high land pressures at higher altitudes. The steepness of the line per species is also an indication of the susceptibility of the species. The steeper the line towards higher altitudes (tsetse free areas) the more susceptible the species are for trypanosomosis. Equines are more susceptible to trypanosomosis than small ruminants whilst small ruminants are more susceptible than cattle [2]. The population distribution maps of 1980 and 1990 have been compared. These maps (Figs 9 and 10) indicate a high increase (%) of population pressure in the highlands, and an expansion of the capital.

The valley specific interactions of the described factors in Fig.6 have been studied using the linear regression module of IDRISI, with a confidential level of 95%, based on the value files of the Didessa valley and the intervention area (disease data) for the three different time periods of pre-intervention, intervention period and post intervention. The resulting correlation coefficients are shown in Table I:

TABLE I. RESULT OF A LINEAR REGRESSION OF DEPENDENT AND INDEPENDENT VARIABLES OF THE DIDESSA VALLEY DURING PRE-, INTERVENTION AND POST INTERVENTION PERIOD

	DISEASE						CULTIVATION						HOUSEHOLD	
period	*pre*		*intervent.*		*post*		*pre*		*intervent.*		*post*		*post*	
	r	n	r	n	r	n	r	n	r	n	r	n	r	n
livestock	X	X	0.187	28	-0.166	28	0.4924	48	0.703	28	0.123	319	0.744	758
cattle	X	X	0.381	28	-0.04	28	0.5034	48	0.756	28	0.302	319	0.656	647
oxen	X	X	0.378	28	-0.06	28	0.5774	48	0.778	28	0.499	319	0.676	572
equine	X	X	0.265	28	-0.254	28	0.4357	48	0.554	28	0.225	319	X	X
small rumin	X	X	0.148	28	-0.222	28	0.4556	48	0.289	28	0.007	319	0.769	597
household	X	X	0.109	28	-0.026	28	0.516	48	0.4128	28	0.135	319	X	X
tsetse	X	X	0.783	7	0.3028	23	0.168	33	X	X	0.186	21	X	X

pre= pre-intervention period
intervent.= intervention period
post = post intervention period
r = correlation
n = number
x= no data
* cultivation in post intervention period = % cultivated of total PA area (km^2); cultivation in pre- and intervention period = % cultivated of cultivable area

Two more regressions have been done but are not specified in the table:
Oxen versus livestock (n=572) r = 0.863
Cultivation versus average altitude zone/pa (n= 319) r = 0.686

The specific valley interactions are not of high significance. It was almost impossible to compare the data-sets of pre-intervention period, mainly based on wereda data-sets (1984 database), the intervention period, consists only of 28 data-sets whilst the post-intervention period (1996) data-base consist of 319 data-sets (of the 764) valuable for cultivation. Disease data were scarce and inconsistent and vary from PCV, prevalence rates to number of trypanosome parasites per blood sample.

Nevertheless a strong relation was found between oxen and cultivated land for all the three periods and can be a good indication of the importance of oxen for cultivation. A less strong relation has been found in the post intervention period due to the different format of the data (in % cultivated of the total pa area, see * table 1) For tsetse versus disease a strong relation was found, but this data set was only based on n = 7. The final tsetse data-set consist of positives and negatives for tsetse presence.

3.2. Agricultural suitability

A series of pair wise comparisons of the relative importance of the factors has been developed and weights, where the sum is one, have been calculated. The calculated eigenvalue is a measure of the consistency of judgement whilst the eigenvector provides the priority ordering of the factors [51]. Factors have been weighted for:

1. soils [45]
2. slope (calculated from DEM)
3. length of growing period (lgp, a measure of agricultural potential, produced by ERGO & TALA Research Group, Oxford)
4. population pressure (Didessa data-set and [54])
5. livestock pressure (Didessa data-set)

All factors have been studied on suitability for agriculture [52] and standardized to a consistent numeric scale before comparison, whereby the mean and standard deviation of each cell/polygon has been calculated, then the mean has been subtracted from each cell and the result has been divided by

the standard deviation. The eigenvector of weights have been set as follows with a consistency ratio of 0.07 to develop the training set through the MCE:

Soils: 0.4678
Slope: 0.1091
LGP: 0.2855
Population: 0.0767
Livestock: 0.0609

The MCE on the factors and their weights resulted in a Boolean suitability map (suitable/not suitable). This map has been used as the training set for the Bayes Theorum analysis (IDRISI module); computing a posterior probability map for each class (suitable) of "*the window*" based on nine environmental variables (predictors), whereby:

$$p(h/e) = p(e/h)*p(h) / \sum_i p(eh_i)*p(h_i)$$

p(h/e) = the probability of the hypothesis being true given the evidence (posterior probability)
p(e/h) = the probability of finding that evidence given the hypothesis being true (derived from training set)
p(h) = the probability of the hypothesis being true regardless of the evidence (prior probability)

A signature for agricultural suitability has been made based on environmental variables (described below; predictors) to create the probability map. The results of the probability prediction (signature per parameter based on 55970 pixels for the predictors NDVImax (index) (standard deviation [sd]: 8.92), NDVImin (index) (sd: 4.69), Tmax (°C) (sd: 5.56), Tmin (°C) (sd: 5.28), Tmean (°C) (sd: 3.52), Rmax (mm) (sd: 6.30), Rmin (mm) (sd: 2.88), Rmean (mm), EVAPmean (mm) (sd: 4.16)) are shown in Fig12. The dark green areas correspond with areas with a high probability to be suitable for agriculture whilst the white areas are predicted not to be suitable for agriculture.

Table II shows the result of a comparison analysis where a variation has been made in the agricultural suitability of parameters. Soil suitability has been categorised into soils suitable for agriculture, soils suitable for agriculture with small inputs (e.g. manure, irrigation), whilst slope suitability has been separated into areas of no erosion control measures and small erosion control measures. The results in table II show the corresponding P-values of Fig.12.

TABLE II. COMPARISON OF VARIATION OF AGRICULTURAL SUITABILITY PARAMETERS WITH THE PROBABILITY MAP OF AGRICULTURAL SUITABILITY

	Soil + input	Slope suitable	Slope + input
Soil suitable	P = 0.65	XXXXXXXX	P = 0.6
Soil + input	XXXXXXXXXXXX	P = 0.65	P = 0.5
Slope suitable	P = 0.7	XXXXXXXXXXX	XXXXXXXXXXX
Slope + input	P = 0.5	XXXXXXXXXXX	XXXXXXXXXXX

P = probability of agricultural suitability
XXX = no data or no analysis

3.3. Disease risk

A Boolean disease training set based on 173 points has been developed. Data sets available on locations of number of trypanosome parasites, packed cell volume (pcv) data and prevalence rates have been combined and a 173 point data set has been developed for trypanosomosis presence. Data sets have been used from the Didessa Valley [35] and other literature resources [39,40,43].

A climate based probability disease risk map for trypanosomosis has been calculated based on the same method and environmental data-sets as for the agricultural analysis described in 3.2. The climatically suitability map has been multiplied by the Boolean tsetse presence map for Ethiopia (disease suitability * vector) to give a final disease risk map.

The predicted areas, climatically suitable for trypanosomosis, are shown in Fig.13 whilst the predicted disease risk areas of Ethiopia, where vector presence and disease suitability have been combined are shown in Fig.14. The dark red areas reflecting a high (90-100%) probability of being exposed to trypanosomes whilst the disease risk will be low in the light blue areas.

3.4. Malaria constraint

Malaria is one of the major life-threatening human diseases in Africa. Therefore, permanently malaria infested areas have been set as a constraint. A constraint, always Boolean in character, excludes areas from consideration as priority area for intervention. A data layer of the distribution of malaria transmission [53] based on climate suitability for stable malaria transmission has been scanned and reclassified for the Didessa Valley and "*the window*". Areas with P >>0.8 have been classified as areas not suitable for intervention (value 0) and used as constraint in the final selection of priority areas.

3.5. Priority areas

Priority areas for tsetse control in Ethiopia have been identified using the followed criteria:
Criterion 1: Areas should have agricultural potential and are suitable for agricultural expansion
Criterion 2: Areas should be suitable for fly suppression
Criterion 3: Areas should be at risk for trypanosomosis
Criterion 4: Areas should be suitable for settlement (using socio-economic data such as access to market and distance to roads).

The priority areas have been set using a logical expression:

[Disease Risk] ≥0.7 AND [Agricultural Suitability]≥0.5 AND [Land Pressure] ≠ High AND [Tsetse] = 2,3 (seasonal and occasional infested) AND[Malaria] = 1

Areas suitable for agriculture (criterion 1) have been identified and the probability of agricultural suitability has been set to P = 0.5. This value has been based on comparison analysis where a variation has been made in the suitability of parameters (Table II). The areas suitable for fly suppression (criterion 2) have been set as areas of seasonal and/or occasional infestation. The risk of the probability of being exposed to trypanosomosis (criterion 3) has been chosen to be at least 70%. Areas suitable for settlement (criterion 4) have been studied through the data-sets used to set L.U.P.'s and the UNEP population map [54] and reclassified into land pressure classes. Areas of high land pressure have been excluded from the priority areas. A distance map of 'distance to road' and 'distance to town' has been created to study the accessibility of the selected land pressure classes. All areas selected fell within a radius of 20 km from the nearest town (market) and/or within a radius of 5 km to the nearest road and therefore the accessibility has not been taken into account in the logical expression above. The malaria statement selects areas not permanently malaria infested (malaria = 1).

The result of this expression (Fig.15) shows the priority areas (purple) for tsetse control in Ethiopia.

3.6. Validation

All data layers will be ground-truthed and corrected with a 228 random sample set (minimum calculated control set) for "*the window*".

4. DISCUSSION

To identify areas where trypanosomosis has a negative effect on agricultural development, the first areas to look at are those areas suitable for agriculture and those areas that are tsetse infested (permanently, seasonally or occasionally). The identified farming systems and the figures (Figs 7 and 8) suggest a strong inverse link between tsetse infestation and population density since number of households are strongly correlated (r=0.744) with the livestock population.

The highest impact or economical benefit can be expected in those areas where control measures can suppress the fly population significantly and, at the same time, those areas suitable for agricultural expansion. The permanently tsetse infested have therefore been excluded as priority areas for tsetse control measures. To expand agricultural activities into these permanently infested areas, control measures will be less economically viable and other techniques such as SIT (eradication technique) can be considered.

The dynamics of tsetse as described by Slingenbergh [36] hinders agricultural development in many areas and makes control strategies more complex. Because tsetse is found more often at higher altitudes (above 1600m) (re-) invasion risk of control areas increases as well. The flies can bridge areas of less then 10 km. In Fig.16, areas at risk for fly-bridging have been identified. The bottleneck shown in Fig.16 has been confirmed through farmer interviews (1999) as a point where tsetse flies enter the valley. The tsetse bridge identified between the Ghibe and Didessa Valley helps to confirm an incidental Ghibe specific tsetse species found at the river basin in the Didessa Valley (Slingenbergh).

Bottleneck points or highland fly bridges should be identified and taken into account during the selection process. Malaria can be a severe constraint for agricultural development as people prefer the seasonally infested or malaria free areas for settlement and cultivation. The permanently malaria infested areas have therefore been considered as not suitable for intervention areas for tsetse control. The priority areas for tsetse control cover a wide area of the South Western Valley and therefore a priority selection should be made (by e.g. the donor, government or specialist) on the economic suitability (cost/benefit analysis) of the area selected. The following criteria can be used as a guideline for selection:

- Administrative organisation
- Accessibility
- Size of the enclosed area
- Method of control
- Number of beneficiaries (livestock and human population)
- Approach (e.g. valley approach)
- Available expertise

Fig.17 gives an example using only two criteria, size of the enclosed area and administrative organisation. With the IDRISI module GROUP, enclosed areas have been identified. The numbered squares shown in Fig. 17 are the prioritisation result of the grouping exercise, where number 1 has the highest priority and number 14 the lowest, based on the administrative organisation of Ethiopia (Fig.1).

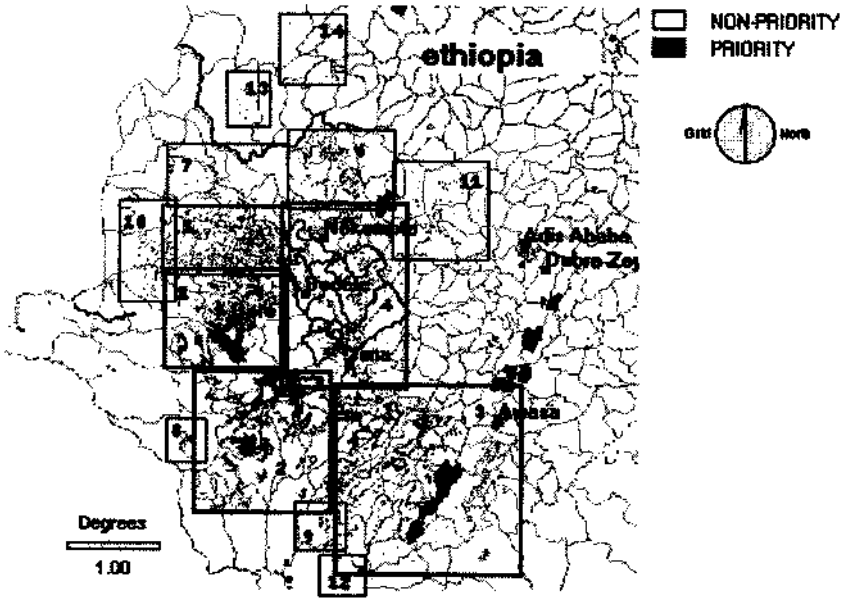


FIG. 17. Graphic representation of a possible method of priority selection of intervention areas

This model has been very specific for the Ethiopian situation. Tsetse flies have not been found in the dryer low-lands of Eastern and Northern parts of Ethiopia. Only the fertile part of Ethiopia, the mountainous areas with high ranges in environmental parameters has been infested and is therefore a typical altitude related tsetse problem. Extrapolation of this model to other geographical areas or countries will not be suitable. The signatures (training sets) developed are highly specific for the altitude related dynamic tsetse situation. Hendrickx [27] tried to extrapolate data of Togo (with a 100% training set) to neighbouring countries such as Burkina Faso but he also showed that the specificity of (micro-) climate, geography and e.g. cattle species, influence the reliability of the predictions.

ACKNOWLEDGEMENTS

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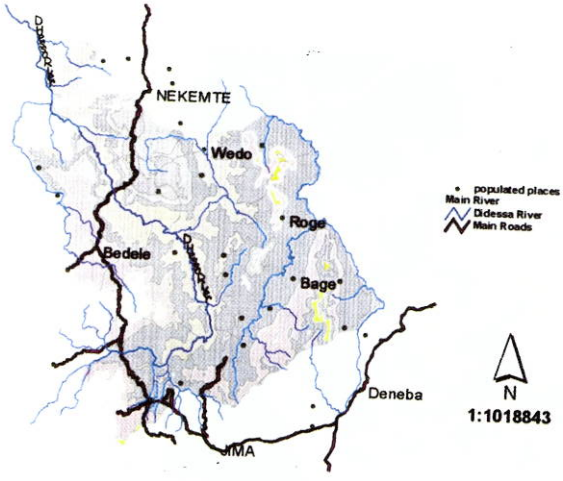

FIG.2. The Didessa Valley infrastructure; hypsography, drainage, main populated places & roads.

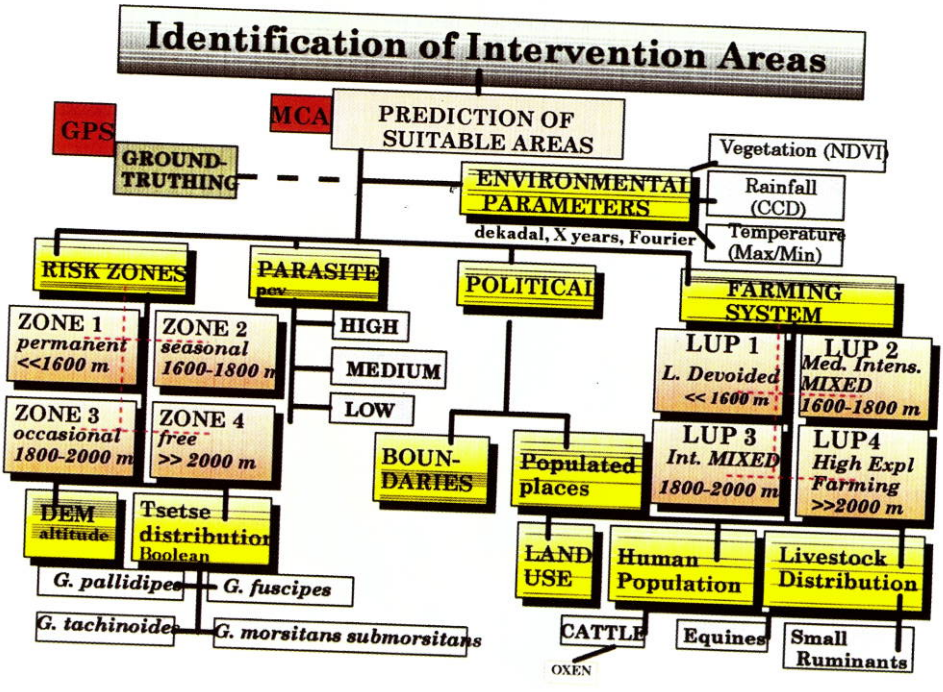

FIG. 3. Graphic representation of the geographical information system for the selection of intervention areas for the control of tsetse transmitted trypanosomosis. (GPS: Global Positioning system, MCA: Multi-Criteria Evaluation, DEM: Digital Elevation Model, LUP: Land Utilisation Patterns, NDVI: Normalised Difference Vegetation Index, CCD: Cold Cloud Duration, PCV: Packed Cell Volume)

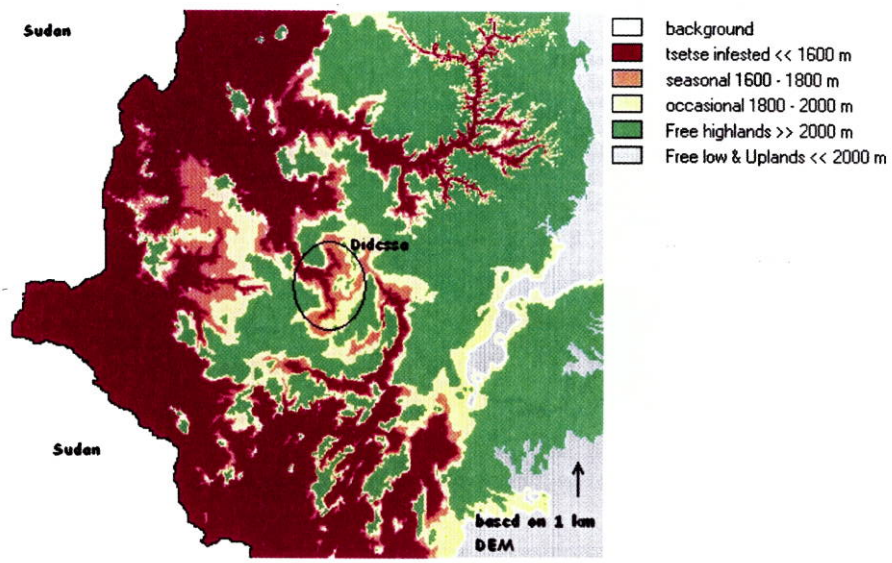

FIG. 4. Ethiopia: Tsetse distribution versus altitude reclassified into four main zones of tsetse presence

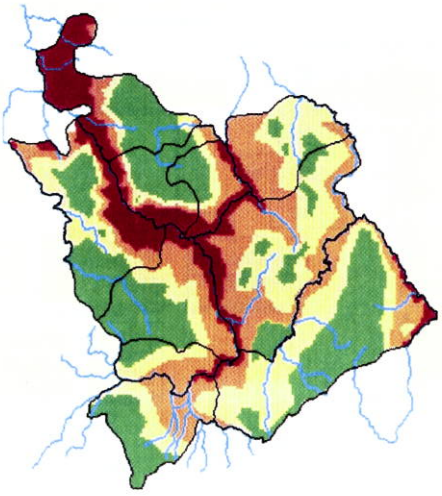

FIG. 5. Didessa Valley: Tsetse distribution versus altitude reclassified into four main zones of tsetse presence

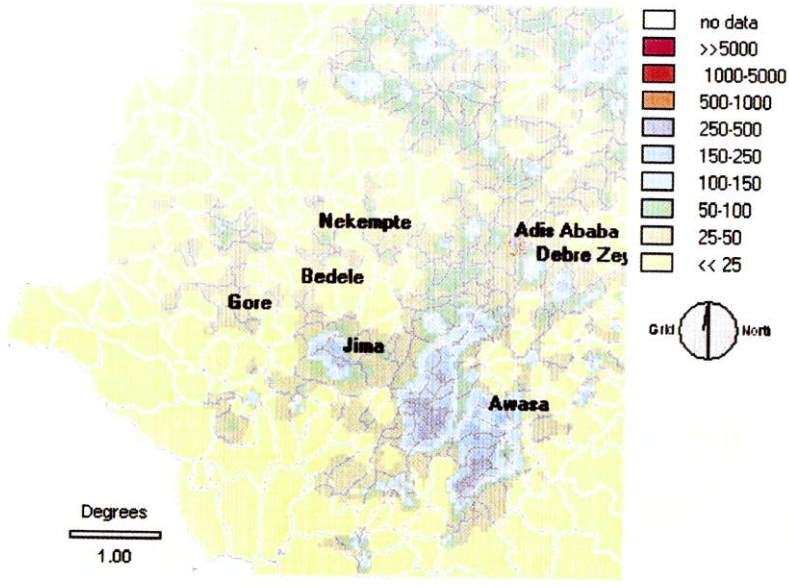

FIG. 9. Population density (pd) classes (no/km^2) 1980 of South-western Ethiopia [database:UNEP]

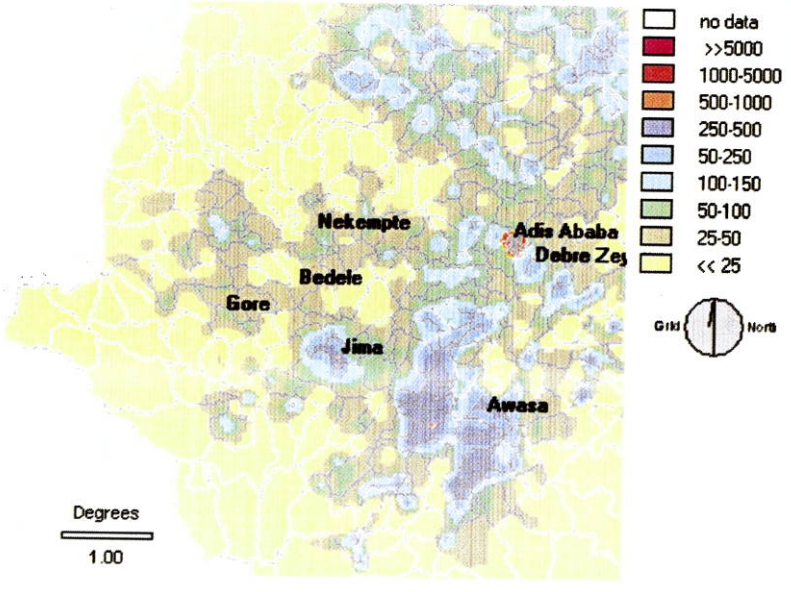

FIG. 10. Population density(pd) classes (no/km^2) 1990 of South-western Ethiopia [database:UNEP]

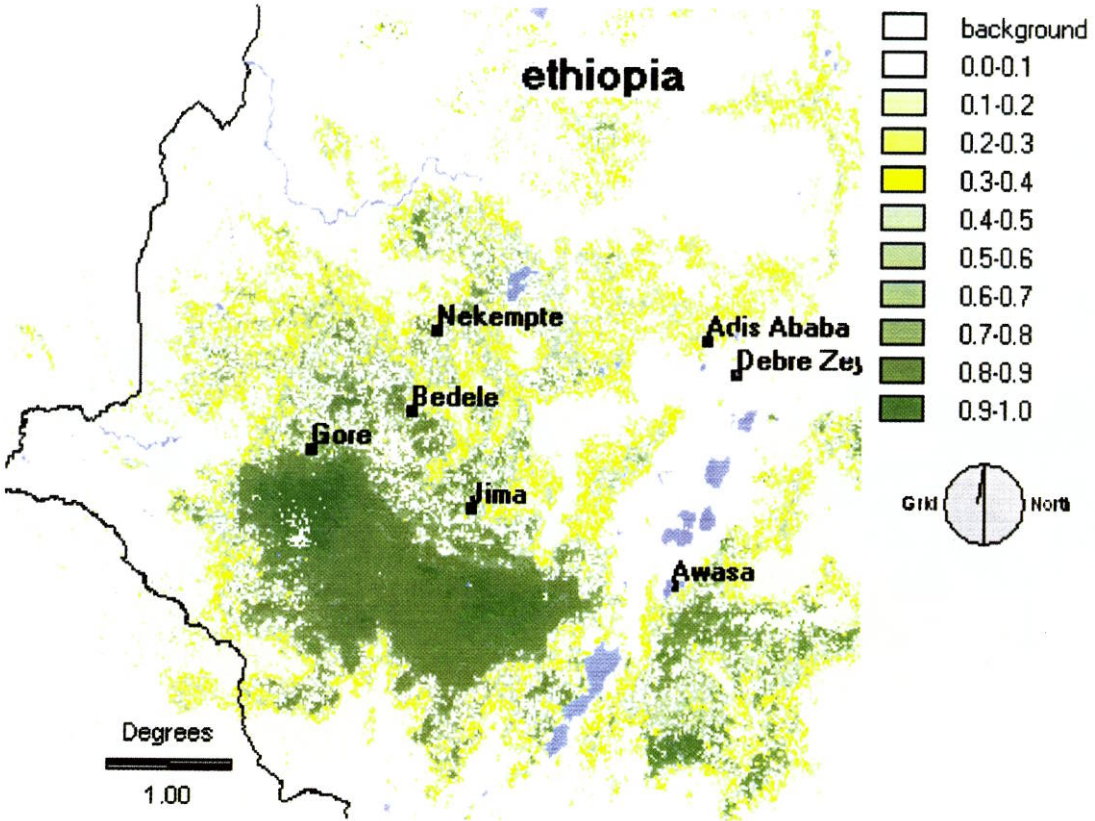

FIG. 12. Results of a probability analysis for agricultural suitability based on a 9 variables environmental prediction set.

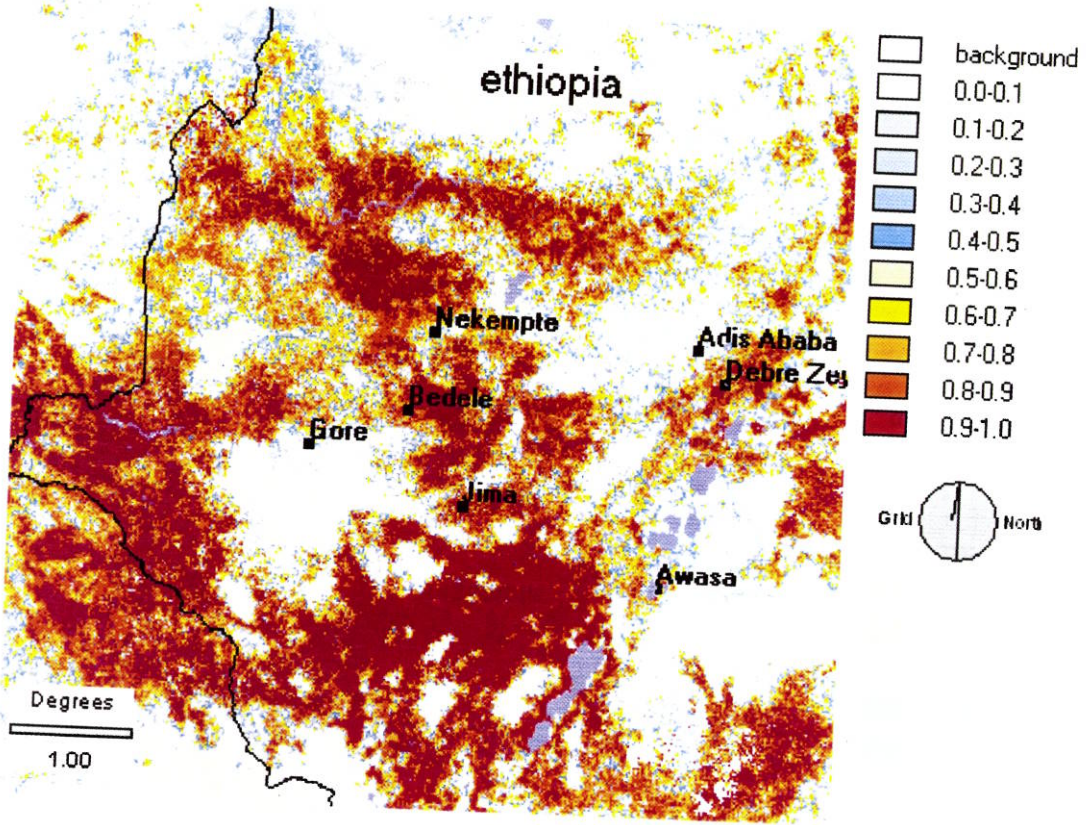

FIG.13. Results of a probability analysis for climatic disease suitability based on a 9 variables environmental prediction set

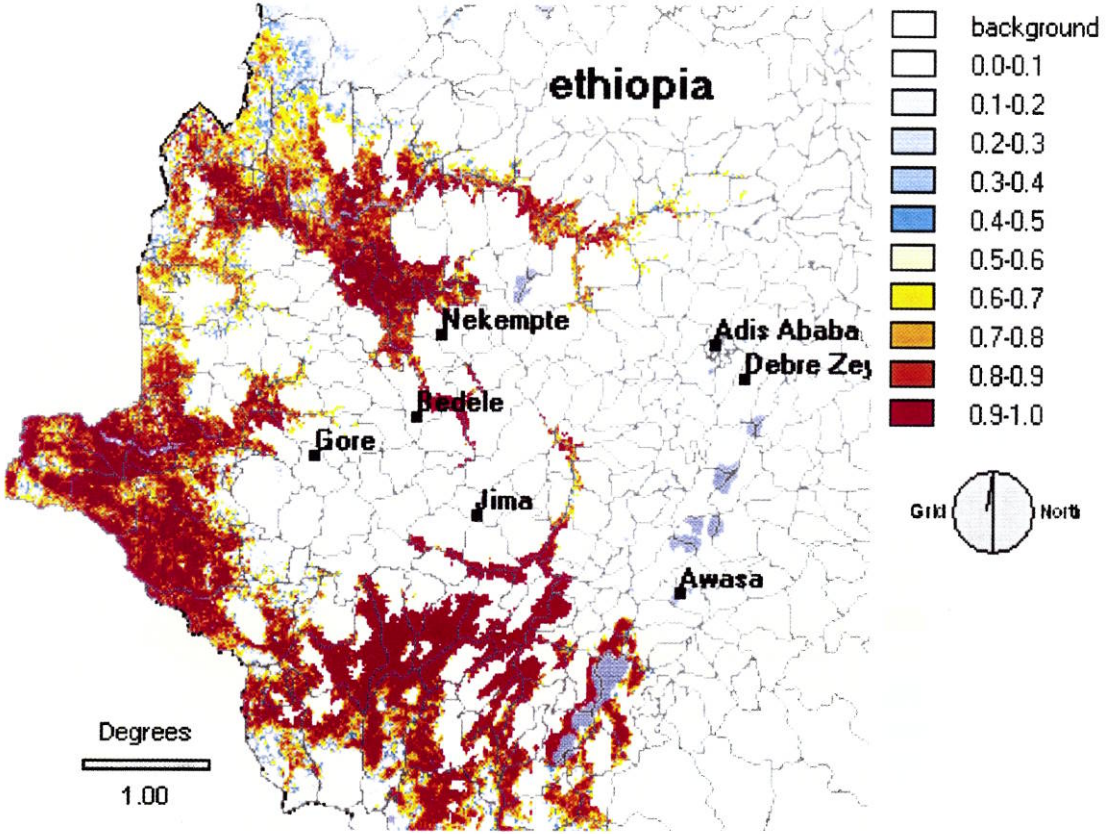

FIG.14. Trypanosomosis disease risk probability map, a combination of vector presence and climatic disease suitability

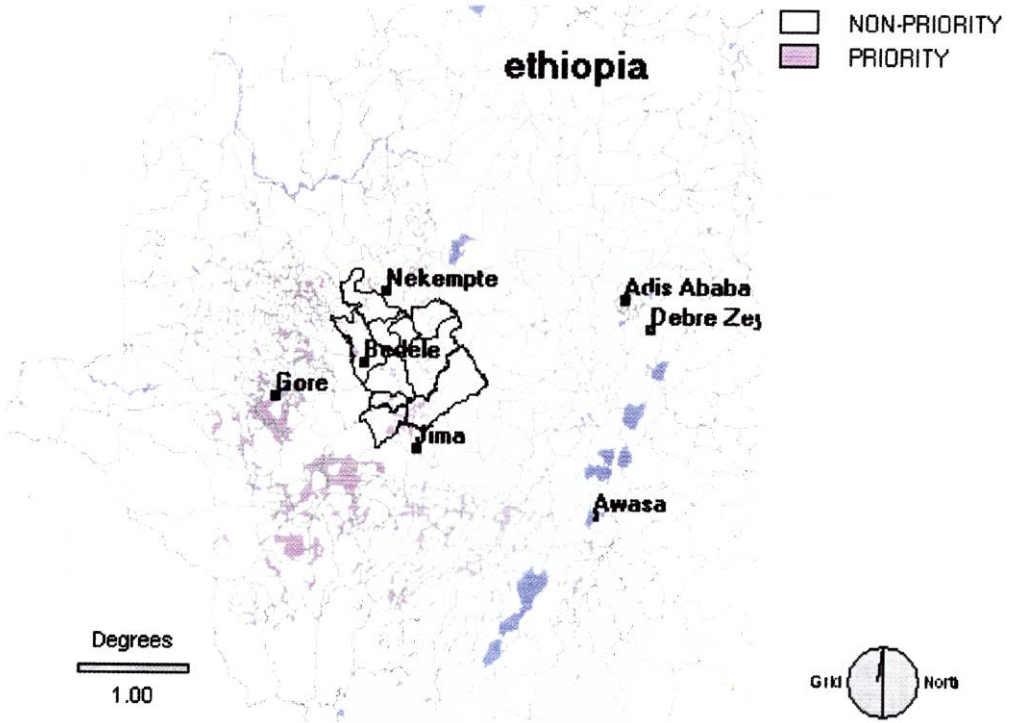

FIG. 15. Boolean presentation of priority areas for tsetse control, Ethiopia

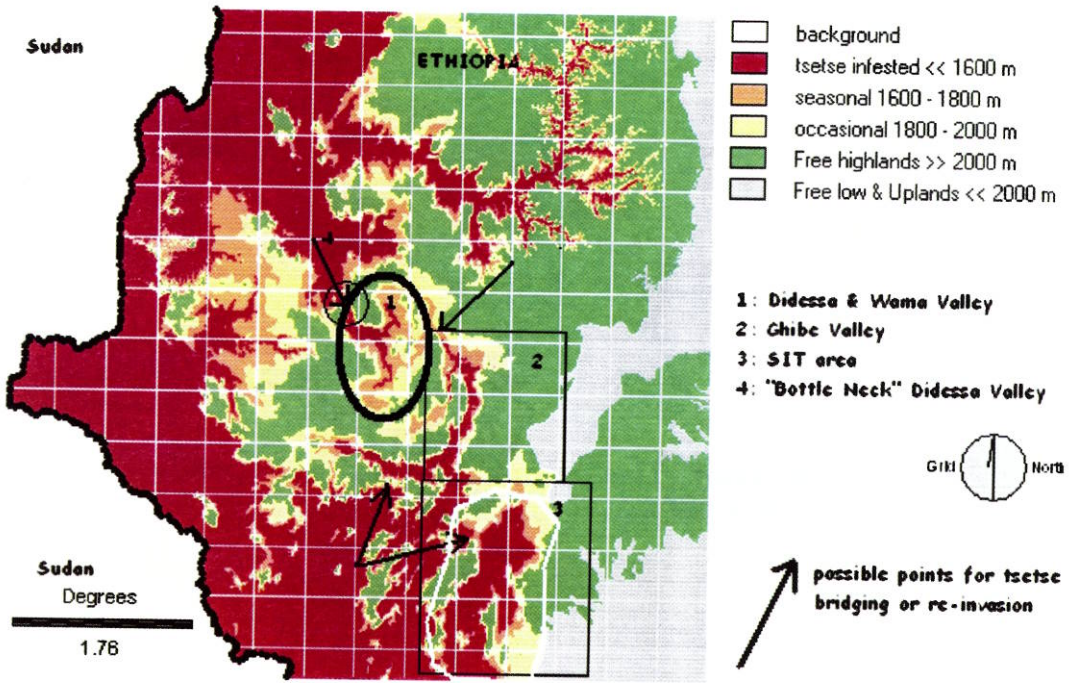

FIG. 16. Possible locations for tsetse fly bridging and re-invasion risk in Ethiopia

TRANSFER OF *TRYPANOSOMA* ELISA TECHNOLOGY TO ONDERSTEPOORT VETERINARY INSTITUTE, SOUTH AFRICA

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Abstract

TRANSFER OF *TRYPANOSOMA* ELISA TECHNOLOGY TO ONDERSTEPOORT VETERINARY INSTITUTE, SOUTH AFRICA.

Following a brief historical overview of trypanosomosis in South Africa, the present day role is explained of the Onderstepoort Veterinary Institute (OVI) in assisting disease diagnosis and surveillance. Production and distribution of ELISA kits will be initiated at OVI, initially for brucellosis and at a later stage for trypanosomosis.

1. BACKGROUND OF TRYPANOSOMOSIS IN SOUTH AFRICA

The early history of trypanosomosis (nagana) in cattle in KwaZulu-Natal dates back tothe 19th century when Sir David Bruce identified trypanosomes as the cause of the notorious "tsetse-fly disease" in northern KwaZulu-Natal (then Zululand). Both *Trypanosoma brucei* and *T. congolense* were found 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 harbored trypanosomes [1; 2]. Later Curson found in 1924 [cited in 3] another pathogenic trypanosome, *T. vivax*, in domestic animals in Zululand. The main vector was *Glossina pallidipes*, although *Glossina austeni* and *Glossina brevipalpis* also occurred in the area. 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 [3]. From 1952 - 1987 only isolated cases of nagana were reported and 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 a different habitat than the eradicated savanna tsetse flies, they will never become a serious economic problem [3]. However, human and animal populations have steadily increased, resulting in closer contact between cattle and the shaded thicket and riverine habitats of these flies and in 1990 bovine trypanosomosis re-emerged in this area [4].

An initial survey at the time, using thick and thin blood smears from 20 emaciated cattle selected at diptanks, revealed that 5-15 % of cattle in three districts (Ingwavuma, Ubombo and Hlabisa) were infected with either *T. congolense* and/or *T. vivax*. By the end of 1993 more than 115 000 cattle had been treated, costing nearly US$ 115 000, in treatment alone (Carter 1994, personal communication). The costs increase substantially when the cost of administration of the drug (equipment, travel, personnel, time, etc), change in dipping compound, etc, are added. Accurate data on production losses and actual mortality are not available but it 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 nagana problem seems to be confined to some 16 000 km^2 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 has also recently become involved to provide limited assistance under a technical cooperation programme for the long-term control/eradication of trypanosomosis in KwaZulu-Natal.

African animal trypanosomosis constrains agricultural production in areas that hold the continent's greatest potential for expanding agricultural production. It has been estimated that about 46 million cattle are at risk of contracting tsetse-transmitted trypanosomosis in an area of about 8.7 million km^2 in Africa.

An essential part of any trypanosomosis/tsetse control programme is the availability of good and reliable diagnostic techniques. The ELISA technique is most suitable for large scale surveys to characterize trypanosomosis risk areas and to monitor, over time, the impact of control programmes. In this respect the International Atomic Energy Agency (IAEA) has been instrumental in establishing a reliable, robust indirect ELISA technique for the detection of *Trypanosoma* antibodies in cattle. This

test has been evaluated in a number of laboratories in Africa with good results [5]. Since the Joint FAO/IAEA Co-ordinated Research Programme on using ELISA techniques for the diagnosis and control of trypanosomosis has reached its objectives, the sustainability of ELISA kit supply for the region will be guaranteed by the transfer to Onderstepoort Veterinary Institute (ARC-OVI) in South Africa of the production and distribution.

2. ARC-OVI AS CENTRE TO PROVIDE DIAGNOSTIC KITS

The successful commercialization of livestock production in South Africa has been made possible through the effective control of the unparalleled diversity of infectious diseases that confronts livestock producers in the region. If livestock production in the rest of the continent is to become similarly commercialized, much more attention need to be paid to the efficient control of these diseases. An essential part of such a control programme will be the ability to accurately diagnose these diseases.

Laboratory diagnosis and disease surveillance is dependent to a large extent on serological diagnosis and today the ELISA seems to be the predominant technique employed. The ARC-OVI has developed an array of these tests and are thus able to provide a comprehensive diagnostic service. However, thus far there has been little attempt to assist other African countries in this process and exploit the opportunities that are being created by international aid agencies assisting African countries with disease control.

ELISA technology has already been established in many African animal health laboratories mainly through the facilitation of IAEA as part of rinderpest eradication programmes. The result is that the ability to conduct ELISA testing is well established in most African countries. On the other hand, these countries mostly do not have the ability to develop "in-house" tests and, therefore, there is an opportunity for the ARC-OVI to commercialize the tests that have been developed for a range of infectious diseases by producing and marketing packaged ELISA kits.

Important diseases for which the ARC-OVI is in a position to produce such kits are bovine brucellosis, Newcastle disease, foot and mouth disease, African swine fever, African horse sickness, Rift Valley fever, rabies and contagious bovine pleuropneumonia (CBPP).

3. ROLE OF OVI IN THE PRODUCTION AND DISTRIBUTION OF KITS TO OTHER COUNTRIES IN THE REGION

It is envisaged that production of ELISA kits for the wide range of diseases affecting livestock in sub-Saharan Africa will be undertaken on a systematic basis. Due to the national importance of bovine brucellosis, the large local market and the results so far obtained with the ELISA validation exercise currently in progress, production of the *Brucella* kit was identified as the first stage of the project. Other kits will follow in due course and it is envisaged that the *Trypanosoma* ELISA will form part of a range of products offered to laboratories in the region.

4. ASPECTS OF *TRYPANOSOMA*-ELISA KIT PRODUCTION AND DISTRIBUTION

It is important that the ARC-OVI should not only provide diagnostic kits, but also provide a backup and trouble shooting service as is clear from past experience by the IAEA that this is an essential service to achieve acceptable diagnostic proficiency and to ensure that reliable and accurate results are obtained. It is, however, also crucial that the ARC-OVI, should still be able to rely on technical support from the IAEA in trouble shooting the *Trypanosoma* ELISA as well as for external quality assurance after transfer of the reagents.

Transfer of the technology has been initiated during a training period of one month of a scientist from ARC-OVI at the IAEA laboratories in Seibersdorf, Vienna. The details of antigen production, ELISA technology, kit assembly and troubleshooting were reviewed. It is envisaged that the transfer of the technology will be completed in the near future.

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COST-BENEFIT ANALYSIS OF THE INTRODUCTION OF ELISA FOR THE DIAGNOSIS OF ANIMAL TRYPANOSOMOSIS IN AFRICA

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Abstract

COST-BENEFIT ANALYSIS OF THE INTRODUCTION OF ELISA FOR THE DIAGNOSIS OF ANIMAL TRYPANOSOMOSIS IN AFRICA.

Socio-economic data was requested by questionnaires from researchers in 15 different National Agricultural Research Systems (NARS). The results of the survey were analysed and used for a socio-economic cost-benefit analysis, comparing the costs of 'diagnosis, treatments and drug-resistance' in the two alternatives 'with' ELISA and the 'without' situation. The major assumptions of the cost-scheme used are: 1) an increase in the occurrence of drug-resistance if nothing changes in the current practice of drug-use; 2) large scale diagnosis in test & treatment practice, combined with the use of pour-on's, would lead to the abolishment of the current practice of administering prophylactic drugs. In order for this to be a feasible option, the development and subsequent promotion of ag-ELISA and pour-on's is recommended. The first alternative, with BCT, has a slightly better cost-benefit ratio (1 : 53) than the second alternative, with ag-ELISA (1 : 44). However, the latter is still considered the only feasible option because of the applicability of pen-side ELISA on local level and the low cost allowing for cost-price savings. The budgetary restrictions for the use of BCT and its labour-intensiveness explain the relatively small amount of diagnoses in current practice.

1. INTRODUCTION

A socio-economic cost-benefit analysis was conducted to evaluate the efforts made by 'the project', i.e. a succession of research programmes as co-ordinated by the Joint FAO/IAEA Division and financed by the Directorate General of International Co-operation (DGIS) of the Netherlands, concerning further development and validation of ELISA, and improving the capability of African NARS laboratories to conduct these tests.

The practice of drug usage without previous diagnostic testing was an important consideration in this study. It is of great importance to tackle this problem in order to prevent further development of drug-resistance. Therefore, in the cost-benefit analysis potential changes of testing and drug-use were considered. For these changes to be a viable scenario, alternatives to prophylactic drug-use should be promoted and adopted on a large scale. This is already one of the objectives of many research programmes. For the further development of pour-on's and also pen-side ELISA tests (i.e. in the form of a dip-stick), an additional budget of US$ 5 million was put on the cost side of the cost-benefit analysis, together with the estimated total of local budgets of the NARS spent on trypanosomosis research. The savings in the costs of diagnoses, treatments and drug-resistance in the alternatives 'with' ELISA as compared to the 'without' situation, could be considered the 'benefit' arising from international research as co-ordinated by 'the project'.

2. MATERIALS AND METHODS

2.1. Questionnaires

A questionnaire was designed and dispatched to 15 researchers involved in the Co-ordinated Research Programme (CRP). Responses came from twelve out of fifteen countries. Some additional data were requested at a later stage from several Research Contract Holders (RCH's) and the project co-ordinator.

The questionnaires were analysed and the results are presented below. Subsequently, the data were used in the calculation of cost-schemes of the 'costs of diagnosis, treatments and drug-resistance' in the 'without' situation and in two 'with' alternatives, as described below.

2.2. Cost-benefit analyses

Only the costs of 'the project' could easily be summed up. The benefits are not as unambiguous, because the project beneficiaries are the NARS and indirectly the local veterinarians and livestock owners living in the tsetse infested areas of Africa.

The 'Socio-Economic' approach to Cost Benefit Analysis we used, is a refinement of the (National-) Economic approach which looks at a project from the viewpoint of a society as a whole, taking into account the interest of country's producers, consumers and the state [1, 2]. The socio-economic variant additionally considers that the utility of extra money is greater to someone with a low income than to someone with a high income. We expected the major *benefit* of 'the project' to be its contribution to changes in livestock-herd management and in the application of diagnoses and drugs by local farmers, hence augmenting their income, which justifies a socio-economic approach.

The 'costs of diagnosis, treatments and drug-resistance' were calculated for the 'without' situation, suggesting increasing occurrence of drug-resistance, and for two 'with' alternatives. Both 'with' alternatives suggest the use of antibody-detection ELISA (Ab-ELISA) at full scale in surveys and monitoring. For testing in 'test and treatment practice', in the first alternative the large-scale use of the buffy coat technique (BCT) was suggested, although the feasibility of this option was questioned, and in the second alternative the large-scale adoption of a pen-side antigen-detection ELISA (Ag-ELISA) was considered.

The time span we used was based on the confident anticipation that overall control techniques will be ready for implementation well within five years, and the assumption was made that within 10 (or 20) years the degree of political and civil stability will be sufficient to enable complete control [3]. We assumed that complete control would in place from year 11 onwards, and focused on the intermediate 10 years that demand critical attention, because of the current risk of drug-resistance. One of the major assumptions made under the 'with' alternatives is that enabling large scale diagnosis in test and treatment practice, combined with the large scale use of pour-on's, would lead to the abolishment of the current practice of administering prophylactic drugs. As the course of this process is as indefinite as the actual implementation of overall control after year 10, we have not bothered to make the levels of adoption of the different changes time dependent. Hence, the full benefit of the eventual 'with' alternatives, have been implemented from year 1. However, in the 'without' situation, we have followed a more conservative approach, the costs of increasing drug-resistance (mortality of cattle) are increasing to the assumed maximum of 25% in year 10.

The total 'costs of diagnoses, treatments and drug-resistance' of the two 'with' alternatives were compared. This can be seen as a cost-efficiency comparison between two alternatives to slow down the occurrence of drug-resistance in a time span presumed to be ten years, until complete control and eradication can be implemented. Furthermore, the *avoided losses* due to the 'with' alternatives were considered to be their *benefit*, and were compared with the cost of the research in the cost-benefit analysis.

3. RESULTS

3.1. Questionnaires

The data provided by the respondents on populations, regional herds, trypanosome infection rates and risk areas, was used as long as regional herds were specified and additional information on infection rates was provided. The data from three countries (Burkina-Faso, Cameroon and Kenya) was used to calculate the potential need for diagnosis in prevalence studies using models based on assumptions on future surveys and monitoring control and eradication operations in the 3 countries. The estimated average sample need 1.4% of the total susceptible cattle at risk, was used for all Africa, to obtain an estimated total need of 500.000 tests to access disease prevalence.

Information was gathered on local prices of trypanocidal drugs, on some aspects of the drug-use practice, such as the frequency of intervention by veterinarians, and on the signalled occurrence of drug resistance. On the basis of the RCH's estimates, the average prices for curative and prophylactic drugs were estimated for West & Central Africa and for East & Southern Africa. However, overall African average prices were later used in the cost-benefit analysis: respectively US$ 1,15 (curative

drugs) and US$ 1,95 (prophylactic drugs) per treatment. It was estimated that veterinary intervention was the case in 50% of drug-use practice and its cost at 20% of the cost of the drugs used.

On the basis of the data on the current tests-practised in some NARS laboratories, the average number of tests per laboratory was estimated at 7640 per month. This would mean that some 240.000 diagnoses would be practised if each tsetse-infected country presently had a similar veterinary laboratory. Furthermore, weighted averages of the country specific repartitions of diagnoses according to their purpose of use, suggested the following overall repartition: 46% of the test are used for prevalence studies, 26% in evaluation of control, 10% for decisions on how to treat and 9% for the evaluation of treatments.

The investigated price of the usual parasitological test protocol (with BCT) varies from US$ 0.20 to 1.65. But it was clear that the true cost-price were not known to the respondents, and the prices they gave were the prices 'as charged'. They might reflect the costs of the supplies used, but do not include any remuneration for the overhead cost.

The answers on questions concerning the direct and indirect influence of 'the project' were all laudatory. Some of the most mentioned influences are: the creation of awareness of disease importance; capacity building through provision of appropriate equipment; capacity building through training and the organisation of international meetings; enabling prevalence studies and monitoring of control. We investigated the contributions by 'the project' to the NARS institutions of the RCH's. Those consisted of three parts: A yearly financial contribution of about US$ 6000, equipment supplied and training. The RCH's perception of the training received was again laudatory. They were asked to estimate the replacement value of the skills they obtained and the skills they passed on to their personnel. With that, the total monetary replacement value of the training could be estimated at US$ 1.152.500. With the data on the 'equipment supplied' and the 'percentage-use for other purposes' we computed that the total monetary value of the equipment provided, in its usefulness to the NARS laboratories was US$ 471.054. The total financial contributions made in two succeeding project terms were some US$ 550.000. The yearly RC-contributions were on average 31% of an institute budget spent on trypanosomosis research. The portion of the total institutes budget spent on trypanosomosis research ranged from 1 to 40%, and on average is only 2.5%.

The respondents' perception of the use of antibody- and antigen-ELISA's was investigated. At the time of the questionnaire (autumn 1998), Ag-ELISA had been used in a process of test-validation and was successfully used in Ghana, Kenya and Uganda for screening and monitoring trypanosomosis control. The work on evaluation of Ab-ELISA was in progress in most of the countries. Promising results had already been recorded which pointed at the tests usefulness in epidemiological screening and surveillance, and in proving the tsetse-free status of areas after initial eradication. The most important problems encountered during the distribution of ELISA-kits were all related to local conditions, such as transportation and taxes.

About the potential future use of ELISA's, all but two institutes stated as their objective to use ELISA's routinely for diagnostic purposes. On the question whether ELISA's could eventually be replacing the parasitological test protocol, 7 out of 12 respondents agreed that because of their poor specificity the ELISA's could never be sufficient on their own, but because of their higher sensitivity they would complete the parasitological test protocol. Only 3 respondents thought that high levels of specificity and sensitivity could eventually be achieved.

Most of the respondents stressed the high cost of serology and the restrictions to its use due to laboratory requirements and a certain level of technical expertise required. One third of the respondents mentioned the need to simplify the ELISA test and/or the usefulness of a pen-side test. Considering the adoptability of a future pen-side test, it was stressed that such a test should detect current infections in order to be adopted. Hence, it should be an antigen-ELISA test.

The proposed reasonable market prices for pen-side test in order for them to be adopted are remarkably concurring with the true costs of a diagnosis with ELISA, as proposed by IAEA (US$ 0.33 – 0.70 for testing one sample twice). Based on the current practice the lowest prices were suggested in West-Africa: US$ 0.10 – 0.15 (Burkina-Faso), US$ 0.15 (Ivory Coast) and US$ 0.20 (Ghana). Gambia, Kenya, Uganda and Zambia all suggested US$ 0.50 to be a reasonable price, and Nigeria and Tanzania only stated an upper limit of about US$ 1.

3.2. Cost-benefit analysis

Both the *'with'* alternatives assumed the use of Ab-ELISA in surveys and monitoring. We computed that this need would be some 500.000 tests yearly in tsetse-infested Africa. The potential need of tests in 'test and treatment practice' would be much more important, we estimated it at 8.750.000 (BCT or Ag-ELISA) tests.

We used an estimate of 35 million trypanosomosis susceptible cattle in the tsetse-infested areas of Africa. The total number of treatments in tsetse areas is usually estimated at 30 or 35 million doses of trypanocidal drugs [4, 3]. We assumed that in the *'without'* situation 15% of these doses are used for curative treatments of cattle pre-selected by farmers-own-judgement, hence 5.250.000 doses. We valued these at the average price of curative drugs: US$ 1,15 (estimated from result of the questionnaire). Resulting in a total cost of some US$ 6 million spent on curative treatments. Eighty five percent of the estimated 35 million doses remain for prophylactic treatments. Assuming the practice of administering prophylactic drugs twice during the rainy season implies that only 42.5% of the 35 million cattle under challenge are given prophylactic treatments (twice). Hence 29.750.000 doses of prophylactic drug, valued at the estimated average price of prophylactic drugs: US$ 1,95 gives the total of US$ 58 million spent on prophylactic drugs in the initial *'without'* situation. We let the total cost of prophylactic treatments decrease every year inversely proportionate to the increase of drugs-resistance. We computed this with a one-year delay, considering that a logic reaction of farmers behaving rational to the perception that part of the prophylactic drugs they administered last year did not prevent their cattle from getting the disease.

The most rigorous assumption made in the *'without'* situation is that drug-resistance will increase by 2.5% yearly to a maximum of 25% ten years from now. Due to this, the cost of mortality of infected cow treated with ineffective curative drugs will increase considerable. Valued at US$ 100 per bovine, this cost increases from some US$ 9 million in year 1 to almost US$ 90 million in year 10. The indirect losses of the production of the lost cattle were not taken into account. In the *'with'* alternatives the direct losses due to mortality do not occur, thus they are 'avoided losses' which are benefits.

Furthermore, it was estimated that if the overall infection rate is 10%, in the *'with'* alternatives cattle owners would pre-select 15% of their herd to be tested for the decision on whether and how to treat. Later the 10% infected cattle would be tested again for evaluation of the treatment they received. Hence a total of 8.750.000 tests were needed, and they were valued at US$ 1.0 (BCT) or US$ 0.5 (Ag-ELISA) with an extra 20% of these prices reserved for the intervention by a veterinarian (even though with a pen-side test this might not be necessary). The total yearly cost of diagnosis in test and treatment practice in the two 'with' alternatives was respectively US$ 10.500.000 and US$ 5.250.000.

The total 'cost of diagnosis, treatments and drug-resistance' per year in the various scenarios were first discounted and subsequently summed-up. The sum-totals are given in Table 1. Furthermore two ratios are computed by dividing the total cost in the 'without'-change situation by the total cost in the various 'with'-ELISA alternatives. The first ratio expresses that if the practice suggested by the first alternative could be realised, the total actual costs and potential losses in the next ten years could be reduced with two thirds (1: 4,2). The second ratio expresses that if the second alternative could be realized, then only one ninth of these costs and losses would remain (1: 9,2).

The discounted totals in Table I can also be used to conduct a cost-effectiveness comparison of the two 'with' alternatives. The desired 'effect' would be: avoiding (or reducing) the risk of drug-resistance occurring in the presumed ten years until full control and eradication might be implemented. The cost-effectiveness ratio of 2.2 : 1 (or 197 / 90) confirms the simple fact that the second alternative, with the use of Ag-ELISA in test and treatment practice, is the cheapest option.

When the total cost from the 'with' alternatives, are deducted from the total cost in the 'without' situation, the figures that remain are 'avoided cost' or 'avoided losses', hence the benefit of the 'with' alternatives. The IAEA's contributions in the cost of 'this project' were known or calculated, the local budgets spent on trypanosomosis and ELISA research were estimated and added.

TABLE I. TOTAL COST OF DIAGNOSIS, TREATMENTS AND DRUG-RESISTANCE OVER A TEN YEAR PERIOD

	*Discount rate: 5%	(In US$)		Ratio's
Without	Sum total (ten years)	$	1.058.985.938	
	Sum of discounted total* (ten years)	$	834.878.935	
With alternative 1	Sum total (ten years)	$	243.300.000	
	Sum of discounted total* (ten years)	$	197.263.301	4,2 : 1
With alternative 2	Sum total (ten years)	$	112.080.188	
	Sum of discounted total* (ten years)	$	90.872.617	9,2 : 1

In Table II the benefits from the first 'with' alternative are compared to the total expenditures for 'this project'. But it would not be right to compare the benefits arising from the alternative with ag-ELISA, only with the development cost made until today. Because the Ag-ELISA has not yet been successfully developed into a pen-side test. Therefore we budgeted an extra US$ 5 million as the cost of development and promotion of Ag-ELISA (and pour-on's). Both resulting cost-benefit ratios: 1 : 53 and 1 : 44 are very promising. Even more because we started our calculations from very conservative assumptions.

TABLE II. COST-BENEFIT RATIOS

Comparing the 'gross benefit' of saved cost in the with antibody-detection ELISA alternatives to the direct-cost of the diagnosis research project: *Discount rate: 5%*

		Ratio's
Cost:		
Total expenditures research project (rounded)	$ 12.000.000	
Total expenditures + extra research on Ag-ELISA	$ 17.000.000	
Benefit:		
WITH alternative 1 Sum of 'benefit' (ten years)	$ 815.685.938	
Sum of discounted 'benefit' (ten years)	$ 637.615.633	1: 53
WITH alternative 2		
Sum of 'benefit' (ten years)	$ 946.905.750	
Sum of discounted 'benefit' (ten years)	$ 744.006.317	1: 44

We also conducted a 'sensitivity analysis' varying the main magnitudes surrounded by uncertainty, in order to study their impact on the outcome of the cost-benefit analysis. The variables that have the greatest impact on the results are: the assumed increasing level of drugs-resistance occurring and the price of cattle. We used three different options for the maximum level of drug-resistance: 10%, 25% and 50%. The price for cattle that we used first was an incontrovertible low estimate of US$ 100. We then increased it to US$150. Using this higher yet still reasonable price for cattle, boosted the ratio of the total cost of the 'without' over the 'with' ELISA alternatives. And even if we used the smallest estimate of 10% for the maximum level of drug-resistance in year 10, the cost-benefit ratio's were still very promising: respectively 1 : 37 and 1 : 33.

4. DISCUSSION

In the absence of this project, the current malpractice in drug-use will continue without change. Local livestock owners will be faced with increasing mortality of cattle when drug-treatments become ineffective. Furthermore, the NARS laboratories would miss an important incentive if the project was not contributing to their research through financial assistance, training and the provision of equipment. Without ELISA, the monitoring of ongoing control programmes would demand a considerable amount of labour and be more expensive. The efficiency gained and the costs saved in control by the introduction of ELISA, will contribute to diminishing the losses in overall agriculture due to trypanosomosis estimated at 4,5 billion annually [3]. However, one should avoid attributing too many (or too few) positive effects to a project. Hence, it is important to define the boundaries of the project. Therefore, we chose to limit the comparison to the more direct and quantifiable implications of changing diagnosis- and drug-use practice.

The cost-benefit considerations suggested that the costs made in the research and development of ELISA may be regarded as cost effective when compared to the potential *savings* in 'costs of diagnoses, treatments and drug-resistance'. Including the reduced costs of diagnoses and treatments of other domestic animals would strengthen the conclusions on the benefit of 'with'-ELISA alternatives as compared to the present situation.

The cost-benefit ratio computed for the first alternative, with ab-ELISA & BCT was very promising (1 : 53). Suggesting that, if it was possible to have BCT used on a large scale in test and treatment practice, it would be very worthwhile to promote. Unfortunately, this is not considered a feasible option. Considering the cost-benefit ratio (1 : 44) of the second alternative, with Ab-ELISA and Ag-ELISA, although being lower than that of the first 'hypothetical' alternative, it is still regarded as very promising. Therefore, the second alternative is recommended, i.e. the further development and promotion of Ag-ELISA, preferably in a dip-stick.

Furthermore, we considered two main aspects of changing diagnosis: the precision and the price. The precision of a test is not that important when just investigating the disease prevalence [5]. However, its sensitivity is important when the test is used in test and treatment situations. As large-scale use of BCT is not easily to realize, here lies a role for ag-ELISA. Due to institutional constraints the budgets of local laboratories are usually not so flexible, and form an economic constraint on the number of diagnoses performed. Because the true costs-price of ELISA is probably 1 to 5 times cheaper than BCT, the large-scale distribution of pen-side ag-ELISA could probably be achieved with maintaining the present level of local overhead-costs. And the cost-price advantage would be considerable due to the potential 8.750.000 tests demanded annually.

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