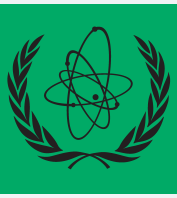
IAEA-TECDOC-1559

Developing Methodologies for the Use of Polymerase Chain Reaction in the Diagnosis and Monitoring of Trypanosomosis

Final Results of a Coordinated Research Project, 2001–2005

Prepared under the Framework of an RCA Project with the Technical Support of the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture



IAEA
International Atomic Energy Agency

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The originating Section of this publication in the IAEA was:

Animal Production and Health Section
Joint FAO/IAEA Division
International Atomic Energy Agency
Wagramer Strasse 5
P.O. Box 100
A-1400 Vienna, Austria

DEVELOPING METHODOLOGIES FOR THE USE OF
POLYMERASE CHAIN REACTION IN THE
DIAGNOSIS AND MONITORING OF TRYPANOSOMOSIS

IAEA, VIENNA, 2007
IAEA-TECDOC-1559
ISBN 978–92–0–105507–1
ISSN 1011–4289

Printed by the IAEA in Austria
June 2007

FOREWORD

The Animal Production and Health Section of the Joint FAO/IAEA Division in the IAEA has promoted the use of modern nuclear based techniques in diagnosis and control of livestock diseases for the past twenty years. Support for methods exploiting the polymerase chain reaction (PCR) began in 1997 with a coordinated research project (CRP) to develop PCR methods to study and diagnose a range of transboundary diseases affecting livestock. Trypanosomes produce a variety of diseases affecting both animals and man. The agents for the disease and immunology pathogen/host are very complex. Diagnosis of the disease has relied on more conventional methods such as direct assessment of organisms through a microscope and serological tests, such as complement fixation and haemagglutination, looking at the serum from animals to detect antibodies. The sensitivity and specificity of the tests have never proven ideal to allow either sufficiently low levels of organisms to be identified or to determine exactly which strain of Trypanosome were causing the disease.

The work described in this publication examins many features of the use of PCR in the detection of Trypanosomes. These include drug treatment of animals and man where it is imperative to understand whether an individual is infected at all, and to what extent that infection has progressed. The PCR offers a solution to the detection of organisms since theoretically it has an incredible sensitivity, since minute amounts of nucleic acid in samples can be amplified. The specificity of PCR also resides in the identification of absolutely specific parts of a genome and their detection. Theoretically the PCR offers the maximum diagnostic sensitivity and specificity profile. In practice there are many factors that affect the theoretical limits of the PCR. Sample taking, handling, extraction and processing all affect the sensitivity from field samples and reduce the diagnostic potential. The high specificity inherent in using specific probes means that the test is expensive where many probes have to be used for ultimate identification of a Trypanosome. Working protocols for the use of specific probes have been determined. The handling and extraction of samples has been optimized. The use of universal primers for the detection of all Trypanosomes has been examined with promising results. Validation of methods is paramount and this has been addressed. One of the key benefits from this CRP, as in all others, has been the cooperation generated between scientists from many countries. The links between more established laboratories with expertise and those just starting were forged and enabled accelerated development. The quality of work in all the laboratories has increased generally through the CRP. Such PCR-based tests will allow an unequivocal estimation of the affect of interventions in the eradication of Trypanosomal diseases, such as those involving the sterile insect technology (SIT) in the tsetse control programmes of the IAEA.

The officer responsible for compiling this publication was J.R. Crowther of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

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SUMMARY

INTRODUCTION

The coordinated research project (CRP) was initiated to try and improve the diagnosis of trypanosomoses using molecular based methods. A Coordinated Research Project provides funds to individuals (Research Contracts) to help research in a specific scientific area and usually lasts over 5 years. The themes for CRPs are set by peer review and look towards developing solutions to emerging problems. Most CRPs have between 8 and 12 Research Contract holders. Agreement Holder contracts are also awarded, cost free, to individuals with expertise to help the scientists in the CRP. Technical contracts are awarded where reagents and materials can be provided to members of the CRP. A major element in sustaining and reviewing work under the CRP is the Research Coordination Meeting (RCM, three of which are held in a 5 year cycle. Here, the research and agreement holders met with the technical officer from IAEA to present data and coordinate workplans.

The Joint FAO/IAEA Division has promoted the use of modern nuclear-based techniques in diagnosis and control of livestock diseases for over twenty years. Trypanosomes produce a variety of diseases in animals and man and diagnosis has relied on more conventional methods for detecting the parasites directly, such as microscopy, and serological tests to detect evidence of infection through measuring antibodies. Detection is vital for effective diagnosis as well as to measure the efficacy of control programs in general, such as those dealing with the eradication of vectors, e.g. sterile insect technologies. The sensitivity and specificity of such tests has always proved less than ideal to allow either sufficiently low levels of organisms to be identified or to determine exactly which strain is present. The polymerase chain reaction (PCR) offers a solution to these problems since it is theoretically extremely sensitive, where minute amounts of nucleic acid can be amplified; as well as being specificity defined through the characterisation and use of specific primer sets. The advantage of molecular methods, in particular the PCR technologies was realised in this field but there were many gaps. The CRP research was geared to using PCR to provide solutions to the diagnostic problems through the development and validation of new methods. This was successful in that the specificity and applicability of the the primers was established in many different countries. Problems were apparent the area of quality control of diagnostic tests using molecular methods. This is not uncommon in any field of diagnosis and the CRP was able to improve the perception and methodologies. The IAEA has been a leader in stimulating better laboratory practice and this is extended in this CRP. The crucial results of the CRP were the demonstration of the use of ribosomal internal transcribed spacer (ITS) primers for the detection of a wide range of Trypanosomes; the improvement in protocols and standardization of methods for specific primer set use; the development of a method to dispense with the need for separation gel systems to identify PCR products and the coordination of methodologies designed to achieve defined fitness of purpose. This was brought about by a set of scientists with some experience in molecular methods who related well and produced good results.

Research areas of the CRP

- The development of a standardized PCR to detect Trypanosomal DNA in blood samples.

- Modification of techniques to an easy-to-use, more sensitive, standardized format.
- Development of a "pan-Tryp" test to amplify DNA of all pathogenic Trypanosomes to assist decision makers in focusing and implementing appropriate disease control programmes.
- Evaluation of the PCR for the identification of sleeping sickness using human blood samples.
- Improvement of the monitoring of control/eradication programmes using a more sensitive diagnostic technique.

Major points from the research coordination meetings (RCMs) held in Antwerp and Rio de Janeiro respectively, are discussed below and the TECDOC presents data of relevance to these areas as summed in up the final RCM in Hanoi. The points made are important in the overall development of the PCR in diagnosis and in validating methods.

1. Role of PCR

It was agreed that clear ideas as to the role of the PCR must be worked out before development of tests to establish whether the PCR is in fact, the most useful and appropriate test to be used. Elements of the high relative cost, sample collection and storage, sensitivity, specificity, need for training, establishment of good laboratory facilities and contamination, should be considered in the context of what exactly is required in any study at the national level. These should be compared to what is already available in any country. It was generally agreed that the PCR should be a component of any testing and not aim to totally replace existing assays.

2. Methods and protocols

It was agreed that each PCR protocol (based on methods and primers used), should be written and that a standard format should be used. A protocol from Dr. Peter-Henning Clausen was examined in detail and additions made. The participants received copies of the protocols to act as guides. It was agreed that some basic background information should also be included with regard to reagent formulation.

3. DNA Bank

A reference bank of DNA was started at the FAO/IAEA laboratory, Seibersdorf. The DNA required was from Trypanosomes; control DNA from various livestock and insect species and DNA from relevant organisms likely to complicate the PCR diagnostic potential with regard to Trypansomoses. The strains selected were well characterized and reliable in terms of their pedigree. Certain participants prepared approximately one milligram of DNA and sent this to the laboratory. A typical withdrawal of approximately 200 ng was envisaged. The DNA was characterized with respect to the PCR products produced using standardized protocols through sequencing studies.

4. GLP

It was agreed that the setting up of a PCR laboratory was fundamental. Each laboratory examined closely their set up and reported deficiencies. A good laboratory practice (GLP) document for PCR was provided.

5. Primers

Extensive discussions on which primers could be used to obtain products for use in various aspects of the study of Trypanosomoses were made. These are reflected in the publications of the Research contract holders and Agreement holders.

6. Comparative testing and sensitivity/specificity

There was a continuous discussion on aspects of sensitivity and specificity of all tests including PCR. There was some confusion as to what exactly is being measured and compared between tests and protocols. There is a need to define sensitivity in terms of both analytical and diagnostic potential of the PCR. There are drawbacks to estimating analytical sensitivity from" spiked" samples by dilution of material. There is also a difficulty where field samples are examined and the effect of the matrix on PCR analysis.

7. Antibody assays (ELISA)

An indirect ELISA developed at Seibersdorf was used in the first phases of the CRP and kits supplied. This assay used pre-coated plates with either *T.vivax* or *T. congolense*, where the whole Trypanosomes were completely denatured. Other ELISA systems to detect antibodies against *T.evansi* were also reported.

8. PCR-ELISA

The need to develop such methods was discussed extensively up to the second RCM. It was concluded that there was no direct benefits from the approach in most laboratories and that the advantages of high through put were not really required.

Research Coordination Meetings

First RCM Antwerp, 26–30 March 2001.

Research contract Holders	
Bolivia	Civera
Brazil	Dávila
Burkina Faso	Sidibe
China	Xu
Côte d'Ivoire	Solano
Kenya	Masiga
South Africa	Viljoen
Thailand	Tuntasuvan
Uganda	Enyaru
Vietnam	Le Ngoc
Agreement Holders	
Belgium	Buscher
Germany	Clausen
Netherlands	te Pas
United Kingdom	Luckins
Others	
IAEA	Diallo
IAEA Technical officer	Crowther

Second RCM, Rio de Janeiro, Brazil, April 7–11 2003.

Research contract Holders	
Bolivia	Gonzales
Brazil	Dávila
Burkina Faso	Sidibe
Chile	Solari
Côte d'Ivoire	Solano
Kenya	Njiru
South Africa	Viljoen
Thailand	Tuntasuvan
Uganda	Enyaru
Vietnam	Nguyen
Agreement Holders	
Belgium	Claes
Germany	Clausen
Netherlands	te Pas
United Kingdom	Luckins

Third RCM, Hanoi, Vietnam, 20–24 June 2005.

Research contract Holders	
Bolivia	Gonzales
Burkina Faso	Sidibe
Côte d'Ivoire	Solano
Kenya	Kinyua
South Africa	Romito
Thailand	Nopporn
Uganda	Enyaru
Vietnam	Nguyen
Agreement Holders	
Belgium	Claes
Germany	Mebratu for Henning-Clausen
Netherlands	te Pas
United Kingdom	Luckins
Others	
IAEA Techncial officer	Crowther
Company-Belgium	Deborggraeve

Technical contacts awarded

(1) Characterization of ITS primers (sequencing) Brazil.

(2) Development of TRYPSTICKs (Belgium).

General outline of developments

(1) Contracts awarded late 2000.

(2) First RCM developed ideas (March 2001).

(3) Work on protocols for sample handling, DNA extraction using specific primers.

(4) Characterization and production of universal ITS primers (Technical contract).

(5) Use of universal ITS primers.

(6) Establishing final protocols for specific tests.

(7) Evaluation of ITS primers results for animal and human diagnosis.

(8) Development of specific TRYPSTICK tests for development of PCR without electrophoresis (Technical contract).

(9) Ring test for TRYPSTICK.

FINAL COMMENTS ON CRP

The proven ability of PCR amplification methods to provide large amounts of nucleic acid from minute quantities possible in well controlled samples, has led to the generalised opinion that PCR is the method of choice in all situations in providing the most sensitive tool available. Although the theortical limits of the PCR are high, the true performance of a PCR on field samples is often over estimated and poorly validated.

Laboratory based diagnosis is the process of confirming or clarifying clinical findings though examination of samples from the field. The ability of a test to diagnose as disease accurately (correctly) relies on the statistical basis of sampling, sample condition, handling of the sample before it enters a laboratory environment, the handling of a sample in the laboratory, the methods used to prepare a sample for testing and the test itself in terms of its analytical sensitivity (minimum detection limit for an analyte) and specificity (ability to detect only a specific analytical target). All diagnostic tests are affected by this process. The terms diagnostic sensitivity (DSn) and diagnostic specificity (DSp) refer to the performance of a test with regard to accurately measuring the true status of an animal with regard to the analyte examined from a field situation and with field samples.

The CRP confirmed that the PCR is particularly susceptible to problems DSn and DSp through variation in sample taking, handling and manipulation, as well as extraction of nucleic acid. Great precautions are necessary through the use of protocols that ensure the protection of nucleic acid at all stages; that allow a maximum yield of nucleic acid from samples and that are not affected by too great an amount of nucleic acid or the innate exquisite sensitivity of primers sets. Examples of this are shown in the papers of the CRP. What was also apparent was the need for fastidious management of PCR laboratories in terms of sample handling and amplification steps.

Work in the CRP greatly extended the understanding of the use of universal primers sets to allow diagnosis of Trypanosomes in general. Epidemiological studies have indicated that the PCR protocols examined can detect up to 85% of all Trypanosomes. The universal primers offer a relatively cheap option to diagnosis where they are successful in an area, particularly where large scale screening is needed, e.g. where interventions such as SIT and drug treatments, are made.

The IAEA is firmly committed to supporting PCR and a newer generation of technologies for PCR e.g. real time PCR systems, offer vastly improved methods for the

estimation and differentiation of pathogens. An example of this was given in the CRP. Such technologies will be exploited and more tailored to Member States needs in new CRPs developed to look at the problems of Rift Valley Fever and Contagious Bovine Pleuropneumonia. A great deal of work was made in the CRP looking at animal and human Trypanosomes and the papers reflect a greater understanding of diagnosis that will allow other researchers a much higher baseline to begin studies in future.

MOLECULAR DETECTION OF TRYPANOSOMA INFECTION: PCR AND PCR-ELISA TECHNIQUES

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Abstract

Fast, clear and easy diagnostic methods allow accurate detection of Trypanosomes in human and animal species enabling cost effective treatment and prevention of outbreaks within an epidemic. Diagnostic methods should preferably be usable in the field. Presently many diagnostic field methods are time consuming and their limit for detection of parasites should be decreased. Molecular methods offer the promise of detecting lower numbers of parasites, faster. One focus of this RCM was to develop fast and easy diagnostic methods detecting lower infection levels. The original ideas involved PCR-ELISA and based on our previous experience, we confirm that PCR-ELISA is indeed a powerful method able to detect minor quantities of target DNA, i.e. very low parasite copy numbers, down to a single parasite. We also point out that the method has several pitfalls and should therefore be applied with great care. During the first research coordination meeting in Belgium, we presented results with RT-PCR-ELISA and the controls we used to make our results reliable. This manuscript summarizes these data.

1. INTRODUCTION

Why use PCR-ELISA? We have developed RT-PCR-ELISA detection methods to measure the mRNA levels of (1) the Muscle Regulatory Factors (MRF) gene family genes, (2) the pituitary genes of the Growth Hormone (GH) axis, and (3) the muscle-specific and adipocytes-specific Fatty Acid Binding Proteins (FABP). The MRF genes are transcription factors regulating muscle development and growth. Transcription factors usually have a (very) low expression level. The pituitary genes involve a transcription factor too, and the pulsatile profile of the GH gene, which also involves stages with very low expression levels. The FABP genes distribute energy carrying fatty acids along the energy storage and energy consumption axes. The low and variable expression of the genes requires powerful detection methods. At the same time low expression level detection is vulnerable to background signal detection and non-specific signal detection, both of which can have either of two different unwanted effects:

(1) suggest expression where no expression is present, and

(2) suggest a higher expression than is actually present.

Therefore, we placed tight controls in place to ensure correct detection; however, these controls may not always be possible in a field test and the controls make using the PCR-ELISA more expensive.

1.1. The dangers of PCR-ELISA for Trypanosoma diagnosis

If used for diagnosis of Trypanosoma this means that if situation (1) occurs a person is treated while this should not be done, i.e. unnecessary use of drug is expensive and increases the possibility of developing drug-resistant strains, and if situation (2) occurs the infection seems far more severe than the actual situation.

1.2. How to use PCR-ELISA for safe Trypanosoma diagnosis

PCR-ELISA is a very powerful technique able to detect small numbers of parasites — probably even down to a single one! Basically, DNA is extracted from a tissue or blood sample without microscopic determination of Trypanosomes. A PCR reaction is followed by capturing the PCR product to a microtiter plate coated with a probe against the PCR product. A second probe associated with an enzyme is bound to the captured PCR product and the enzyme produces a reaction, e.g. changing the colour of a marker substrate or producing chemoluminescence to detect the PCR product. Alternatively the PCR product could be labelled and the label detected by an antibody associated with an enzyme. The enzyme product is in principle quantitative to the PCR product captured by the microtiter plate so, if the PCR reaction was quantitative the number of the Trypanosomes can be estimated. If a full PCR reaction was performed even very small numbers of parasites, in principle a single Trypanosome, can be detected. However, one should bear in mind the following:

If the PCR product is too small to bind (two) specific probes (or the sequence of the PCR product is not specific enough) interference with the primers may occur endangering false positive results. If the PCR product is too long, over 400 to 600 bp, depending upon the sequence of the PCR product (this has to be determined experimentally) the PCR product will have secondary structure hampering capturing of the PCR product to the microtiter plate and / or binding the second probe. The secondary structure may also hamper binding of the antibody if a labelled PCR product has been produced during the PCR reaction. This may lead to false negative results.

Non-specific PCR reactions can hamper both capturing of the PCR product to the microtiter plate and the correct binding of the second probe or the antibody leading to either false positive or false negative results. As a solution to these problems we set up a set of controls:

Before starting a PCR diagnosis, always run quantitative PCR-ELISA reactions showing that the length and sequence of the PCR product enables meaningful use of the technique for diagnosis.

Always check all PCR reactions on a gel: use only single banded PCR product for ELISA detection.

These controls will reduce the possibilities for using the technique in a field laboratory. However, without these checkpoints in place the method is vulnerable to false positive and false negative results, either resulting in unnecessary use of drug to treat non-patients or not treating patients. With the correct use of the technique it will be a powerful method enlarging the diagnostic capacities to fight Trypanosomosis.

Fig. 1 provides a flow chart for the setup and use of PCR-ELISA. It involves the following steps:

(1) Create PCR primers and an ELISA probe without overlapping sequences. PCR reactions should be between 100 bp and 500 bp. Shorter PCR reactions usually do not provide space for separate probe development, longer PCR products show reduced PCR product probe annealing (empirical result). It is suggested that the PCR product will be labelled during the reaction; otherwise an additional ELISA probe needs to be developed.

(2) Set up a PCR reaction that produces a single band (check with agarose gel electrophoresis)

(3) Setup an ELISA

(4) Run the PCR-ELISA with all samples

(5) Check the PCR products with agarose gel electrophoresis, use only ELISA results of clear single banded PCR reactions.

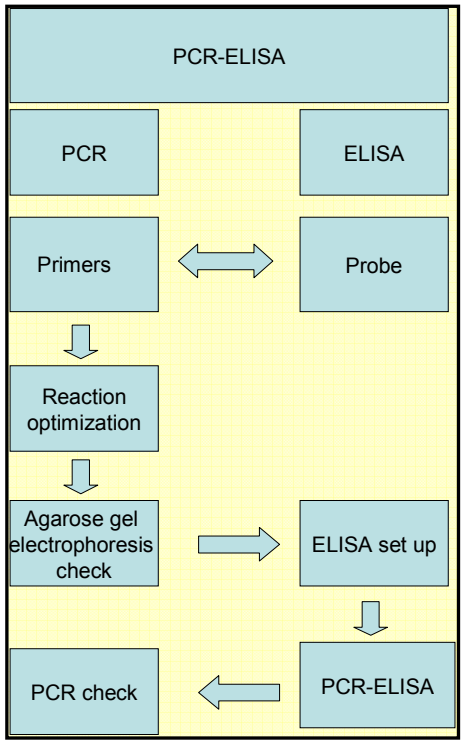

FIG. 1. Flow chart for the setup of a PCR-ELISA.

The following sequential steps are visualized: Creation of two PCR primers and a single ELISA probe without overlapping sequences, followed by the setup of a PCR reaction that produces a single band as checked with agarose gel electrophoresis. Using this PCR product an ELISA reaction is setup and checked for quantitative ness, if needed. After this the PCR-ELISA can be done for all samples. Only the ELISA results of PCR reactions with clear single bands are reliable.

1.3. Future research directions

Treating parasitic infection in general will be increasingly hampered by drug resistance of the parasite populations and it is expected that Trypanosoma are no exception. Without effective treatment more infections will become epidemic; therefore, knowledge on the (genomic) background of drug resistance is of utmost importance. Presently such knowledge is limited to a few genes indicating some pathways of drug resistance in the parasite. It can be expected that more and diverse pathways will be involved and a comprehensive picture of these pathways involved in the development of drug resistance is needed. This will encourage the development of new drugs tackling parasite growth and spread through different pathways and also the development of fast and reliable tests to detect existing drug resistance to allow cost effective use of drugs and use of the correct drugs to prevent the spread of the disease. The use of functional genomics and proteomics technologies enables us to investigate developments in Trypanosoma to search for mechanisms of drug resistance development. With this knowledge more efficient treatment will be feasible.

NEW DIAGNOSTICS FOR THE DETECTION OF ANIMAL TRYPANOSOMOSES — A SHORT REVIEW

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Abstract

The detection of infections with the pathogenic animal Trypanosomoses depends mainly on three criteria: clinical signs and symptoms of disease; detection of the parasites in body fluids and detection of parasite products in body fluids. These diagnostic criteria apply commonly in respect of all parasitic or infectious diseases and the degree to which one or other becomes the chosen diagnostic method depends on the particular disease. Factors that will determine choice include diagnostic sensitivity and specificity, ease of use and cost, but most important in ensuring that a technique is adopted is the demand from the clinician to be provided with a convenient and efficient way of diagnosis. The past hundred years has seen the regular introduction of new technologies for disease diagnosis. Many of these initiatives derive from laboratory-based scientists whose interest in perfecting a test may differ from the field investigator. In the field, the clinician requires a test that will provide evidence of current infection so that decisions can be made on measures required for treatment and control. The laboratory scientist may opt for tests with high analytical sensitivity and specificity without due consideration of their diagnostic attributes.

Animal Trypanosomosis is often difficult to detect parasitologically so the isolation of the agent is not the most sensitive method of diagnosis. This has led to much effort to develop methods that indirectly indicate the presence of infection with increased sensitivity. Unfortunately, there has been reluctance, or at least a lack of interest, in including these technologies into tsetse and Trypanosomosis control programmes. Hence although there have been many reports of their use, there a relatively few accounts of their application in major control programmes. Consequently, classical light microscopy still plays an important role in Trypanosome diagnostics. There are a number of reasons why this is so; sometimes tests have not been fully validated and standardized and required specialist laboratories to operate; the cost of the tests may have been too high; the advantages of including new diagnostics was not clearly defined. Given the situation in many tsetse-infested areas, it is easy to understand the reluctance to introduce new technology. In areas of high Trypanosomosis risk it is easy to detect infections in cattle by parasitological means. Also, if drug treatment is administered on a herd basis it is not necessary to identify every infected individual since all will treated anyway.

This scenario is beginning to change however. Post eradication monitoring based on serological assays has been used following clearance of tsetse flies on Zanzibar Island, and in Ethiopia, work is on-going monitoring serological prevalence of infection after the clearance of tsetse flies using sterile males. In addition, in areas where non tsetse- transmitted Trypanosomoses caused by *Trypanosoma evansi* and *T.vivax* exist, control of haematophagous flies is problematic and there is a need to identify and treat infected animals individually. Foremost amongst the candidate tests for serological monitoring are enzyme immunoassays for detection of Trypanosomal

antibodies. The FAO/IAEA Joint Division has been instrumental in producing and validating a number ELISA tests for bovine Trypanosomosis. These tests have been used to diagnose infections in individuals and herds, monitor the efficacy of control programmes and monitor the effects of Trypanosomosis on productivity [1].

Although there is a drive to introduce ever more sophisticated technology it is always worth keeping a realistic perspective when deciding how to approach diagnosis. In the end, the diagnostic performance of a test will determine how effective it will be under the prevailing ecological conditions. Parasitological diagnosis will give an indication of the presence of infection, but cannot indicate the extent of Trypanosomosis in the population since there is liable to be seasonal fluctuations in the parasitological prevalence of infection [2]. The use of an enzyme immunoassay will give a more consistent result, less dependent on seasonal changes in fly challenge and providing an historical picture of the extent of the population's exposure to Trypanosomosis [2]. Enzyme immunoassays were also used to provide a more comprehensive evaluation of the occurrence of Trypanosomosis caused by *T.evansi* in Indonesia [3].

Although serological assays have not been widely used in Trypanosomosis control programmes, they have been used in number of small-scale surveys. Bengaly et al [4] examined the serological prevalence of infection in cattle following tsetse control. They found that although the parasitological prevalence was zero and that anaemia was no longer present in the cattle, there was little change in antibody prevalence 12 months after control had been initiated. This result underlines the necessity of understanding the attributes of a test in order to use it effectively — it is well known that antibodies persist in the serum long after Trypanosomes have been removed by treatment, and this must be taken in to account and an appropriate strategy devised to compensate. The presence of antibodies was also shown to be valuable in identifying cattle with a low productivity in the Gambia [5].

Although serological assays have not been used widely in control programmes it likely that in future they will become an integral part of any programme from the initial phase. Guidelines currently being discussed [6] on the measures that should be taken to declare an area free from Trypanosomosis envisage enzyme immunoassays being used in the pre-control estimates of prevalence and thereafter in the follow-up post intervention and monitoring phases. These will include strategies such as the introduction of new, parasite free sentinel animals, since it is known that the resident population will continue to harbour antibodies for some time after eradication. Such methods were used in Zanzibar [7] to assess the seroprevalence after eradication of tsetse flies. The CRP report details the testing of more technological developments in Trypanosomosis diagnosis; these tests are based on the detection of parasite antigens using polymerase chain reaction (PCR). Such tests have been shown to have very high analytical sensitivity and are potentially capable of identifying individual infected animals. Whether or not they will become part of the increasingly sophisticated technology that is now available for use in the control of the pathogenic animal Trypanosomoses in great part depends on clear rational thought in determining exactly what role they can play. All tests have limitations and their use may involve compromise; PCR should not necessarily seek to replace serological assays (in Trypanosomosis they are only just beginning to be used for monitoring) but to integrate and enhance and add to the information that can be obtained from serology.

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MOLECULAR MARKERS FOR THE DIFFERENT (SUB)-SPECIES OF THE TRYPANOZOON SUBGENUS

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Abstract

During the past years, species specific PCRs for identifying the different taxa within the Trypanozoon subgenus have been developed by our laboratory. For the detection of the two human pathogenic Trypanosomes, PCR-SRA for *T.b.rhodesiense* [1] and PCR-TgsGP gene for *T.b. gambiense* [2] exist now. For animal Trypanosomiasis, a *T.evansi* specific PCR based on the RoTat 1.2 VSG was developed [3]. Only for *T.b.brucei* and *T.equiperdum*, no specific markers could be identified. However, results examine here indicate that *T.equiperdum* is more closely related to *T.b.brucei* than to *T.evansi* and even might be a particular strain of *T b.brucei* [4].

1. INTRODUCTION

Diagnosis of Trypanosomosis usually starts with clinical suspicion or with the detection of antibodies in the blood of the examined animal. Conclusive evidence of infection relies on detection of the parasite in the blood or tissue fluids of infected humans or animals. Unfortunately, parasitological techniques cannot always detect ongoing infections as the level of parasitaemia is often low and fluctuating, particularly during the chronic stage of the disease [5].

As an alternative to parasitological tests, DNA detection based on PCR is being investigated. *Trypanozoon* specific primers have been designed previously: TBR primers which target a 177 bp repeat by [6]; pMUTEC primers targeting a retrotransposon by [7]; ORPHON primers which target the spliced leader sequence by [8] and the PCR-ESAG6/7 which target the transferrin receptor [9]. PCR tests for diagnosis of *T.congolense* and *T.vivax* infections exist as well [7]; [10]. PCR assays for differentiating the different members of the *Trypanozoon* subgenus, however, remained a challenging issue. In this manuscript we will give an overview of the work carried out the last four years at the Institute of Tropical Medicine, Antwerp, in this field.

2. MATERIALS AND METHODS

2.1. Trypanosome populations

A large collection of Trypanosomes, including clones and stocks from *T. b. gambiense, T.b.rhodesiense*, *T.b.brucei*, *T.evansi* and *T.equiperdum, T.congolense* and *T.vivax* was used in the different experiments. They are isolated from different host and regions, all over world (Table I). All populations were kept as cryostabilates in liquid nitrogen.

2.2. DNA extraction

Trypanosome populations were purified as in [11]. Twenty µL of Trypanosome pellet (approximately 2 x 10^7 cells) was resuspended in 200 µL of phosphate buffered saline (PBS) (8.1 m*M* $Na_2HPO_4.2H_20$, 1.4 m*M* $NaHPO_4$, 140 m*M* NaCl, pH 7.4) and the Trypanosome DNA was extracted using the commercially available QIAamp DNA mini kit (Westburg, Leusden, The Netherlands), resulting in pure DNA in 200 µL of TE buffer. The typical yield of DNA extracted from a 20 µL pellet was 150 ng/µL or 30 µg total DNA. Obtained extracts were diluted 200 times in water and divided into aliquots of 2 mL in microcentrifuge tubes for storage at -20°C.

2.3. PCR-SRA [1]

Primers were derived from the sequence of the serum resistance associated gene (SRA) (accession number Z37159). Primer sequences were as follows:

PCR-SRA-*f* $^{5'}$ATA GTG ACA AGA TGC GTA CTC AAC GC $^{3'}$

PCR-SRA-*r* $^{5'}$AAT GTG TTC GAG TAC TTC GGT CAC GCT $^{3'}$

These amplify a 284 bp fragment between nucleotides 383-667 of the *SRA* gene. All PCR amplifications were made using 10 ng of the DNA extracted from purified parasites.

The DNA templates were amplified in 50 µL of PCR reaction mixture containing 1 x PCR buffer (20 m*M* Tris-HCl pH 8.7, 100 m*M* KCl, 50 m*M* $(NH_4)_2SO_4$), 1.5 m*M* $MgCl_2$, 200 µ*M* of each of the four dNTPs, 1 µ*M* of each of the primers and 2.5 units of HotStar Taq DNA polymerase (Qiagen, Westburg, Leusden, The Netherlands).

All PCR amplifications were made on a Biometra T3 Thermocycler (Westburg, Leusden, Netherlands)). PCR conditions were as follows: sample incubation for 15 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at 68°C and 1 min at 72°C, and a final extension at 72°C for 10 min.

A 20 µL sample of each PCR product was analysed by electrophoresis in a 2% agarose gel. The gels were stained with ethidium bromide (1µg/mL) (Sigma) and analysed on an Imagemaster Video Detection System (Pharmacia, UK).

2.4. PCR-TgsGP [2]

The primers were derived from the sequence of the *T.b.gambiense* specific glycoprotein (TgsGP) [12]. Using the Genbank homology search program, the primer sequence was derived from the region lacking any significant similarity with already known DNA sequences. Primer sequences were as follows:

PCR-TgsGP-*f* 5' GCT GCT GTG TTC GGA GAG C 3’

PCR-TgsGP-*r* 5’ GCC ATC GTG CTT GCC GCT C 3’

All PCR amplifications were made using 10 ng of extracted DNA. Amplification was made using 50 µL of a reaction mixture containing 1 x PCR buffer (20 m*M* Tris-HCl pH 8.7, 100 m*M* KCl, 50 m*M* $(NH_4)_2SO_4$, Q solution), 1.5 m*M* $MgCl_2$, 200 µ*M* each of the four dNTPs, 1 µ*M* each of the primers and 2.5 units of

HotStar Taq DNA polymerase (Qiagen, Westburg, Leusden, The Netherlands). All PCR amplifications made using a Biometra T3 Thermocycler (Westburg, Leusden, Netherlands). The PCR amplification using TgsGP derived primers was performed by incubating the samples for 15 min at 95°C followed by 45 cycles of 1 min at 94°C, 1 min at 63°C and 1 min at 72°C, and a final extension at 72°C for 10 min. A 20 µL sample of each PCR product was analysed by electrophoresis in a 2% agarose gel. Gels were stained with ethidium bromide (0.5µg/mL) (Sigma, USA) and analyzed on an Imagemaster Video Detection System (Pharmacia, UK). Additionally, the detection limit was increased by a second PCR amplification that was performed using 1 µL of the first one.

2.5. PCR-RoTat 1.2

Primers were derived from the RoTat 1.2 VSG sequence (AF317914), recently cloned and sequenced by [13]. Primer sequences were identified within the region (608-812 bp) lacking homology with any other known VSG sequences. Primer sequences (in 5'-3' direction) and annealing temperatures were as follows: PCR-RoTat 1.2-*f* GCG GGG TGT TTA AAG CAA TA, $T_{ann.}$ 59°C and PCR-RoTat 1.2-*r* ATT AGT GCT GCG TGT GTT CG, $T_{ann.}$ 59°C. Twenty µL of extracted DNA were mixed with 30 µL of a PCR-mix containing: 1 U Taq DNA recombinant polymerase (Promega, UK), PCR buffer (Promega, UK), 2.5 m*M* $MgCl_2$ (Promega, UK), 200 µ*M* of each of the four dNTPs (Roche, Mannheim, Germany) and 0.8 µ*M* of each primer (Gibco BRL, UK). All amplifications were carried out in a Biometra T3 thermocycler (Westburg, Leusden, Netherlands). Cycling conditions were as follows: denaturation for 4 min at 94ºC, followed by 40 amplification cycles of 1 min denaturation at 94ºC, 1 min primer-template annealing at 59ºC and 1 min polymerization at 72ºC. A final elongation step was carried out for 5 min at 72°C. Twenty µL of the PCR product and ten µL of a 100 bp size marker (MBI Fermentas, Germany) were subjected to electrophoresis in a 2% agarose gel (25 min at 100V). Gels were stained with ethidium bromide (0.5 µg/mL) (Sigma, USA) and analyzed on an Imagemaster Video Detection System (Pharmacia, UK).

2.6. Multiple-endonuclease genotyping approach (MEGA)

A fine-scale genotyping approach involving multiplex endonucleases in combination with a pair of cognate adapters was used according to [13]. Briefly, 100-250 ng genomic DNA were digested for 4 h using 10 U each of *Bgl*II, *Bcl*I, *Acs*I and *Mun*I endonucleases in two successive double digestion reactions. The final digestion products were precipitated and reconstituted in 10 µL distilled water. Ten µL of a buffer containing 660 m*M* Tris HCl, 50 m*M* $MgCl_2$, 10 m*M* Dithiothreitol, 10 m*M* ATP, pH7.5, and 20 p*M* of each *Bgl*II and *Mun*I adapters were added. The *Bgl*II adapter also ligated to the overhang sites created by *Bcl*I, while *Mun*I adapter also ligated to the *Acs*I site. One µL (400U) of T4 DNA ligase (New England Biolabs) was added and the mixture incubated for 2 h at 25ºC. Pre-selective amplification was performed in a total volume of 20 µL containing 4 µL of 1:1-diluted ligation product, 1 U of Taq polymerase (Roche Molecular Biochemicals, Almere, The Netherlands), 10X PCR buffer (100 m*M* Tris HCl pH 9.0, 50 m*M* KCl, 1% triton X-100, 0.1% w/v gelatin), 2.5 m*M* $MgCl_2$, 200 µ*M* of each dNTP and 5 p*M* of each *Bgl*II

8($^{5'}$-GAGTACACTGTCGATCT) and *Mun*I ($^{5'}$-GAGAGCTCTTGGAATTG) primers. The reaction mix was incubated for 2 min at 95ºC, and subjected to 20 cycles of PCR

(30 s at 95ºC, 30 s at 56ºC and 2 min at 72ºC). Four µL of 1:20-diluted pre-selective products were used as template for selective reaction with *Mun*-0/*Bgl*-A selective primer combination (in which the *Mun* primer was fluorescently labelled). The PCR program was essentially the same as for pre-selective amplification, except that the last cycling step was followed by 30 min incubation at 60ºC. The final products were diluted 1:1 with TE, and Genescan-500 internal lane standard (PE Applied Biosystems) was added. One µL of the mix was resolved in a 7.3% denaturing sequencing gel using a model ABI 373A automated DNA sequencer. Gels were routinely prepared by using ABI protocols and electrophoresed for 5 h. Gel patterns were collected with GenScan software (PE Applied Biosystems) and sample files were transferred to GelCompar II software (Applied Maths, Kortrijk, Belgium).

3. RESULTS

3.1. PCR-SRA

The expected 284 bp *SRA*-PCR product was obtained with the DNA of the 24 different populations considered as *T.b.rhodesiense*. All other 72 non-*T.b.rhodesiense* populations were negative, thus confirming the specificity of the *SRA*-PCR for the *T.b.rhodesiense* subspecies. The PCR-SRA results with different human African Trypanosomiasis strains (*T.b.rhodesiense* and *T.b.gambiense*) are shown in Fig. 1.

3.2. PCR-TgsGP

In total, 73 different Trypanosome populations have been analysed by the TgsGP-PCR. A specific PCR product was obtained with 13 out of 15 *T.b.gambiense* populations. All other 58 non-*T.b.gambiense* populations remained negative, thus confirming the specificity of the TgsGP-PCR for *T.b.gambiense* within the collection of tested Trypanosoma sp. After a single PCR reaction, the detection limit reached 1000 Trypanosomes/mL blood. This detection limit was lowered to 10 Trypanosomes/mL blood when the TgsGP-PCR was repeated using an aliquot of the first PCR reaction product. The PCR-TgsGP results with different human African Trypanosomiasis strains are shown in Fig. 2.

3.3. PCR RoTat 1.2

As shown in Fig. 3 the RoTat 1.2 PCR yielded a 205 bp amplicon in the positive control (lane 1) as well as in all other *T.evansi* populations (lanes 3-8). Moreover, the same fragment was found in 7/9 of the *T.equiperdum* populations tested. Only the *T.equiperdum* BoTat 1.1 (lane 10) and the *T.equiperdum* OVI strain (lane 11) were PCR negative. All other tested Trypanosome populations, including six *T.b.brucei*, eight *T.b.gambiense*, five *T.b.rhodesiense,* two *T. congolense*, one *T.vivax* and one *T.theileri,* were negative. (Lanes 18-40). As a negative control a PCR-mix without template DNA was included (lane 2). The PCR was able to detect as few as 10 Trypanosomes per PCR reaction, which corresponds with a lower detection limit of 50 Trypanosomes per mL. In principal, this limit can still be lowered if a blood sample of 200 µL extracted with the QIAamp DNA mini kit is eluted in less than 200 µL.

3.4. MEGA, a modified AFLP

The UPGMA clustering data obtained from the modified AFLP analysis, all *T.evansi* are grouped in one cluster with a similarity of 85-95%, together with eight out

of ten *T.equiperdum* strains. The *T.b.brucei* group appeared as a heterogenous cluster, including the *T.equiperdum* BoTat 1.1 and OVI strains. Based on the modified AFLP data, the level of similarity of these two latter strains was calculated at 74%. In this analysis, OVI seems closely related to *T.b.brucei* KETRI 2494 (83%), while BoTat 1.1 shares more homology with *T.b.brucei* AnTat 2.2 (6%), (Fig. 4).

4. DISCUSSION

The *SRA*-based PCR was shown to be specific for *T.b.rhodesiense*, as a 284 bp specific PCR product was generated with 24 of the 25 *T.b.rhodesiense* used in this study, whereas this fragment was never detected in other subspecies and species. Other recently used analytical techniques such as isoenzyme analysis and RFLP failed to identify an unequivocal criterion to differentiate *T.b.rhodesiense* from *T.b.brucei* [14]; [15]; [16]. As a large variety of game and domestic animals serve as a reservoir for both subspecies, our PCR test could be used for identification of human infective and non-human infective Trypanosomes within the animal reservoir and the vector [14]; [15]; [17]; [18]. Using the *T.b.gambiense* specific marker showed that the expected 308 bp specific PCR product was generated solely with *T.b.gambiense* parasites and that no cross-reactivity occurred with any other DNA templates used in this study. Interestingly, two *T.b.gambiense* strains i.e. ABBA and LIGO scored negative in PCR amplification. These strains had previously already been classified as being distinct from the conventional *T.b.gambiense* parasites and were grouped together with the Nigerian *T.b.brucei* subspecies [19]. This classification had been made based on the cluster analysis of the restriction enzyme polymorphism pattern using the ribosomal non-transcribed spacer region [19]. As both strains were negative by TgsGP-based PCR, but were previously characterized as human infective, we have re-tested their resistance to normal human serum. Both strains appeared to be completely human serum sensitive and as such were presumed to be non-infective to human, possibly representing a *T.b.brucei* subspecies. On the other hand, two other *T.b.gambiense* strains used in this study i.e. OUSOU and KOBIR that were previously classified together with the ABBA and LIGO strains, did contain the TgsGP gene and were confirmed to be completely resistant to normal human serum. Besides the diagnostic value, the developed TgsGP based PCR test can also serve as a useful tool for disease, vector and reservoir control, indeed rendering possible the differentiation of *T.b.gambiense* from *T.b.rhodesiense* and *T.b.brucei.* As such, the TgsGP-based PCR can be used for epidemiological purposes as well.

A new marker for all *T.evansi*. Species-specific markers for *T.evansi* have been developed based on kDNA mini-circle sequences [20]; [21]. These assays; however, could not detect dyskinetoplastic *T.evansi* strains since their kDNA is severely reduced or even absent. Work by [22] and [23] already identified the RoTat 1.2 VSG as an interesting candidate for species-specific diagnosis of *T.evansi*. Several serological antibody detection tests have been developed and tested both on camels [24] and water buffaloes [25]. Results indicated the usefulness of the RoTat 1.2 VSG for diagnostic purposes of *T.evansi*. Regarding the results of this study, a problem with species-specificity appears unless we accept that those *T.equiperdum* reacting with RoTat 1.2 VSG (gene) are misclassified *T.evansi*. In that case, the RoTat 1.2 VSG (gene) is a potential marker for all *T.evansi* strains, including the dyskinetoplastic strains. Data provided in this study indicate that the *T.equiperdum* collection is not as homogenous as previously believed and the generally followed concept that *T.equiperdum* is very closely related to *T.evansi* and more distant from *T.b.brucei* seems to be incorrect. If we accept the presence of RoTat 1.2 gene to be a specific *T.evansi* marker, only two strains

of *T.equiperdum, in casu* the BoTat 1 and the OVI strain, are non *T.evansi* and are more closely related to *T.b.brucei* than to *T.evansi*. Thus, the problem of differentiating *T.equiperdum* from the rest of the *Trypanozoon* species shifts from *T.evansi* (the general belief) to *T.b.brucei* (the new concept).

TABLE I. DIFFERENT TRYPANOSOME POPULATIONS, PRESENT IN THE INSTITUTE OF TROPICAL MEDICINE, ANTWERP

Species	**Sub sp.**	**Trypanosome populations**	**Parasite form**	**Origin**	**Isolation year**	**Original host**
T. brucei	*brucei*	AnTat 1.8	bloodstream	Uganda	1966	Bushbuck
T. brucei	*brucei*	AnTat 2.2	bloodstream	Nigeria	1970	Tsetse
T. brucei	*brucei*	AnTat 5.2	bloodstream	Gambia	1975	Bovine
T. brucei	*brucei*	AnTat 17.1	bloodstream	D.R.Congo	1978	Sheep
T. brucei	*brucei*	Ketri 2494 ITMAS 270881	procyclic	Kenya	1980	Tsetse
T. brucei	*brucei*	J10 ITMAS 250500A	bloodstream	Zambia	1973	Hyena
T. brucei	*brucei*	TSW 196 ITMAS 300500A	bloodstream	Côte d'Ivoire	1978	Pig
T. brucei	*gambiense*	AnTat 9.1 ITMAP 1788	bloodstream	Cameroon	1976	Man
T. brucei	*gambiense*	LiTat 1.3	bloodstream	D.R.Congo	1952	Man
T. brucei	*gambiense*	AnTat 11.6	bloodstream	D.R Congo	1974	Man
T. brucei	*gambiense*	AnTat 22.1	bloodstream	Congo/Brazza.	1975	Man
T. brucei	*gambiense*	JUA ITMAS 010799	bloodstream	Cameroon	1979	Man
T. brucei	*gambiense*	BAGE ITMAP 2569	procyclic	D.R.Congo	1995	Man
T. brucei	*gambiense*	NABE ITMAP 2569	procyclic	D.R.Congo	1995	Man
T. brucei	*gambiense*	PAKWE ITMAP 2570	procyclic	D.R.Congo	1995	Man
T. brucei	*gambiense*	SEKA ITMAP 2568	procyclic	D.R.Congo	1995	Man
T. brucei	*gambiense II*	KOBIR ITMAS 260600	bloodstream	Côte d'Ivoire	1982	Man
T. brucei	*gambiense II*	OUSOU ITMAS 220600	bloodstream	Côte d'Ivoire	1982	Man
T. brucei	*gambiense II*	ABBA ITMAS 190600A	bloodstream	Côte d'Ivoire	1983	Man
T. brucei	*gambiense II*	LIGO ITMAS 190600B	bloodstream	Côte d'Ivoire	1984	Man
T. brucei	*rhodesiense*	AnTat 25.1	bloodstream	Rwanda	1971	Man
T. brucei	*rhodesiense*	0404	procyclic	Rwanda	1970	Man
T. brucei	*rhodesiense*	STIB 847 ITMAS 050399A	bloodstream	Uganda (Busoga)	1990	Man
T. brucei	*rhodesiense*	STIB 848 ITMAS 190399	bloodstream	Uganda (Busoga)	1990	Man
T. brucei	*rhodesiense*	STIB 849 ITMAS 050399B	bloodstream	Uganda (Busoga)	1991	Man
T. brucei	*rhodesiense*	STIB 850 ITMAS 050399C	bloodstream	Uganda (Busoga)	1990	Man
T. brucei	*rhodesiense*	STIB 851 ITMAS 080399C	bloodstream	Uganda (Tororo)	1990	Man
T. brucei	*rhodesiense*	STIB 882 ITMAS 080399A	bloodstream	Uganda	1993	Man
T. brucei	*rhodesiense*	STIB 883 ITMAS 080399B	bloodstream	Uganda	1994	Man
T.cong		TRT 17 ITMAS 020699	bloodstream	Zambia	1997	Bovine
T.evansi		AnTAR 3 ITMAS 180274A	bloodstream	South America	1969	Capybara
T.evansi		AnTat 3.1 ITMAS 070799	bloodstream	South America	1969	Capybara

Species	Sub sp.	Trypanosome populations	Parasite form	Origin	Isolation year	Original host
T.evansi		AnTat 3.2 ITMAS 270280A	bloodstream	South America	1969	Capybara
T.evansi		AnTat 3.3 ITMAS 161189A	bloodstream	South America	1969	Capybara
T.evansi		AnTat 3.4 ITMAS 301189A	bloodstream	South America	1969	Capybara
T.evansi		AnTat 3.5 ITMAS 310180A	bloodstream	South America	1969	Capybara
T.evansi		RoTat 1.2 ITMAS 020298	bloodstream	Indonesia	1982	W. buffalo
T.evansi		MHRYD/BR/86/E18 ITMAS 020297	bloodstream	Brazil	1986	Capybara
T.evansi		CAN 86 K ITMAS 140799B	bloodstream	Brazil	1986	Dog
T.evansi		Stock Colombia ITMAS 150799	bloodstream	Colombia	1973	Horse
T.evansi		Stock Vietnam WH ITMAS 101298	bloodstream	Vietnam	1998	W. buffalo
T.evansi		STIB 816 ITMAS 140799A	bloodstream	China	1978	Camel
T.evansi		KETRI 2479 ITMAS 100883A	bloodstream	Kenya	1980	Camel
T.evansi		KETRI 2480 ITMAS 110297	bloodstream	Kenya	1980	Camel
T.evansi		KETRI 2481 ITMAS 010883C	bloodstream	Kenya	?	?
T.evansi		KETRI 2485 ITMAS 080981B	bloodstream	Kenya	?	Camel
T.evansi		stock Philippines ITMAS 060297	bloodstream	Philippines	1996	W. buffalo
T.evansi		stock Kazakstan ITMAS 060297	bloodstream	Kazachstan	1995	Camel
T.evansi		Merzouga 56 ITMAS 120399D	bloodstream	Morocco	1998	Camel
T.evansi		Merzouga 94 original stab.	bloodstream	Morocco	1998	Camel
T.evansi		Merzouga 93 ITMAS 150399C	bloodstream	Morocco	1998	Camel
T.evansi		Zagora I.3 ITMAS 010399B	bloodstream	Morroco	1997	Camel
T.evansi		Zagora I.5 ITMAS 040399A	bloodstream	Morroco	1997	Camel
T.evansi		Zagora I.10 ITMAS 220299	bloodstream	Morroco	1997	Camel
T.evansi		Zagora I.17 ITMAS 040399B	bloodstream	Morroco	1997	Camel
T.evansi		Zagora I.28 ITMAS 040399	bloodstream	Morroco	1997	Camel
T.evansi		Zagora I.31 ITMAS 120399A	bloodstream	Morroco	1997	Camel
T.evansi		Zagora I.75 ITMAS 010399C	bloodstream	Morroco	1999	Camel
T.evansi		Zagora I.81 ITMAS 010399D	bloodstream	Morroco	1998	Camel
T.evansi		Zagora I.86 original stab.	bloodstream	Morroco	1997	Camel
T.evansi		Zagora II.28 ITMAS 150399B	bloodstream	Morroco	1997	Camel
T.evansi		Zagora II.42 original stab	bloodstream	Morroco	1998	Camel
T.evansi		Zagora II.52 ITMAS 120399B	bloodstream	Morroco	1998	Camel
T.evansi		Zagora II.111 original stab	bloodstream	Morroco	1998	Camel
T.evansi		Zagora II.114 original stab	bloodstream	Morroco	1998	Camel
T.evansi		Zagora II.115 original stab	bloodstream	Morroco	1998	Camel
T.evansi		Zagora III.25 ITMAS 120399C	bloodstream	Morroco	1998	Camel
T.equiperdum		AnTat 4.1 ITMAS 210983A	bloodstream	?	?	?
T.equiperdum		Alfort ITMAS 241199A	bloodstream	?	?	?
T.equiperdum		SVP ITMAS 241199B	bloodstream	?	?	?
T.equiperdum		Hamburg ITMAS 251199A	bloodstream	?	?	?
T.equiperdum		ATCC 30019 ITMAS 020301	bloodstream	France	1903 ?	Horse
T.equiperdum		ATCC 30023 ITMAS 280201	bloodstream	France	1903 ?	Horse
T.equiperdum		STIB 818 ITMAS 010999	bloodstream	P. R. China	1979	Horse
T.equiperdum		American ITMAS 220101	bloodstream	?	?	?
T.equiperdum		Canadian ITMAS 290101	bloodstream	?	?	?
T.equiperdum		OVI ITMAS 241199C	bloodstream	South Africa	1975	Horse
T.equiperdum		BoTat 1.1 ITMAS 240982A	bloodstream	Morocco	1924	Horse

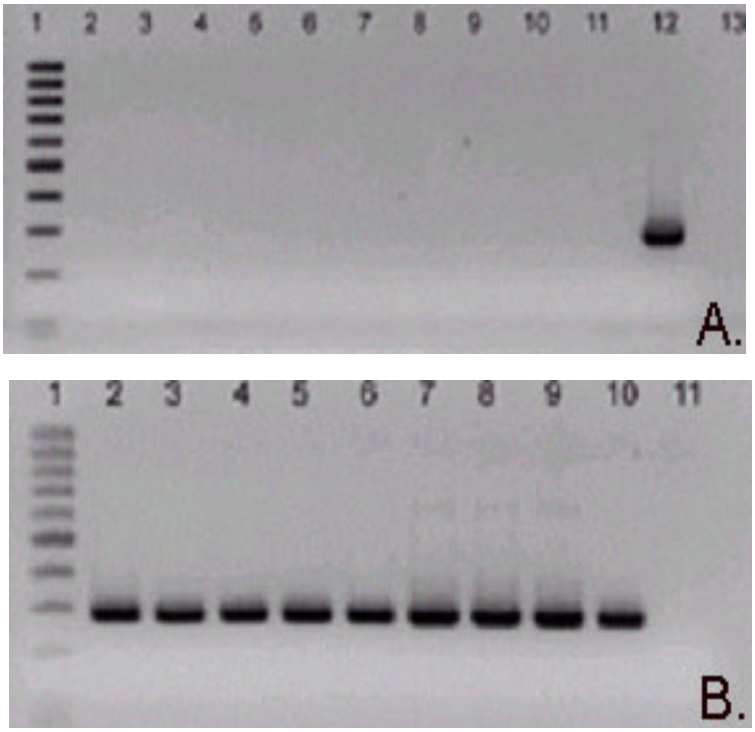

FIG. 1. PCR-SRA, a T.b.rhodesiense specific PCR.

Part A. 1 Marker; lanes 2-11 are *T.b. gambiense*, respectively JUA, AnTat 11.17, AnTat 22.1, SEKA, AnTat 9.1, AnTat 11.6, LiTat 1.6, LiTat 1.3, BAGE, NABE; lane 12 *T.b.rhodesiense* STIB 850; lane 13 white blood cells. Part B. lane 1 Marker; lanes 2-10 are *T.b.rhodesiense*, respectively STIB 883, STIB 882, AnTat 12.1, AnTat 25.1, STIB 847, STIB 884, STIB 851, STIB 850, STIB 849; lane 11 is *T.b.gambiense* JUA.

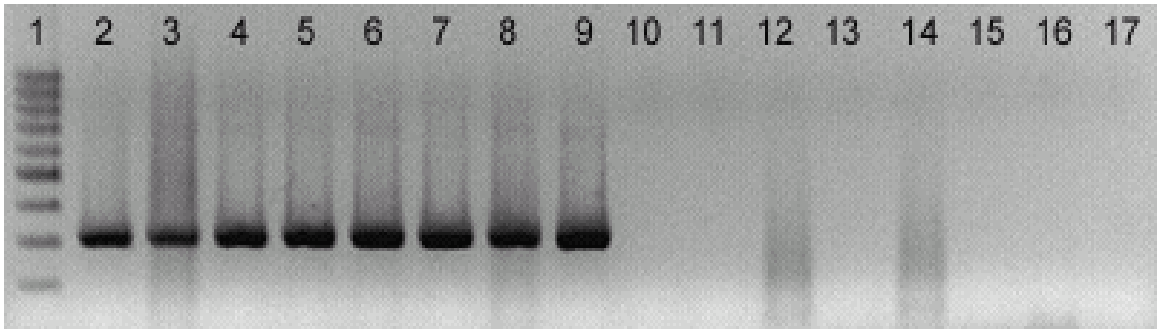

FIG. 2. PCR-TgsGP, a T.b.gambiense specific PCR.

Lane 1 Marker; lanes 2-9 are *T. b. gambiense*, respectively JUA, LiTat 1.3, AnTat 11.17, AnTat 22.1, SEKA, AnTat 9.1, BAGE, LiTat 1.6; lanes 10-17 are *T.b.rhodesiense*, respectively STIB 883, STIB 882, AnTat 12.1, AnTat 25.1, STIB 847, STIB 884, STIB 851, STIB 850.

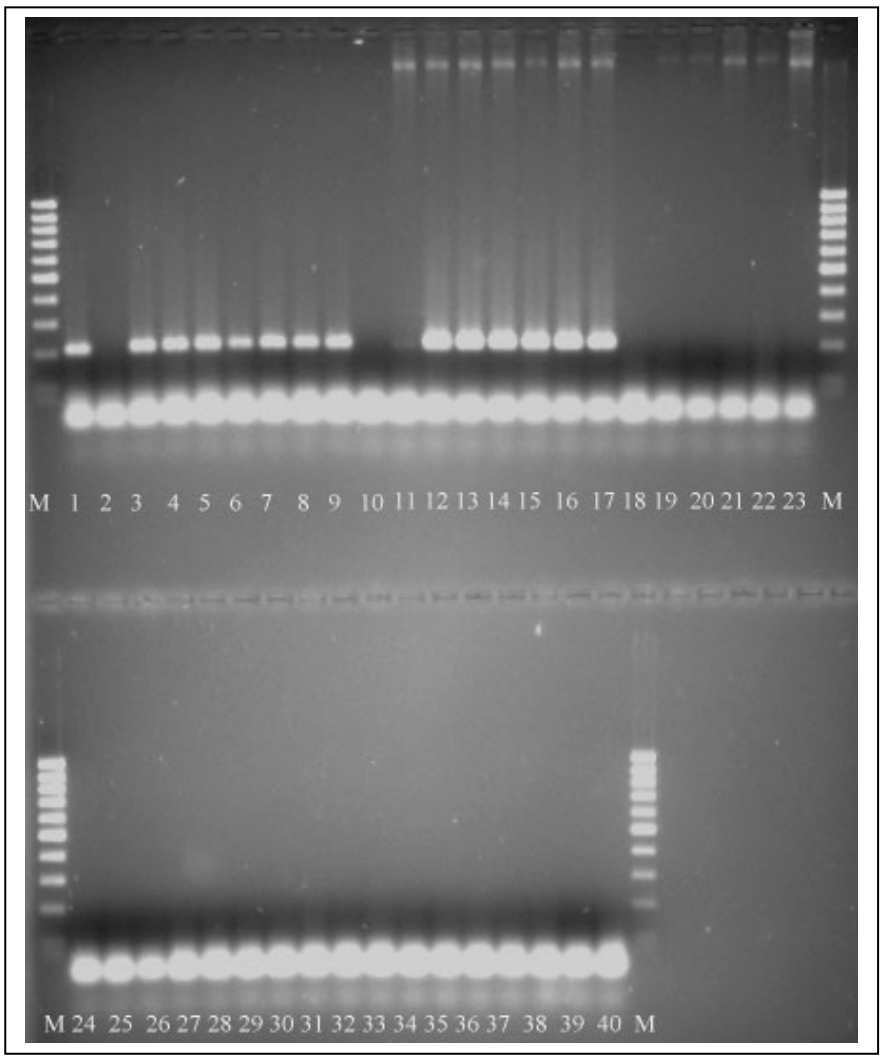

FIG. 3. PCR RoTat 1.2 specificity results for different Trypanosoma (T.) species and subspecies.

Lane 1 pos. control RoTat 1.2. Lane 2 neg. control. Lanes 3-8 (T.evansi) are respectively, AnTat 3.1, STIB 816, Zagora I.17, Colombia, Merzouga 56, CAN 86K; Lanes 9-17 (T.equiperdum) are respectively, AnTat 4.1, BoTat 1.1, OVI, STIB 818, Alfort, Hamburg, SVP, Am. Strain, Can. Strain; Lanes 18-23 (T.b .brucei) are AnTat 1.8, AnTat 2.2, AnTat 5.5, KETRI 2494, TSW 196, STIB 348; Lanes 24-31 (T.b.gambiense) are respectively, AnTat 9.1, AnTat 11.6, AnTat 22.1, NABE, SEKA, ABBA, LIGO, LiTat 1.6; Lanes 32-36 (T.b.rhodesiense) are STIB 884, STIB 850, AnTat 25.1/S, Etat 1.2/S, AnTat 12.1/S; Lanes 37-38 (T.congolense) are IL1180, TRT 17; Lane 39 (T.vivax) is ILRAD 700 and Lane 40 (T.theileri) is MELSELE; Lanes M 100 bp molecular marker (MBI Fermentas, Germany).

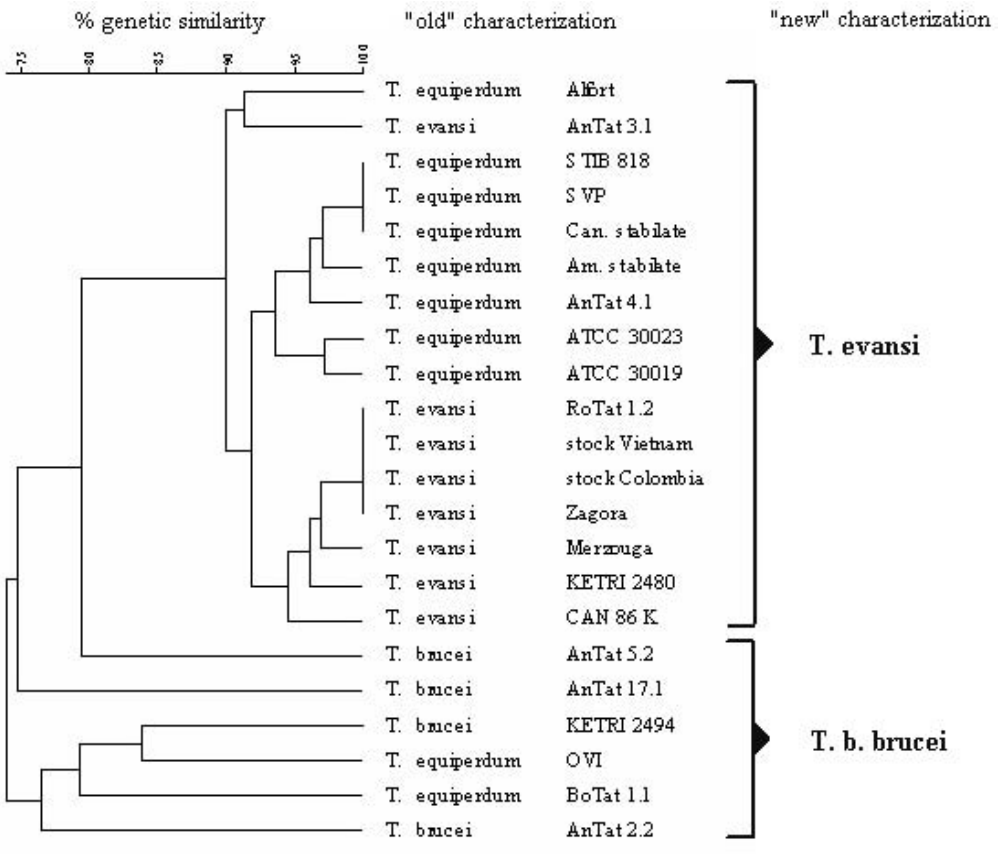

FIG. 4. UPGMA cluster analysis based on the MEGA results.

All T.evansi and 8 out of 10 T.equiperdum cluster out in one group with a 90-100% genetic similarity. All these strains also contain the RoTat 1.2 VSG gene. Thus, in our newly proposed characterization, this group are T.evansi while the other strains can be characterized as T.b.brucei.

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APPLICATION OF PCR/CSF FOR STAGE DETERMINATION AND THERAPEUTIC DECISION IN HUMAN AFRICAN TRYPANOSOMIASIS IN CÔTE D'IVOIRE

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Abstract

In Human African Trypanosomosis (HAT) two disease stages are defined: the first, or hemo-lymphatic stage, and the second, or meningo-encephalitic stage. Stage determination forms the basis of therapeutic decision and is of prime importance, since the drug used to cure second stage patients has considerable side effects. However, the tests currently used for stage determination have low sensitivity or specificity. Two new tests for stage determination in the cerebrospinal fluid (CSF) were evaluated on 73 patients diagnosed with HAT in Côte d'Ivoire. The polymerase chain reaction detecting Trypanosome DNA (PCR/CSF) is an indirect test for Trypanosome detection, whereas the latex agglutination test detecting IgM (LATEX/IgM) is an indicator for neuro-inflammation. Both tests were compared with classically used tests, double centrifugation (DC) and white blood cell count (WBC count) of the CSF. PCR/CSF appeared to be the most sensitive test (96%), and may be of use to improve stage determination. However, its value for therapeutic decision appears limited, as patients whose CSF was positive with PCR were successfully treated with pentamidine. This result confirms those of previous works that showed that some patients with Trypanosomes in the CSF could be treated successfully with pentamidine. LATEX/IgM, which depending on the cut-off, showed lower sensitivity of 76 and 88%, but higher specificity of 83 and 71% for LATEX/IgM 16 and LATEX/IgM 8 respectively, appears more appropriate for therapeutic decision making.

1. INTRODUCTION

Human African Trypanosomiasis (HAT) remains an important public health problem in sub-Saharan Africa. About 55 million people are daily exposed to the risk of infection and it is estimated that there are about 500,000 infected but untreated people [1]. The control of the disease heavily relies on the detection and treatment of patients. HAT evolves from a hemo-lymphatic phase (first or early stage) during which the parasite proliferates in the blood and the lymph, to a meningo-encephalitic phase (second, late or advanced stage) corresponding to invasion of the central nervous system

(CNS). The disease is lethal if untreated. The nature of treatment classically depends on the stage of the disease. For the chronic form of the disease occurring in West and Central Africa, caused by *Trypanosoma brucei gambiense*, the drug currently available for treatment of early stage is usually pentamidine, while melarsoprol is used for advanced-stage cases. However, melarsoprol is extremely toxic and is associated with encephalopathy in about 10% of treated patients [1]; therefore, reliable diagnosis of this stage is required. A broad clinical diversity is observed in HAT [2] and neurological signs and symptoms can be observed during the first stage, becoming more frequent and pronounced in the second stage [1]. Stage determination is therefore generally based on direct or indirect evidence of CNS invasion, assessed at the level of the cerebrospinal fluid (CSF) obtained by lumbar puncture. Direct evidence comprises detection of the parasite in the CSF during cell count or after single or double centrifugation [1]; [3]; [4]. Indirect evidence comes from increased cell counts (>5 cell/µL) or protein concentrations (> 37 mg/100 mL) in the CSF, which form the basis of second-stage determination according to WHO criteria [1]; [5]; however, these criteria are controversial [6]; [7]. Trypanosome detection techniques have low sensitivity, whereas increased cell counts or total protein concentrations in CSF are not necessarily specific for Trypanosomosis [8]; moreover, the latter is only rarely determined.

Recently, PCR methods have been developed for HAT diagnostic purposes. When performed on blood, high sensitivity and specificity have been reported [9] [10]. Report [11] shows the use of PCR on CSF (PCR/CSF) after a simple DNA purification with an anion chelating resin. They observed a good correlation between the PCR and the WHO criteria on a limited number of patients (n = 15), with PCR being more sensitive than parasite detection by double centrifugation.

On the other hand, an elevated IgM concentration in the CSF, characteristic of the meningo-encephalitic stage of HAT [12] can now be easily detected through a latex agglutination test (LATEX/IgM) which combines sensitivity, specificity and simplicity and which is applicable in the field [13]; [14].

The aims of this study were first to evaluate on a larger number of patients the value and the contribution of these two new tests in the improvement of the stage determination (discrimination between the first stage P1, and the second stage P2) and secondly, to evaluate their contribution for therapeutic decision-making. The two new tests were compared with classical parameters (parasite detection and cell count in CSF).

2. MATERIALS AND METHODS

2.1. Field study and collection of samples

During April and May 2000, active and passive surveillance were carried out in a sleeping sickness focus in Central-West Côte d'Ivoire by the National Control Programme in collaboration with Institut Pierre Richet (IPR), Project de Recherches Cliniques sur la Trypanosomiase (PRCT) in Daloa and Base de Santé Rurale (BSR) in Bouaflé respectively. Blood and lymph node aspirates from suspected sleeping sickness patients were examined for the presence of Trypanosomes by the miniature anion-exchange centrifugation technique (mAECT) and wet thin smear respectively.

In order to determine the stage of the disease, a lumbar puncture was performed. In each case the cerebrospinal fluid was examined for the presence of Trypanosomes

after double centrifugation (DC) [3] and the cells were counted in a Malassez counting chamber [15]. For each patient, blood and CSF samples were taken prior to treatment, at the end of the treatment, and at 1, 3, 6 and 12 m after treatment. In case of direct evidence of Trypanosomes in blood (mAECT) and/or in the CSF (DC) after treatment, the patient was considered as relapsed. Unlike WBC, PCR/CSF, LATEX/IgM8 and LATEX/IgM16 were not systematically performed after the beginning of the treatment and corresponding data were not included.

In this study and according to WHO recommendations [5]; patients with more than 5 cells/μL or with Trypanosomes in the CSF were considered in the second stage of the disease. However, according to the prevailing recommendations in Côte d'Ivoire, "intermediate stage" patients with =< 20 cells/μL and no Trypanosomes in CSF were not treated with melarsoprol but with the first stage drug, pentamidine [6].

LATEX/IgM titration of CSF samples was carried out as described by [14]. Briefly, the lyophilized latex reagent was re-suspended in 1.0 mL of phosphate buffered saline (PBS, 0.01 *M*, pH 7.4) and 2-fold serial dilutions of CSF were prepared with the same PBS buffer. Latex reagent (20 μL) was then mixed with diluted or undiluted CSF (20 μL) and spread over the white reaction zone (1.5 cm diameter) on the card.

The card was rocked at 70 rpm for 5 min on a horizontal rotator and examined for presence of agglutination. The end titer was expressed as the highest dilution of CSF that was positive with the agglutination test. The two cut-off end titers used in this study, that is 1:8 and 1:16 corresponding to LATEX/IgM 8 and LATEX/IgM 16 were considered positive if the CSF end titers were $\geq$1:8 and $\geq$1:16 respectively.

Cerebrospinal fluid (2 mL) remaining after WBC count, DC and LATEX/IgM titration was stored at –20°C for subsequent PCR analysis at IPR. Trypanosome DNA detection in the CSF by PCR was done according to [11].

2.2. Extraction of DNA for PCR

Briefly, an anion chelating resin was used in order to remove PCR inhibitors from the sample [16]. An amount of 500 μL of CSF was transferred to a 0.6 mL Eppendorf conical tube and centrifuged at 13000 *g* for 20 min. By means of a pipette, 450 μL of the supernatant was gently removed and discarded.

The pellet was resuspended in the remaining liquid and vortexed for 5 min. Subsequently, 100 μL of 1% Chelex solution in sterile purified water (Chelex 100 Resin, Bio-Rad Laboratories, CA, USA) was added to each tube.

The tubes were then vortexed for 1 min and incubated at 56°C for 1 h. This was followed by a second incubation at 95°C for 30 min. After incubation, the tubes were again centrifuged for 5 min at 13000 *g*. The supernatant, now containing the DNA, was used for PCR.

2.3. Polymerase Chain Reaction

PCR was performed according to the method described in [9]; using TBR1-2 primers TBR1: $^{5'}$-CGA-ATG-ATT-AAA-CAA-TGC-GCA-G-$^{3'}$; TBR2: $^{5'}$-AGA-ACC-ATT-TAT-TAG-CTT-TGT-TGC-$^{3'}$) specific for *Trypanosoma brucei sensu lato* [17].

The amplification conditions were as follows: initial denaturation at 94°C for 3 min, 40 cycles with the denaturation step at 94°C for 1 min, the annealing step at 56° for 1 min and the polymerization step at 72°C for 1 min. The final elongation was at 72°C for 5 min. Samples of 10 µL of each reaction product were run in 2% agarose gel with a 0.5 µg/mL solution of ethidium bromide before being visualized under ultraviolet light. A positive control (purified *T.brucei* DNA), a negative control (without DNA template) and a molecular weight marker (Marker IV, Eurogentec) were run in each PCR test. When the expected 177 bp product was visible, the PCR was considered positive.

2.4. Data analysis

The DC allows direct assessment of the presence of Trypanosomes in the CSF; it was used as the reference test for the calculation of sensitivity and specificity of PCR/CSF, LATEX/IgM 8, LATEX IgM/16, and WBC count. Then, PCR/CSF, LATEX/IgM 8 and 16 were compared with combined results for DC and WBC count reflecting the current way stage determination is performed. As sample sizes were too small for the approximate confidence interval based on the normal distribution to be reliable, confidence intervals were computed based on the exact theoretical distribution of a proportion, the binomial distribution.

To take account that biological tests were carried out on the same subjects, we performed McNemar Chi square. This procedure yields a p value that quantifies the probability that the difference in the new test response is due to chance, rather than actual difference between the new and the reference test.

3. RESULTS

Seventy-seven patients were diagnosed during two medical surveys in the area of Bonon, of whom 65 were treated and subsequently included in this study. The remaining 12 patients did not come to the treatment center. Twenty-seven other patients presenting themselves spontaneously for examination were also included in the study. For correct testing, hemorrhagic CSF, i.e. CSF contaminated with red blood cells detected during lumbar puncture, WBC count or after DC, was excluded from the tests.

Out of the 92 included patients, each of the four techniques (DC, WBC count, PCR/CSF and LATEX/IgM) could only be performed on 73 patients. Of these 73, 31 had WBC between 0 and 5 cells/µL and were negative in DC (see Table I). All patients of this first group were treated with pentamidine and no relapse was observed. Eleven patients had WBC between 6 and 20 cells/µL and were negative in DC. Patients of this second group were also treated with pentamidine and 1 relapse was observed 6 months after treatment. Thirty one patients had > 20 cells/µL (n= 25) or were positive in DC (n= 25). Patients of this third group were treated with melarsoprol and 2 relapses were observed respectively at 2 and 6 m after treatment.

With only the presence of Trypanosomes in CSF as the gold standard, PCR/CSF appeared to be the most sensitive test (0.96) with 95% confidence interval (IC_{95}) = [080-0.99], whereas LATEX/IgM 16 showed the lowest sensitivity (0.76, IC_{95} = [0.6–0.92]). A negative PCR/CSF had a significantly lower probability to occur in DC positive subjects (4%) than in DC negative ones (75%) (McNemar test = 9.31, 1df, $p<0.01$). On the contrary, the results of LATEX/IgM 16 did not depend on the presence of Trypanosome in CSF (McNemar test = 0.28, 1df, $p>0.5$). Sensitivity of LATEX/IgM 8

and of WBC count were 0.88 (IC_{95} = [0.75-1]) and 0.88 (IC_{95} = [0.69-0.97]) respectively. Specificity was highest for LATEX/IgM 16 (0.83; IC_{95} = [0.72-0.94]). The other tests presented lower specificity: 0.75 (IC_{95} = [0.6-0.86]) for PCR/CSF; 0.71 (IC_{95} = [0.58-0.84]) for LATEX/IgM 8 and 0.65 (IC_{95} = [0.49-0.78]) for WBC count. Since therapeutic decision is usually based on both presence of Trypanosomes and WBC count in the CSF, we compared the results of PCR/CSF, LATEX/IgM 8 and LATEX/IgM 16 with the combined results of DC and WBC count (Table I). Within the group of classically first stage patients (negative in DC and <6 cells/µL in CSF) PCR/CSF was positive for 7/31 patients. LATEX/IgM 8 and 16 were positive in respectively 6/31 and 2/31 patients in the same group. All these patients were treated with pentamidine and no relapses were observed. Among the 11 'intermediate stage' patients (negative in DC and 6 to 20 cells/µL), two were positive in both PCR/CSF and LATEX/IgM 16. A third was positive in LATEX/IgM 8 alone. The latter patient relapsed, while all other 10 'intermediate stage' patients seemed to be treated successfully with pentamidine.

Among the 31 late stage patients with >20 cells/µL or positive DC, PCR/CSF was negative in 4 cases. Within this group, LATEX/IgM 8 and 16 were negative in respectively 4 and 8 of the 31 tested patients.

4. DISCUSSION

In most sleeping sickness cases, the presence of Trypanosomes is associated with increased WBC numbers in the CSF thus leaving no doubt about disease stage and appropriate treatment. However, in those patients where elevated cell count is not confirmed by detection of the parasite and *vice versa*, correct stage determination and consequent treatment is not guaranteed. Mechanisms leading to discordance between cell count and Trypanosome presence may be of different origins, e.g. low sensitivity of Trypanosome detection techniques, limited accuracy of cell counting techniques, very recent CNS invasion by the parasite, low virulent parasite strains not inducing an intrathecal immune response, CNS inflammation without actual Trypanosome invasion, CNS inflammation caused by other infections.

Recently, two new techniques have been developed for assessment of Trypanosome presence in CSF [10]; and for assessment of CNS inflammation [13]; where the value for stage determination and therapeutic decision making in HAT were evaluated.

When compared with DC, the PCR/CSF appeared the most sensitive test which is in agreement with results from other studies [10]; [18]. Its specificity seems rather low when compared to DC, which most probably is due to the higher analytical sensitivity of PCR/CSF. On the other hand, false positive PCR/CSF results cannot be totally excluded, since PCR/CSF does not necessarily detect living Trypanosomes in CSF, but rather their DNA. False positive results might be due to low primer specificity [19] or to the presence of Trypanosome DNA in the CSF without actual presence of the parasite. The latter possibility has been postulated to explain false positivity of PCR performed on blood samples where Trypanosome DNA can originate from tsetse bites contaminated with non-human pathogenic Trypanosomes [20]; [21]. Such a phenomenon seems however less evident to explain false positivity in CSF. Here, circulating Trypanosome DNA may leak from the blood through the blood-brain barrier or alternatively, originate from non-surviving parasites as a consequence of the suboptimal CSF survival environment as already demonstrated by [22].

For IgM detection in CSF by LATEX/IgM, cut-off values of 1:16 and 1:8 were tested. Whatever the cut-off used, some DC-positive samples remained LATEX/IgM negative (24% (6/25) for LATEX IgM 16 and 12% (3/25) for LATEX IgM 8) which would lead to some second stage patients treated with pentamidine thus putting them at risk for relapse when only the result in LATEX/IgM is taken into account for stage determination. The combination DC positive and LATEX/IgM negative might indicate that the presence of Trypanosomes in the CSF does not necessarily lead to a host CNS inflammatory reaction, reflected by abnormal number of cells or IgM concentrations in the CSF.

It appears here that the two tests (PCR/CSF and LATEX IgM) have different qualities. The PCR/CSF appears to be a very sensitive test for detection of Trypanosome DNA in the CSF. If therapeutic decision in this study had been based on the PCR/CSF result, 7/31 (23%) of patients with negative DC and with < 5 cells/µL would have been treated unnecessarily with melarsoprol. Indeed, none of them relapsed after treatment with pentamidine of which it is known that low amounts cross the blood-brain-barrier [23]. Moreover, it has been reported that some patients with Trypanosomes in their CSF were successfully treated with pentamidine [24]. If therapeutic decision in this study had been based on LATEX/IgM applying cut-off of respectively 1:16 and 1:8, 2/31 (6.5%) and 6/31 (19%) of patients with negative DC and with < 5 cells/µL would have been treated with melarsoprol. For therapeutic decision, these results rather suggest to use LATEX/IgM at 1:16 cut off, combined with WBC count to avoid unnecessary melarsoprol treatment. On the other hand, LATEX/IgM 8 was the only new test which was positive in the relapsing “intermediate stage” patient who had been treated with pentamidine. If therapeutic decisions had been based on LATEX/IgM 8 positivity, this patient would have been treated with melarsoprol. Hence, combining the results of this study with those of previous studies [22]; [25]; [6], it appears to be increasingly important to make a distinction between stage determination and therapeutic decision: the former should not always condition the latter, especially in view of the severe side effects of melarsoprol which are not necessarily related to the presence of the Trypanosome in the CNS [26]. Some authors have suggested that immunological reactions within the CNS, against either immune complexes or antigens, could be also involved in such reactions independently of the presence of Trypanosome [27]; [28]. In conclusion, this study confirms the complexity of stage determination in sleeping sickness. Sensitive tools to detect the Trypanosome within the CSF, such as DC and PCR are not appropriate for making therapeutic decisions in all cases, neither are sensitive tools for assessment of CNS inflammation, such as WBC count and LATEX/IgM.

TABLE I RESULTS OF PCR/CSF, LATEX/IGM 16 (LI 16) AND LATEX/IGM 8 (LI 8) COMPARED TO WBC COUNT AND DC

	WBC from 0 to 5 (34)		WBC from 6 to 20 (14)		WBC > 20 (25)		Total : 73
	DC +	DC -	DC +	DC -	DC +	DC -	Total
PCR +	2	7	3	2	19 (2*)	3	36
PCR -	1	24	0	9 (1*)	0	3	37
LI 16 +	0	2	2	2	17 (2*)	4	27
LI 16 -	3	29	1	9 (1*)	2	2	46
LI 8 +	2	6	2	3 (1*)	18 (2*)	5	36
LI 8 -	1	25	1	8	1	1	37

* indicates the number of observed relapses in the corresponding group.

ACKNOWLEDGEMENTS

We thank the HAT team of the Institut Pierre Richet in Bouaké and the National Control Program for HAT of Côte d'Ivoire for their excellent technical support. This work was supported by a grant from the Fonds d'Aide à la Coopération du Ministère des Affaires Etrangères, Direction du Développement et de la Coopération, by the Belgian Directorate General for International Cooperation and by International Atomic Energy Agency (IAEA); the Agence Française de la Francophonie (AUF) and the Ministère de l'Education Nationale et de la Recherche. We thank G. Manners for editorial advice.

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EVALUATION OF DIFFERENT PRIMERS AND DNA PREPARATIONS FOR MOLECULAR DIAGNOSIS OF HUMAN AFRICAN TRYPANOSOMOSIS

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Abstract

Dans cette étude, afin dessayer d'améliorer le diagnostic de la Trypanosomose Humaine Africaine (THA, ou maladie du sommeil), nous avons utilisé la PCR sur sang en comparant 2 méthodes différentes d'extraction/purification d'ADN (chelex et DNA easy), et plusieurs amorces PCR (TBR 1-2, TgsGP, ESAG 6-7). Les comparaisons ont porté sur des sujets prélevés lors de prospections médicales dans le centre-ouest de la Côte d'Ivoire et au Bénin: des malades (Trypanosomes vus en parasitologie), des sujets positifs aux tests sérologiques mais négatifs aux tests parasitologiques (appelés séropositifs), des sujets négatifs en sérologie et parasitologie, et des volontaires sains vivant en Europe. La meilleure combinaison a été l'amorce TBR utilisée avec le Chelex. Dans ces conditions, la PCR est un test simple, facilement utilisable dans un laboratoire tropical équipé, plus sensible que les examens parasitologiques utilisés, et pourrait aider à la détection précoce des cas, à la décision de traiter un malade, et au suivi post-thérapeutique pour détecter précocément des cas de rechute. Toutefois, les limites de spécificité et son coût encore élevé restreignent encore son utilisation aux laboratoires de recherche.

1. INTRODUCTION

Dans le cadre de ce CRP, nous avons déjà comparé plusieurs méthodes de prélèvement de sang (sang total et buffy-coat) en utilisant le Chelex comme méthode d'extraction [1]. Les résultats ont montré une bonne sensibilité et une bonne spécificité de la technique PCR utilisée sur le sang pour le diagnostic de la THA, mais des problèmes de reproductibilité ont été rencontrés pour les sujets séropositifs non confirmés parasitologiquement. Il avait alors été proposé de tester d'autres amorces, de préférence spécifiques de *Trypanosoma brucei gambiense*, parasite responsable de la THA en Afrique de l'Ouest.

Nous avons ensuite continué d'utiliser la PCR avec le Chelex, mais sur le liquide cépahalo-rachidien des patients atteints de THA [2]. Nous avons montré que cette méthode était plus sensible que l'examen microscopique du LCR et le comptage des cellules, toutefois son intérêt dans le cadre du choix du traitement à administrer reste à être démontré.

En septembre 2002, une guerre est survenue en Côte d'Ivoire. En particulier des combats ont eu lieu à Bouaké, ville où est sise l'Institut Pierre Richet, et cette ville a dû être évacuée. Après plusieurs semaines de combat, les personnels de l'Institut ont pu être localisés. Des transferts de matériel ont pu être faits début 2003, et mi-2003 l'équipe « THA et glossines » était de nouveau opérationnelle à Abidjan, grâce au dévouement total du personnel et aux risques pris pour démanger les échantillons, et le matériel malgré la situation de guerre qui perdurait. Cette situation explique le retard pris par les activités. Nous présentons cependant ci-dessous les activités qui ont pu être menées malgré ces conditions dans le cadre du projet.

2. MATERIALS AND METHODS

2.1. Zone d'étude

Deux échantillonnages ont été effectués dans le foyer de THA du Centre-Ouest de la Côte d'Ivoire et dans le foyer de l'Atakora du Bénin. Ils ont été réalisés lors de prospections médicales faites en 2002 et 2003 en collaboration avec les Programmes Nationaux de Lutte Contre la THA de Côte d'Ivoire et du Bénin, ainsi que lors de la prise en charge au traitement de malades au niveau du Projet de Recherches Cliniques sur la THA (PRCT) de Daloa, et de la Base de Santé Rurale de Bouaflé, les 2 centres de traitement de la THA dans le foyer du Centre-Ouest ivoirien.

2.2. Echantillonnages

2.2.1. Echantillonnage 1

Une mL de sang hépariné a été prélevé au pli du coude de 38 malades (sujets T+, parasite mis en évidence par les tests parasitologiques), de 250 sujets séropositifs au CATT sur sang total et au CATT sur plasma mais négatifs aux tests parasitologiques (PL+, sujets que l'on qualifie de séropositifs), de 213 sujets positifs au CATT sur sang mais négatifs au CATT sur plasma (C+, sujets que l'on peut qualifier de « suspects » sérologiques), de 73 sujets négatifs en sérologie (C-), et de 30 sujets français volontaires n'ayant jamais résidé en Afrique (sujets sains, H).

2.2.2. Echantillonnage 2

Une mL de sang hépariné a été prélevé au pli du coude de 12 malades (sujets T+, parasite mis en évidence par les tests parasitologiques), de 82 sujets séropositifs au CATT sur sang total et au CATT sur plasma mais négatifs aux tests parasitologiques (sujets Pl+), sur 99 sujets suspects (C+), et sur 32 sujets négatifs en sérologie (C-).

2.3. Extraction de l'ADN, Protocole PCR

2.3.1. Echantillonnage 1

Une purification d'ADN a été réalisée sur résine Chelex (Chelating Ion Exchange Resin) [3]; qui est une résine chélatante ayant une haute affinité pour les ions métalliques divalents. Le Chelex capte ces ions les empêchant d'inhiber la réaction d'amplification. C'est une méthode simple et rapide de purification de l'ADN applicable aux conditions de terrain [1] Pour les échantillons de sang, 1 mL d'eau stérile purifiée est ajouté au 1 mL de sang. Le mélange est maintenu à température ambiante, vortexé toutes les 2 min pour obtenir une lyse complète des cellules puis le mélange est centrifugé à 15000 tr/min pendant 4 minutes [4]. Le surnageant est éliminé, le culot remis en suspension (vortex) dans 100 µL d'une solution de Chelex 1% (Chelex 100 Resin, Bio-Rad Laboratories, CA, USA) puis incubé 1 h à 56°C puis 30 min à 95°C. Les tubes sont ensuite centrifugés 5 min à 15000 tr/min. L'ADN purifié est présent dans le surnageant. La PCR a été effectuée avec 3 couples d'amorces:

TBR1-T BR2, spécifiques de *T.brucei s.l.* [5]; qui amplifient un fragment de 177 pb. La PCR a été réalisée selon la méthode décrite par [4].

ESAG6-ESAG7, aussi spécifiques de *T.brucei s.l.* [6]; qui amplifient un fragment de 286 pb. La PCR a été réalisée selon la méthode décrite par [6].

TgsGP, spécifique de *T. b. gambiense* [7]; qui amplifient un fragment de 308 pb. La PCR a été réalisée selon la méthode décrite par [8].

Pour tester la reproductibilité du protocole, chaque PCR a été réalisée en triplicate.

2.3.2. Echantillonnage 2

Une extraction d'ADN a été réalisée avec le kit DNeasy (Qiagen). L'extraction DNeasy a été effectuée à partir de 0.5 mL de sang en appliquant le protocole fourni avec le kit. Pour cet échantillonnage, les deux méthodes d'extraction utilisées (Chelex et DNeasy) ont été comparées.

3. RESULTS

3.1. PCR échantillon 1

Le couple d'amorce ESAG 6 et 7 n'a été testé que sur les 10 sujets T+ et les 10 sujets S de l'échantillonnage 1. Aucun produit d'amplification n'a été mis en évidence sur les 20 échantillons testés (seuls les stocks de référence ont montré un signal spécifique). Comme suggéré par les auteurs, une seconde PCR a été effectuée à partir des produits d'amplification de la première, mais là encore, seuls les stocks de référence ont donné un résultat positif.

Le couple d'amorce TgsGP a été testé sur l'ensemble des échantillons de l'échantillonnage 1. Là encore, aucun produit d'amplification n'a été mis en évidence sur les échantillons testés (seuls les stocks de référence ont montré un signal spécifique). Comme suggéré par les auteurs, une seconde PCR a été effectuée à partir des produits d'amplification de la première, mais là encore, seuls les stocks de référence ont donné un résultat positif. Avec TBR1/2, tous les échantillons de l'échantillonnage 1 (purification Chelex) ont été testés. Les échantillons de sang apparaissant 3 fois négatifs ou 3 fois positifs sont comptabilisés respectivement dans la colonne - et dans la colonne +, les échantillons dont le résultat diverge selon l'expérience sont comptabilisés dans la colonne +/-.

TABLEAU I. RESULTATS DE PCR TBR1/2 EN TRIPLICATE DES ECHANTILLONS DE SANG (ECHANTILLONNAGE 1)

	PCR (%)		
	+	+/-	-
38 T+	36 (95)	2 (5)	0
250 Pl+	38 (15)	55 (22)	157 (63)
213 C+	9 (4)	32 (15)	172 (81)
73 C-	1 (1)	3 (4)	69 (95)
30 H	0	0	30 (100)

Les 30 sujets H apparaissent négatifs en PCR. Sur les 38 malades (T+), 36 sont positifs lors des 3 essais PCR, et 2 sont douteux (au moins une PCR négative sur les 3 essais). Parmi les 463 sujets négatifs en parasitologie mais ayant présenté une agglutination au CATT (sur sang, et/ou sur plasma), 329 (soit 71%) sont négatifs en PCR, représentant probablement de faux-positifs au CATT. 47 d'entre eux (10%) sont positifs en PCR, ce qui montrerait une plus forte sensibilité que la parasitologie ou de possibles, mais peu probables, infections temporaires par *T.b.brucei*. Sur les sujets séronégatifs, 1/70 apparaît positif en PCR, et 3 douteux, ce qui peut s'expliquer soit par une fausse positivité, soit par des infections récentes où les anticorps ne sont pas encore présents. Sur toutes les catégories de sujets (604 au total), 92 (15%) ont présenté des

discordances, donc un résultat douteux, ce qui n'est d'aucune aide pour la décision thérapeutique. Il faut noter que sur ces 92 douteux, 87 (soit 95%) sont des sujets ayant présenté une réaction positive en sérologie, mais négative en parasitologie.

3.2. Comparaison CHELEX , DNeasy, échantillonnage 2

TABLEAU II. COMPARAISON DNEASY, ECHANTILLONNAGE 2

PCR	Chelex			DNeasy®		
	+	+/-	-	+	+/-	-
12 T+	11 (92)	1 (8)	0	12 (100)	0	0
82 Pl+	7 (9)	14 (17)	61 (74)	5 (6)	20 (24)	57 (70)
99 C+	1 (1)	8 (8)	90 (91)	5 (5)	14 (14)	80 (81)
32 C-	1 (3)	0	31 (97)	0	3 (9)	29(91)

Sur les 12 malades (T+), les 12 ont donné un résultat positif avec le DNeasy, 11 ont été positifs avec le chelex et 1 douteux en triplicate. Sur les séronégatifs, les résultas sont aussi très proches, ainsi que d'ailleurs sur les suspects ou séropositifs non confirmés parasitologiquement, dont les résultats douteux concernent respectivement 10% (chelex) et 16% (DNeasy) des sujets. Là encore, ces résultats discordants se localisent à plus de 90% chez les sujets ayant présenté une réaction positive au CATT mais non confirmés parasitologiquement. Aucune des différences entre les 2 méthodes n'est significative.

4. DISCUSSION AND CONCLUSION

Nos résultats confirment ceux d'études précédentes sur la meilleure sensibilité de la PCR (sur sang) par rapport aux tests parasitologiques [1]; [2]; [6]; [8]; [9], et la meilleure spécificité par rapport au CATT test qui génère beaucoup de faux-positifs. Il faut signaler que la bonne sensibilité de la PCR conduit à son l'utiliser de plus en plus comme méthode de diagnostic dans d'autres maladies parasitaires, par exemple la leishmaniose cutanée et viscérale, le paludisme, la maladie de Chagas. En matière de THA, son intérêt principal pour le diagnostic se situe notamment pour le diagnostic précoce et le suivi post-guérison, étant donnés la présence fréquente de porteurs asymptomatiques, les parasitémies souvent faibles et fluctuantes, et la difficulté d'établir un diagnostic de guérison avec les techniques classiques.

Dans notre étude, la PCR avec les amorces TBR a semblé être un test spécifique puisque tous les sujets sains n'ayant jamais habité en zone d'endémie se sont révélés négatifs, ainsi que 95% des sujets C- (sujets sérologiquement négatifs mais vivant en zone d'endémie). Nous avons observé, à l'instar d'autres études d'autres avant [1] ; [10], que des problèmes de reproductibilité se posent sur les sujets séropositifs non confirmés parasitologiquement, pour lesquels d'ailleurs la décision de traiter ou non fait toujours l'objet de controverses. Une des raisons (mais peut-être pas la seule) des fausses positivités observées avec les amorces TBR pourrait être liée au fait que TBR

est spécifique de *Trypanosoma brucei* sensu lato, y compris *T.b.brucei*, considéré non pathogène pour l'homme, mais qui pourrait causer des infections temporaires. Une autre possibilité serait constituée par un portage de parasites à très faible parasitémie qui ne permettrait pas la détection d'un amplicon à chaque réaction.

Il serait donc souhaitable d'avoir des amorces spécifiques de *T.b.gambiense*, et c'est dans ce sens que nous avons testé 2 amorces récemment décrites (TgsGP et ESAG). Malheureusement aucun échantillon de terrain, y compris provenant de malade, n'a donné d'amplification avec ces amorces. Il n'est pas impossible que la résine utilisée pour enlever les inhibiteurs de Taq Polymerase (Chelex) ne soit pas adaptée à des PCR de ce type, pour des détails que nous ignorons. L'utilisation du DNeasy comparée au Chelex n'a pas entraîné d'amélioration avec les amorces TBR. Un autre inconvénient des amorces décrites spécifiques de *T.b.gambiense* réside dans le fait que 2 PCR successives sont nécessaires pour obtenir un bon résultat selon leurs auteurs. Nous pensons que dans un contexte tropical, et sur une maladie comme la THA qualifiée de 'maladie du bout de la piste', si la PCR doit être utilisée, il faut essayer de simplifier au maximum les procédures de prélèvement, d'extraction, et d'amplification, pour un meilleur prix possible. Actuellement, la combinaison Chelex et TBR semble bien adaptée en tenant compte des paramètres tropicaux y compris le coût, même s'il est souhaitable d'obtenir une meilleure spécificité. Actuellement, cette technique peut constituer une aide à la décision thérapeutique, mais ne peut pas, à elle seule provoquer la décision. De nouveaux marqueurs semblent nécessaires afin d'affiner le diagnostic, ainsi qu'une simplification des procédures de prélèvement sanguin.

ACKNOWLEDGEMENTS

To the National Control Programmes of Côte d'Ivoire and Bénin, and the technical staff of Institut Pierre Richet and Programme de Recherche Clinique sur la Trypanosomiase de Daloa. To IRD and Joint FAO/IAEA Division for support.

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MOLECULAR DIFFERENTIAL DIAGNOSIS OF AFRICAN TRYPANOSOMOSIS IN UGANDA

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Abstract

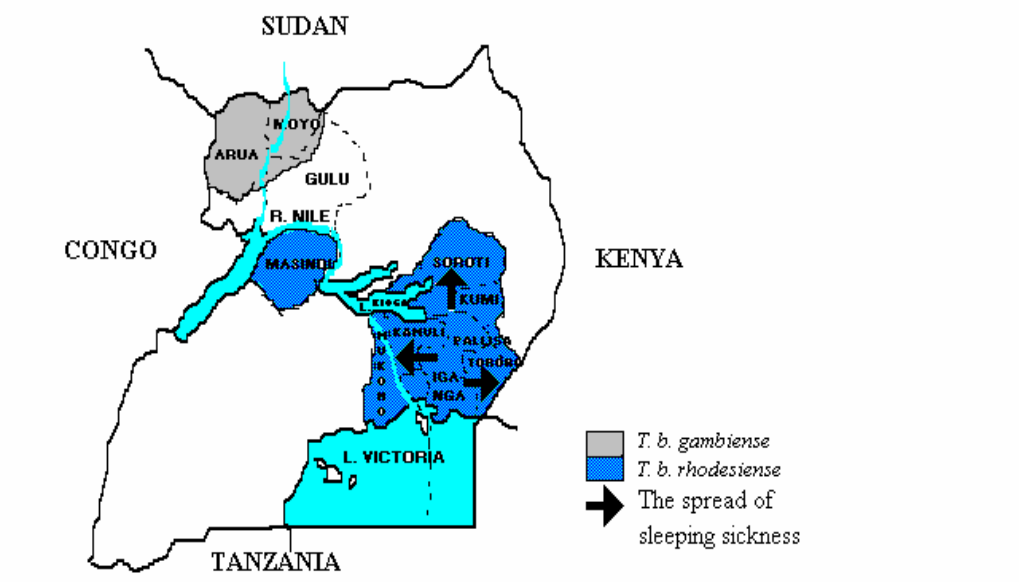
Out of 106 Trypanosomal DNAs from infected domestic animals, 77 (72.6%) were resolved into different Trypanosome species using primers based on ribosomal internal transcribed spacer-1 region in a single PCR (ITS-PCR). The remaining 29 (27.4%) Trypanosomal DNA samples were negative by ITS-PCR, but were positive by TBR-PCR, indicating that they were *Trypanozoon* Trypanosomes where the ITS-PCR could not resolve their identities. This was possibly due to the low amounts of DNA in the extracts which could not be picked up by ITS-PCR. The TBR-PCR was able to resolve all 106 Trypanosome isolates into 88 *Trypanozoon* and 18 others, which were identified and confirmed as *T.congolense* (6) and *T.vivax (*12) Trypanosomes. In addition, a total of 58 (89.2%) of the 65 (53 from cows and 12 from pigs) Trypanosome isolates from domestic animals in *T.b.gambiense* endemic areas in North West Uganda were positive by TBR-PCR, indicating that they were *Trypanozoon* Trypanosomes while 7 (10.8%) of the Trypanosome isolates were TBR-PCR negative, indicating that they were possibly not *T.brucei* ssp and could be *T.congolense, T.vivax* or *T.theileri*. Indeed, the 7 Trypanosome isolates were confirmed to be *T.congolense* (2) and *T.vivax* (5) using species specific primers. Furthermore, 31 of the 58 TBR-PCR positive Trypanosomal DNA samples analysed were TgsGP-PCR negative, indicating that they were not *T.b.gambiense* and hence no domestic animals identified as reservoir of *T.b.gambiense*. Additionally, all the 31 Trypanosomal DNA samples were SRA-PCR negative, indicating that there is probably no mixed infection of the two diseases, *T.b.gambiense* and *T.b.rhodesiense* in North West Uganda. Analysis of Trypanosomes derived from domestic animals from *T.b.rhodesiense* endemic areas in South East Uganda showed that, 79 (90.8%) of the 87 Trypanosomes isolated from cattle were positive by TBR-PCR, indicating that they were *Trypanozoon* while 8 (9.2%) were negative, suggesting that they could be *T.vivax, T.congolense* or *T.theileri*. When subjected to SRA-PCR, 10 (11.5%) of the 87 Trypanosomes isolates derived from cattle were positive, indicating that they could be *T.b.rhodesiense* circulating in cattle which is similar to the percentage of *T.b.rhodesiense* previously obtained in cattle in Serere, Soroti district. Some of the SRA-PCR negative Trypanosomes could be *T.b.rhodesiense* since this technique appears to miss some of the parasitologically confirmed cases of *T.b.rhodesiense* sleeping sickness which could be due to modified SRA genes or loss of the SRA genes from the expression sites in which they reside during the gene rearrangements associated with antigenic variation.

1. INTRODUCTION

The control of African Trypanosomosis in both animals and humans is difficult, partly because of the low sensitivity of the routine parasitological techniques used to diagnose the disease are based on the demonstration of Trypanosomes in the body fluids (blood, lymph node aspirate and cerebral spinal fluid). However, in the seventies concentration methods such as the haematocrit centrifugation technique (HCT) [14]; and the buffy coat technique (BCT) [9]; were developed using a haematocrit centrifuge. These techniques improved the diagnosis of Trypanosomosis and more infected animals and humans with Trypanosomes were detected. Although the specificity of these

techniques was high (over 96%) with very few false positives, the sensitivity was still low with a detection limit reported to be between 100 and 1000 Trypanosomes /mL.

With better methods of isolating minute amounts of DNA from infected blood samples, it may now be possible to detect and distinguish by polymerase chain reaction (PCR) technique human infective Trypanosomes from non human infective Trypanosomes all of which may be present in livestock. PCR has already been applied to the identification of *T.congolense* and *T.brucei* in rodent blood and tsetse flies [7]; and in *T.b.gambiense* ([1]; [4]; [6]. It is noted that PCR has allowed an increase in the



sensitivity and specificity of diagnosis of Trypanosomosis in tsetse flies and in animals. However, because of the diversity of *Trypanosoma* species potentially present in a single host, PCR diagnosis carried out on host material requires several PCR reactions. This has now been circumvented by the development of a multi-species-specific PCR diagnostic protocol for a single PCR [2]; and [3]. The single PCR using Pan-Tryp primers based on the ribosomal internal transcribed spacer-1 (ITS1) region is used in the diagnosis of mixed infections of animal Trypanosomosis in domestic animals [2]. Presently, there are also primers already designed for the differentiation of *T.b.rhodesiense* from *T.b.brucei* using Serum Resistance Associated (SRA) gene [5]; and [11]. Furthermore, subspecies specific primers for *Trypanosoma gambiense* surface glycoprotein (TgsGP) gene has been developed [12]; for the detection and differentiation of *T.b.gambiense* from *T.b.rhodesiense* and *T.b.brucei*. In Uganda, *T.b.rhodesiense* and *T.b.gambiense* sleeping sickness foci have persisted since the colonial times. Despite efforts by Uganda government and Non Governmental Organizations (NGOs) to address this problem, sleeping sickness has persisted and continue to spread to other areas within the country and, sooner than later, the Rhodesiense focus in South East Uganda may merge with this Gambiense focus (Map shown above).

Despite the traditional thinking that man is his own reservoir host for the Gambiense sleeping sickness, there are indications that there are other hosts besides

man. Unfortunately, little has been done in Uganda to study the animal reservoirs of Gambiense sleeping sickness. However, there are many published reports on animal reservoirs of Rhodesiense sleeping in Uganda but nothing has been documented on the role played by domestic animals in the transmission Rhodesiense sleeping sickness in the new focus in Kaberamaido, north east Uganda. To assess the risk presented to the human population by their domestic animals, it is necessary to distinguish human infective *T.b.rhodesiense* from non-human infective *Trypanosoma brucei brucei*, both of which may be present in livestock. Although the PCR technique is highly sensitive and specific in diagnosis of Trypanosomes, it is seldom used in routine diagnosis because it involves carrying out several PCR tests for each sample under test, resulting in high costs of approximately US $8 per test. Therefore a need for a single PCR test that detects several pathogenic animal Trypanosomes was realised in order to reduce costs (approximately $0.5 per test) while maximising on the high sensitivity and specificity of the test. Hence, a PCR assay based on the Internal Transcribed 1 (ITS-1) of rDNA to detect several livestock Trypanosome infections in a single reaction using one pair of primers was developed. The PCR assay based on ITS-1 primers has been optimised and tested in West Africa and was also tested in Uganda using freshly collected field samples in order to diagnose mixed infections of animal Trypanosomosis in the new focus of sleeping sickness in Kabearamaido district.

Furthermore, since *T.b.gambiense* and *T.b.rhodesiense* Trypanosomes show differential drug sensitivity, a correct differential diagnosis of the two parasites is necessary for a successful drug treatment of sleeping sickness. It is, therefore, necessary to further evaluate the serum resistance associated (SRA) gene based PCR and *T.b.gambiense* surface glycoprotein (TgsGP) gene based PCR for the detection and identification of *T. b.rhodesiense* and *T.b.gambiense* respectively in different parasite foci. The two PCR tests were tested using freshly collected field Trypanosome isolates to confirm the reliabilities of the two molecular techniques in the diagnosis of sleeping sickness.

2. MATERIAL AND METHODS

2.1. Experimental design

2.1.1. Animal and human Trypanosomosis surveys in South East Uganda

A total of 35 Trypanosomes from humans and 87 from domestic animals were isolated and Trypanosomal DNA extracted for PCR analysis (Table I).

TABLE I. DNA EXTRACTS FROM TRYPANOSOMES ISOLATED FROM DOMESTIC ANIMALS AND HUMANS IN SOUTH EAST UGANDA

Origin	DNA extracts from Trypanosomes isolated from		Total
	Domestic animals	Humans	
Serere	46	11	57
Tororo	6	22	28
Kaberamaido	30	2	32
Sembabule	5	0	5
Total	87	35	122

2.1.2. Animal and human Trypanosomosis field survey in North West Uganda

Three animal and one human Trypanosomosis field surveys were made in Arua and Moyo districts to isolate Trypanosomes from infected domestic animals and humans. A total of 1,398 domestic animals (981 cattle, 192 goats, 84 pigs, 89 sheep and 60 dogs) had their blood examined by the wet thin smear and the haematocrit centrifugation technique (HCT) for the presence of Trypanosomes. All the Trypanosome positive animals were treated with samorin. Each infected blood sample was divided into 3 portions: one portion was inoculated into *Mastomys natalensis* rats for propagation, the second portion was used for DNA extraction and the third portion was stabilated using 10% glycerol in phosphate buffered saline glucose, pH 8.0. The stabilate was stored in liquid nitrogen for subsequent transport to Tororo laboratory. Trypanosomal DNAs were extracted from the infected blood samples for characterization using TBR, ITS-1, SRA and TgsGP primers by polymerase chain reaction technique. Similarly, 1,686 persons who came for screening were examined by HCT for the presence of Trypanosomes and 12 were diagnosed with sleeping sickness. All diagnosed sleeping sickness cases were referred to treatment centres.

2.1.3. Preparation of DNA

The QIAamp DNA blood Mini kit extraction protocol (Qiagen) was used in preparing DNA for PCR by mixing 200 µL of blood and 200 µL lysis buffer (Qiagen) in a microcentrifuge to which had 20 µL of proteinase K and vortex for 15 s.

The mixture was incubated at 56^{o}C for 10 min followed by addition of 200 µL of ethanol (100%); vortex for 20 s.

After mixing, the mixture was centrifuged at 6,000 g (8,000 rpm) for 30 s to remove drops from the inside of the lid.

The mixture was carefully applied on to the QIAamp spin column in a 2 mL collection tune and centrifuged at 6,000 g (8,000 rpm) for 1 min, the supernatant discarded and column transferred into another clean microcentrifuge tube.

The QIAamp spin column was carefully opened and 250 µL buffer AW1 were added, centrifuged at 6,000 g (8,000 rpm) for 1 min and the filtrate discarded. This procedure was repeated with AW2 buffer, but centrifuged at 20,000 x g (14,000 rpm).

To the QIAamp spin column in a clean microcentrifuge was added 200 µL AE elution buffer, incubated at room temperature for 1 min and centrifuged 6,000 x g (8,000 rpm) for 1 min.

The DNA collected into 200 µL AE elution buffer (Qiagen) was precipitated using 20 µL of 3 *M* sodium acetate pH 5.2 and 400 µL of pre-chilled 100% ethanol and centrifuged at 14,000 rpm for 3 min. The pellet was re-suspended in 100 µL of distilled water.

2.1.4 Polymerase chain reaction (PCR) using ITS1 primers

A polymerase chain reaction that amplifies DNA of all the five pathogenic mammalian Trypanosomes using ITSC-F and ITSB-R primers was used, as described in the protocol sent by Dr. Alberto Davilla. The primers were

ITS1C-F = $^{5'}$ CCG GAA GTT CAC CGA TAT TG $^{3'}$ and

ITS1-BR = $^{5'}$ TTG CTG CGT TCT TCA ACG AA$^{3'}$

Briefly, the PCR was carried out in 25 µL reaction mixture containing as final concentrations of 10 m*M* Tris-HCl pH 8.3, 75 m*M* KCl, 3.5 m*M* $MgCl_2$, 200 µ*M* each of the four deoxynucleotide triphosphates (dNTPS), primers at 0.4 µ*M*, 2.25 µg BSA and 0.75 U of Taq DNA polymerase. The amplification cycle was for initial step at 95^{o}C for 5 min, followed by 40 cycles of denaturation at 95^{o}C for 1 min, annealing at 58^{o}C for 1min and extension at 72^{o}C for 1 min with a final elongation at 72^{o}C for 10 min. Fifteen µL of each sample was analysed by gel electrophoresis using 2% agarose gels stained with ethidium bromide and viewed under UV illuminator.

2.3. Polymerase chain reaction for *T.brucei* subspecies

The DNAs from Trypanosome isolates from domestic animals were amplified by TBR1 using 2 primers:

TBR1

$^{5'}$ CGA ATG AAT ATT AAA CAA TGC GCA GT $^{3'}$

TBR2

$^{5'}$ AGA ACC ATT TAT TAG CTT TGT TGC $^{3'}$

For *T.brucei* subspecies, to ascertain whether the Trypanosomes derived from domestic animals were, indeed, *T.brucei* subgroup Trypanosomes before PCR involving TgsGP primers specific for *T.b.gambiense* was done. It also served as away of estimating the amount of DNA in the solution and hence what volume to use in the PCR. The PCR amplification was carried out as described by [10]; in 25 µL reaction mixture. The reaction mixture contained final concentrations of 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1% (v/v) Triton X-100, 200 µ*M* each of dATP, dCTP, dGTP, and dTTP, 0.1 µ*M* each of the 5' and 3' primers, 50 ng DNA template or 2 µL crude DNA preparation and 1 unit of Taq DNA polymerase. The amplification started with the initial denaturation step at 94^{o}C for 3 min and subjected to 35 cycles involving denaturation for 1 min at 94^{o}C, annealing at 56^{o}C for 1 min, and extension at 72^{o}C for 1 min and final elongation of 5 min at 72^{o}C. The absence of contaminants was routinely checked by inclusion of negative control samples in which the DNA sample was replaced with sterile water. Fifteen microlitres of each sample alongside control and marker DNA were electrophoresed in 2.0% w/v agarose gel containing 1 µg/ml ethidium bromide.

2.4. Polymerase Chain Reaction using primers specific for *T.b.gambiense*

A PCR assay using primers specific for *T.b.gambiense* surface glycoprotein (TgsGP) gene was conducted to detect the presence/absence of *T.b.gambiense* in domestic animals derived Trypanosomes as described in [12].

The primer used were: Tbg-R = $^{5'}$ GCT GCT GTG TTG GGA GAG C $^{3'}$

and Tbg-F = $^{5'}$ GCC ATC GTG CTT GCC GCT C $^{3'}$

PCR amplification was performed using 50 ng of DNA extracted from purified *T.b.gambiense* Trypanosomes as control DNA, or 10 µL of DNA extracted from

domestic animal blood. The DNA amplification was carried out in 25 µL reaction mixture containing final concentrations of 20 m*M* Tris-HCl, pH 8.7, 100 m*M* KCl, 50 m*M* $(NH4)_2$ SO_4, 1.5 m*M* $MgCl_2$, 200 µ*M* each of dATP, dCTP, dGTP and dTTP, 0.5 µ*M* each primers and 2.5 units of HotStar Taq DNA polymerase. TgsGP-PCR was performed using a GeneAmp PCR System 9700 from Applied Biosystems with the initial incubation for 15 min at 95^{o}C, followed by 45 cycles involving denaturation for 1 min at 94^{o}C, annealing at 63^{o}C for 1 min, extension at 72^{o}C for 1 min; and a final extension for 10 min at 72^{o}C. The absence of contaminants was routinely checked by inclusion of negative control samples in which the DNA sample was replaced with sterile water. All samples were subjected to an initial PCR, followed by a second PCR using 1 µL of the first PCR product in order to increase sensitivity. Twenty microlitres of each sample were electrophoresed in a 2% agarose containing 1 µg/mL ethidium bromide. The amplified products were observed by UV transillumination.

2.5. Polymerase Chain Reaction using *T.b.rhodesiense* specific primers

PCR assay using primers specific for *T.b.rhodesiense* serum resistance associated (SRA) gene was conducted to identify *T.b.rhodesiense* as described by [11].

Primers were:

SRA-R = $^{5'}$ AAT GTG TTC GAG TAC TTC GGT CAC GCT $^{3'}$

SRA-F = $^{5'}$ ATA GTG ACA AGA TGC GTA CTC AAC GC $^{3'}$

PCR amplification was performed using 50 ng of DNA extracted from purified *T.b.rhodesiense* Trypanosomes as control DNA, or 10 µL of DNA extracted from domestic animal blood. The DNA amplifications was carried out in 25 µL reaction mixture containing final concentrations of 20 m*M* Tris-HCl, pH 8.7, 100 m*M* KCl, 50 m*M* $(NH_4)_2SO_4$, 1.5 m*M* $MgCl_2$, 200 µ*M* each of dATP, dCTP, dGTP, and dTTP, 0.5 µ*M* each primers and 2.5 units of HotStar Taq DNA polymerase. SRA-PCR was performed using a GeneAmp PCR System 9700 from Applied Biosystems with the initial incubation for 15 min at 95^{o}C, followed by 45 cycles involving denaturation for 1 min at 94^{o}C, annealing at 68^{o}C for 1 min, extension at 72^{o}C for 1 min; and a final extension for 10 min at 72^{o}C.

The absence of contaminants was routinely checked by inclusion of negative control samples in which the DNA sample was replaced with sterile water. All samples were subjected to an initial PCR, followed by a second PCR using 1 µL of the first PCR product in order to increase sensitivity. Twenty microlitres of each sample were electrophoresed in a 2% agarose containing 1 µg/ml ethidium bromide. The amplified products were observed by UV transillumination.

3. RESULTS

3.1. Animal and human Trypanosomosis surveys

The infection rates of animal Trypanosomosis varied from different domestic animal species in each district in North West Uganda with a total of 57 cattle, 26 pigs, 3 sheep and 3 dogs diagnosed positive for Trypanosomes (Table II). In each district pigs appeared to be more susceptible to Trypanosome infection with infection rates of 39.6% in Arua-Omugo, 25.0% Arua-Koboko and 13.0% in Moyo-Metu.

TABLE II. SUMMARY OF THE SCREENING RESULTS FOR THE PRESENCE OF TRYPANOSOMES IN DOMESTIC ANIMALS FROM ARUA AND MOYO DISTRICTS, NORTH WEST UGANDA

Field work	District	Screen	Cattle	Goats	Pigs	Sheep	Dogs
1st Survey	Arua-Omugo	No. examined	259	160	53	63	36
		No. Positive Tryps	34	0	21	3	2
		Infection rate%	13.1	0	39.6	4.8	5.6
2nd Survey	Arua-Koboko	No. examined	236	4	8	12	8
		No. Positive Tryps	15	0	2	0	0
		Infection rate%	6.4	0	25.0	0	0
3rd Survey	Moyo-Metu	No. examined	486	28	23	14	16
		No. Positive Tryps	8	0	3	0	1
		Infection rate%	1.6	0	13.0	0	6.2
Total		No. examined	981	192	84	89	60
		No. Positive Tryps	57	0	26	3	3
		Infection rate%	5.8	0	30.9	6.4	5.0

3.2. Polymerase chain reaction using ITS-primers

A total of 106 Trypanosomal DNAs were extracted from 106 Trypanosome infected blood samples from domestic animals and analysed by ITS-PCR and TBR-PCR techniques (Table III). Twenty nine DNA samples were negative by ITS-PCR but positive by TBR-PCR possibly due low amount of DNA in the extract which could be picked up by TBR-PCR which is very sensitive, may be second ITS-PCR could using aliquot of the first PCR could be positive. However, TBR-PCR was able to resolve all the 106 Trypanosome isolates in 88 *Trypanozoon* and 18 others, which were identified and confirmed as 6 *T.congolense* and 12 *T.vivax* Trypanosomes.

TABLE III. RESULTS OF SCREENING DOMESTIC ANIMALS FOR ANIMAL TRYPANOSOMOSIS BY ITS-PCR

Domestic animals		ITS-PCR								TBR-PCR	
Species	No	+							-	+	-
		Tb	Tv	Tc	Tb+Tc+Tv	Tb+Tv	Tb+Tc	Tv+Tc			
Dogs	2	2	0	0	0	0	0	0	0	2	0
Pigs	22	18	3	1	0	0	0	0	0	18	4
Cattle	82	34	9	5	1	4	0	0	29	68	14
Total	106	54	12	6	1	4	0	0	29	88	18

3.3. Optimization of polymerase chain reaction techniques

Twenty-four Trypanosomal DNA extracts (22 *T.b.gambiense* and 2 *T.b.rhodesiense*) were used for optimizing the PCR condition for the 3 primers (TBR, TgsGP and SRA), Table IV. All the 24 DNA extracts were strongly TBR-PCR positive, indicating that they were *T.brucei* while only 21 DNA samples from *T.b.gambiense* were TgsGP-PCR positive, demonstrating the specificity of these primers in detecting and differentiating *T.b.gambiense*. Only the 3 DNA samples from *T.b.rhodesiense were*

SRA-PCR positive and negative with all DNA samples from *T.b.gambiense* (Table IV and Fig. 1).

3.4. Trypanosomes from domestic animals from North West Uganda

A total of 58 (89.2%) of the 65 (53 from cows and 12 from pigs) Trypanosome isolates from domestic animals were positive by TBR-PCR, indicating that they were *Trypanozoon* Trypanosomes; and 7 (10.8%) Trypanosome isolates were TBR-PCR negative, indicating that they were possibly not *T.brucei* ssp and could be *T.congolense, T.vivax* or *T.theileri*. From the 58 *Trypanozoon* Trypanosomes, 31 Trypanosome isolates from 25 cows and from 6 pigs were TgsGP-PCR negative, suggesting they were not from *T.b.gambiense* Trypanosomes (Table V and Fig. 2). Additionally, all the 31 Trypanosomal DNA samples were also SRA-PCR negative, indicating that they were not from *T.b.rhodesiense* Trypanosomes.

TABLE IV. RESULTS OF PCR STANDARDIZATION USING 3 DIFFERENT PRIMERS FOR AMPLIFICATION OF TRYPANOSMAL DNA

Serial No	Code No	Inoculum	Origin	Host	Tryp Species	TBR Primers	TgsGP primers	SRA primers
1	19	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
2	20	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
3	21	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
4	22	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
5	23	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
6	29	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
7	30	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
8	31	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
9	33	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
10	35	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
11	36	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
12	37	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
13	39	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
14	43	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
15	44	Procyclics	Adjumani	Human	*T.b.g*	+++	+++	-
16	M1	Blood stage2	Moyo	Human	*T.b.g*	+++	++	-
17	M2	Blood	Moyo	Human	*T.b.g*	+++	+	-
18	M3	Blood	Moyo	Human	*T.b.g*	+++	+	-
19	M4	CSF	Moyo	Human	*T.b.g*	+++	+	-
20	M5	CSF	Moyo	Human	*T.b.g*	+++	++	-
21	M6	Blood	Moyo	Human	*T.b.g*	+++	+	-
22	S1	Blood	Serere	Human	*T.b.rh*	+++	-	+++
23	S2	Blood	Serere	Human	*T.b.rh*	+++	-	+++
24	S3	Blood	Tororo	Human	*T.b.rh*	+++	-	+++

T.b.g. = *Trypanosoma brucei gambiense*; *T.b.rh* = *Trypanosoma brucei rhodesiense*
TBR = Primers for (Trypanozoon) *Trypanosoma brucei*
TgsGP = Primers for *Trypanosoma gambiense* surface glycoprotein gene
SRA = Primers for Serum resistance Associated gene
+++ = Very strongly positive;
++ = Strongly positive;
+ = weakly positive;
- = negative

TABLE V. SUMMARY OF THE RESULTS OF POLYMERASE CHAIN REACTION USING TBR, TGSGP AND SRA PRIMERS FOR THE AMPLIFICATION OF TRYPANOSOMAL DNA ISOLATED INFECTED DOMESTIC ANIMALS IN NORTH WEST UGANDA

Serial No	**Code No**	**Inoculum**	**Origin**	**Host**	**Tryp Species**	**TBR Primers**	**TgsGP primers**	**SRA primers**
1	365	Blood	Koboko	Cow	*T.brucei*	+++	-	-
2	367	Blood	Koboko	Cow	*T.brucei*	+++	-	-
3	364	Blood	Koboko	Cow	*T.brucei*	+++	-	-
4	371	Blood	Koboko	Cow	*T.brucei*	+++	-	-
5	369	Blood	Koboko	Cow	*T.brucei*	+++	-	-
6	368	Blood	Koboko	Cow	*T.brucei*	+++	-	-
7	363	Blood	Koboko	Cow	*T.brucei*	+++	-	-
8	362	Blood	Koboko	Cow	*T.brucei*	+++	-	-
9	370	Blood	Koboko	Cow	*T.brucei*	+++	-	-
10	366	Blood	Koboko	Cow	*T.brucei*	+++	-	-
11	310	Blood	Koboko	Cow	*T.brucei*	+++	-	-
12	361	Blood	Koboko	Cow	*T.brucei*	+++	-	-
13	227	Blood	Koboko	Cow	*T.brucei*	+++	-	-
14	228	Blood	Koboko	Cow	*Others*	-	-	-
15	229	Blood	Koboko	Cow	*Others*	-	-	-
16	230	Blood	Koboko	Pig	*Others*	-	-	-
17	231	Blood	Koboko	Pig	*T.brucei*	+++	-	-
18	232	Blood	Arua	Pig	*T.brucei*	+++	-	-
19	233	Blood	Arua	Pig	*T.brucei*	+++	-	-
20	234	Blood	Arua	Cow	*T.brucei*	+++	-	-
21	235	Blood	Arua	Cow	*T.brucei*	++	-	-
22	236	Blood	Arua	Cow	*T.brucei*	+	-	-
23	237	Blood	Arua	Cow	*T.brucei*	+	-	-
24	238	Blood	Arua	Cow	*T.brucei*	+	-	-
25	239	Blood	Arua	Pig	*T.brucei*	+		-
26	240	Blood	Arua	Cow	*T.brucei*	-	-	-
27	241	Blood	Arua	Cow	*T.brucei*	+	-	-
28	241	Blood	Arua	Cow	*T.brucei*	+	-	-
29	243	Blood	Arua	Cow	*T.brucei*	+	-	-
30	244	Blood	Arua	Cow	*T.brucei*	+	-	-
31	245	Blood	Arua	Pig	*T.brucei*	+	-	-

TBR = Primers for (*Trypanozoon*) *Trypanosoma brucei* subspecies
TgsGP = Primers for *Trypanosoma gambiense* surface glycoprotein gene
SRA = Primers for Serum resistance Associated gene
+++ = Very strongly positive
++ = Strongly positive
+ = Weakly positive
- = Negative

TABLE VI. RESULTS OF PCR AMPLIFICATION OF TRYPANOSOMAL DNA ISOLATED FROM INFECTED HUMANS FROM THREE DIFFERENT AREAS IN SOUTH EAST UGANDA

Origin	Host	No. of isolates examined	Primers used in PCR					
			TBR 1 and 2		SRA(Radwanska)		SRA (Gibson)	
			+	-	+	-	+	-
Serere	Humans	11	11	0	7	4	8	3
Tororo	Humans	22	22	0	20	2	18	4
Kaberamaido	Humans	2	2	0	1	1	1	1
Total (%)		35	35 (100)	0 (0)	28(80.0)	7(20.0)	27(77.2)	8(22.8)

TABLE VII. RESULTS OF PCR AMPLIFICATION OF TRYPANOSOMAL DNA ISOLATED FROM INFECTED DOMESTIC ANIMALS FROM 4 DIFFERENT AREAS IN SOUTH EAST UGANDA

Origin	Host	No. of DNA tested	Primers used in PCR					
			TBR 1 and 2		SRA(Radwanska)		SRA (Gibson)	
			+	-	+	-	+	-
Serere, north east Uganda	Domestic animals	46	39	7	6	40	7	39
Kaberamaido North East Uganda	Domestic animals	30	29	1	1	29	1	29
Sembabule in South Uganda	Domestic animals	5	5	0	0	5	0	5
Tororo in S.E Uganda	Domestic animals	6	6	0	3	3	2	4
Total (%)		87	79 (90.8)	8 (9.2)	10(11.5)	77 (88.5)	10(11.5)	77(88.5)

4. DISCUSSION AND CONCLUSION

ITS1-PCR could not differentiate all the 106 Trypanosome isolates into different Trypanosome species because 29 Trypanosome isolates were negative by ITS1-PCR, but positive by TBR-PCR. The 29 Trypanosome isolates were TBR-PCR positive, indicating that they were *Trypanozoon* Trypanosomes, but could not be amplified by ITS-PCR possibly due to low concentration of DNA in the extracts. The sensitivity of ITS-PCR could be improved by carrying out a second PCR using an aliquot of the first PCR or a bigger volume of infected blood samples is to be used to increase DNA concentration. However, TBR-PCR was able to resolve all the 106 Trypanosome isolates in 88 *Trypanozoon* and 18 others, which were identified and confirmed as 6 *T.congolense* and 12 *T.vivax* Trypanosomes.

Animal Trypanosomosis results from North West Uganda

The combined infection rate of animal Trypanosomosis in Arua and Moyo districts varied from 0 in goats, 5% in dogs, 58% in cattle, 6.4% in sheep and 30.9% in pigs, indicating that pigs were more susceptible to Trypanosome infection. A total of 58 (89.2%) of the 65 (53 from cows and 12 from pigs) Trypanosome isolates from domestic animals were positive by TBR-PCR, indicating that they were *Trypanozoon* Trypanosomes. However, 7 (10.8%) of the 65 Trypanosome isolates were TBR-PCR negative, indicating that they were possibly not *T.brucei* ssp and could be *T.congolense, T.vivax* or *T.theileri*. Indeed, the 7 Trypanosome isolates were confirmed to be *T.congolense* (2) and *T.vivax* (5) using species specific primers. Furthermore, the 31 from the 58 TBR-PCR positive Trypanosomal DNA samples were TgsGP-PCR negative, indicating that they were not *T.b.gambiense* and hence no domestic animals identified as reservoir of *T.b.gambiense*. Additionally, all the 31 Trypanosomal DNA samples so far tested were SRA-PCR negative, indicating that there is probably no mixed infection of the two diseases, *T.b.gambiense* and *T.b.rhodesiense* in this area.

Human Trypanosomosis -*T.b.rhodesiense* identification using SRA gene

The human serum resistance associated (SRA) gene was identified in 28 (80%) of the 35 *T.b.rhodesiense* Trypanosomes from parasitologically confirmed sleeping sickness cases, using the primers designed by [11] and in 27 (77.2%) of the same 35 *T.b.rhodesiense* Trypanosomes using the primers designed by Gibson *et.al* (Table VI). In Serere area 4 of the 11 *T.b.rhodesiense* isolates were SRA-PCR negative using primers as in [11] and three of the same isolates were again SRA-PCR negative using [5] primers (VI).

Similarly in Tororo area, 2 of the 22 *T.b.rhodesiense* isolates were SRA-PCR negative using [11] primers and four including the two of the same isolates were again SRA-PCR negative using [5]; primers (Table I). However, about 20% of the 35 *T.b.rhodesiense* Trypanosomes could not be detected by SRA-PCR even when an aliquot of the first PCR was used in the second PCR, indicating that the SRA gene may be absent in those Trypanosomes or the Trypanosomes could be having another variant of SRA not detectable by these primers since three variants of SRA genes have so far been identified or the amount of Trypanosomal DNA extracted from infected blood was too low to be detected. Since SRA genes resemble variable surface glycoprotein (VSG) genes, it may be that these isolates which are SRA-gene negative are indicative of some *T.b.rhodesiense* Trypanosomes with modified or lack SRA genes. The number of *T.b.rhodesiense* isolates which are SRA gene negative are too many to be accounted for as miss identification, but could be explained by simple loss of the SRA gene from the expression site in which it resides during antigenic variation.

Analysis of Trypanosomes derived from domestic animals showed that, 79 (90.8%) of the 87 Trypanosomes isolated from cattle (Table VII) were positive by TBR-PCR, indicating that they are *Trypanozoon* while 8 (9.2%) were negative, suggesting that they could be *T.vivax, T.congolense* or *T.theileri*.

When subjected to SRA-PCR, 10 (11.5%) of the 87 Trypanosomes isolates derived from cattle were positive, indicating that they could be *T.b.rhodesiense* circulating in cattle which is similar to the percentage of *T.b.rhodesiense* previously obtained in cattle in Serere, Soroti district. Some of the SRA-PCR negatives could be *T.b.rhodesiense* since this technique appears to miss some of the parasitologically

confirmed cases of *T.b.rhodesiense* sleeping sickness which could be due to modified SRA genes or loss of the SRA genes from the expression sites in which they reside during the gene rearrangements associated with antigenic variation.

5. CONCLUSIONS

ITS1-PCR could only resolve 72.6% of the 106-Trypanosome isolates into different Trypanosome species. These samples may require the use of an aliquot of the first PCR in the second PCR or increase the volume of infected blood sample used in the DNA extraction in order to increase the ITS1-PCR sensitivity. Trypanosome infection rate in domestic animals was highest in pigs (30.9%), followed by sheep (6.4%), cattle (5.8%) and dogs (5.0%).From the few isolates so far analysed, TgsGP primers appear to be specific for *T.b.gambiense* from North West Uganda. All the 31 Trypanosomal DNA samples from domestic animals were TgsGP-PCR negative, indicating that they are not *T.b.gambiense*; hence no domestic animals have been identified as harbouring pathogenic parasites to humans. So far no SRA genes found in *T.b.rhodesiense* Trypanosomes were detected in Trypanosomes from domestic animals from northwest Uganda, possibly no mixed infections of the two parasites. Screen the remaining DNA samples for presence of *T.b.gambiense* and isolate more Trypanosomes from domestic animals especially pigs, which appear to have more infections.

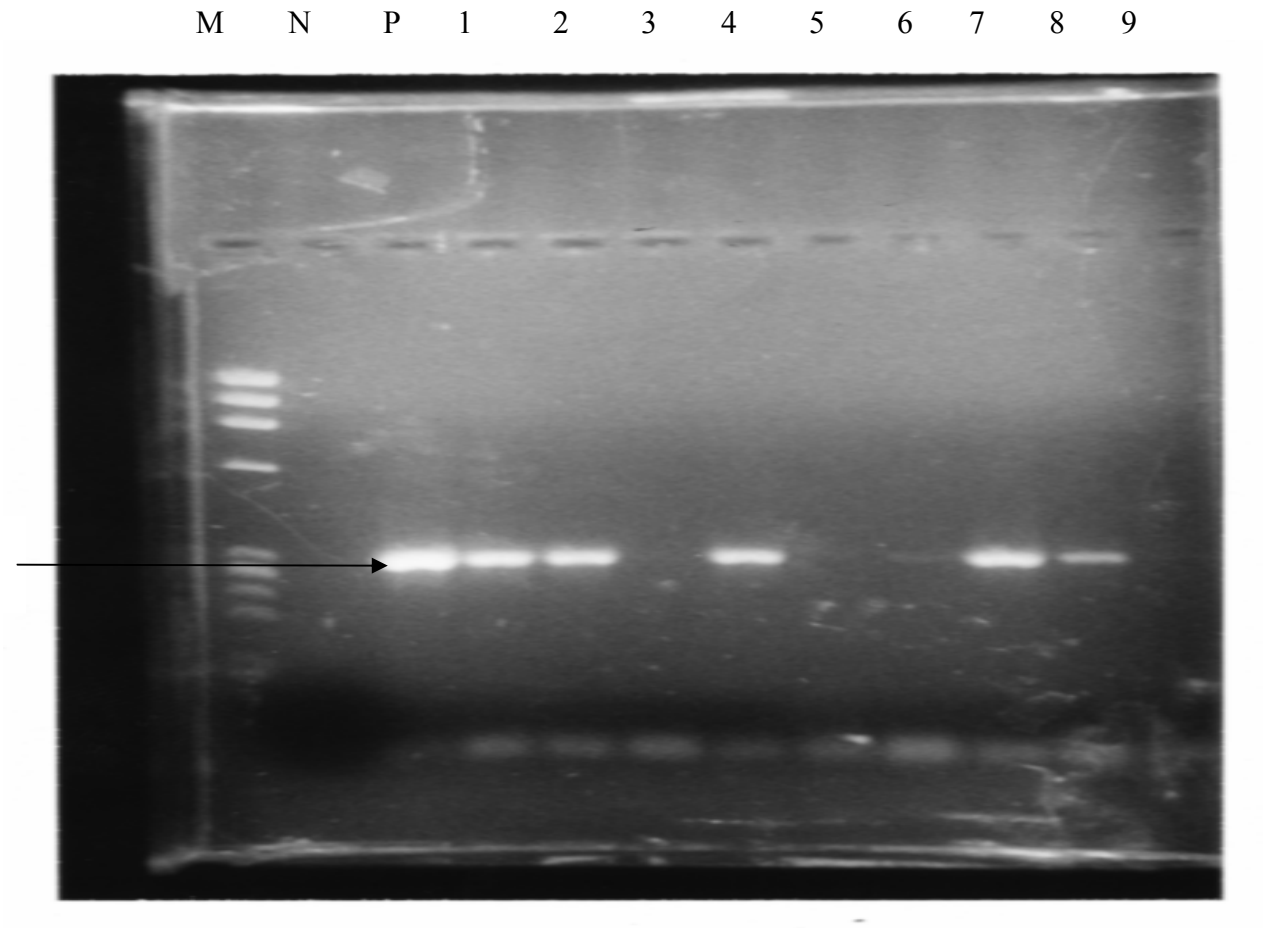


FIG. 1. Agarose gel electrophoresis showing results of standardization of TgsGP-PCR protocol for the detection of T.b.gambiense.

M = Marker; N = Negative control; P = Positive control; lanes 1-9, *T.b.gambiense* (lanes 3, 5, 6 and 9 are weakly positives while lanes 1, 2, 4, 7 and 8 are stronger positives)

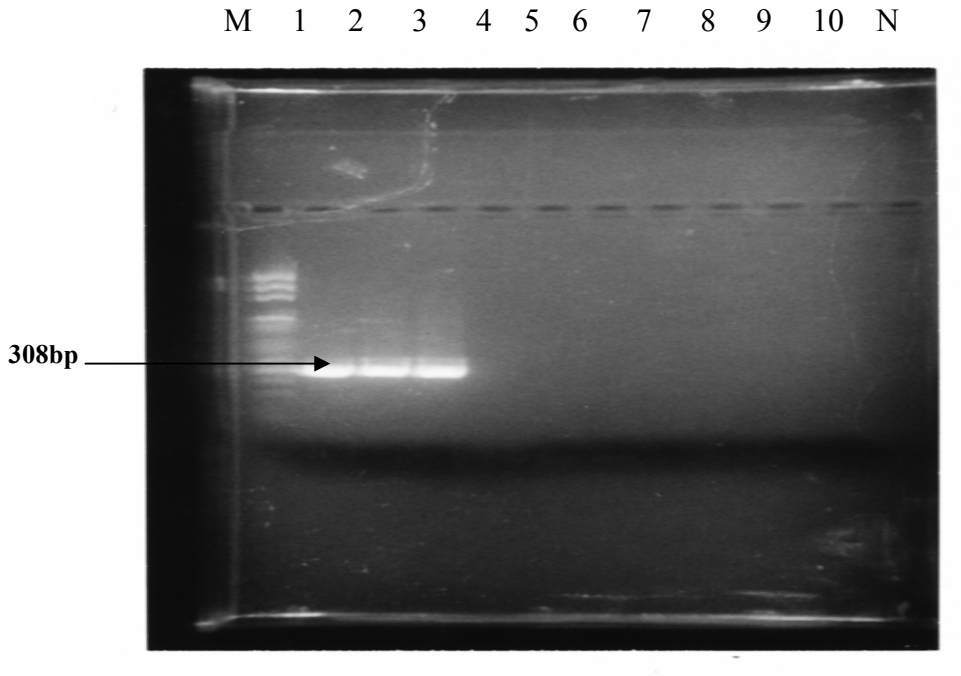


FIG. 2. GP-PCR for T.b.gambiense.
M = Marker; N = Negative control; Lane1-3 = *T.b.gambiense*, positive controls- Lanes 4-10 *T.brucei* from cattle (Arua)

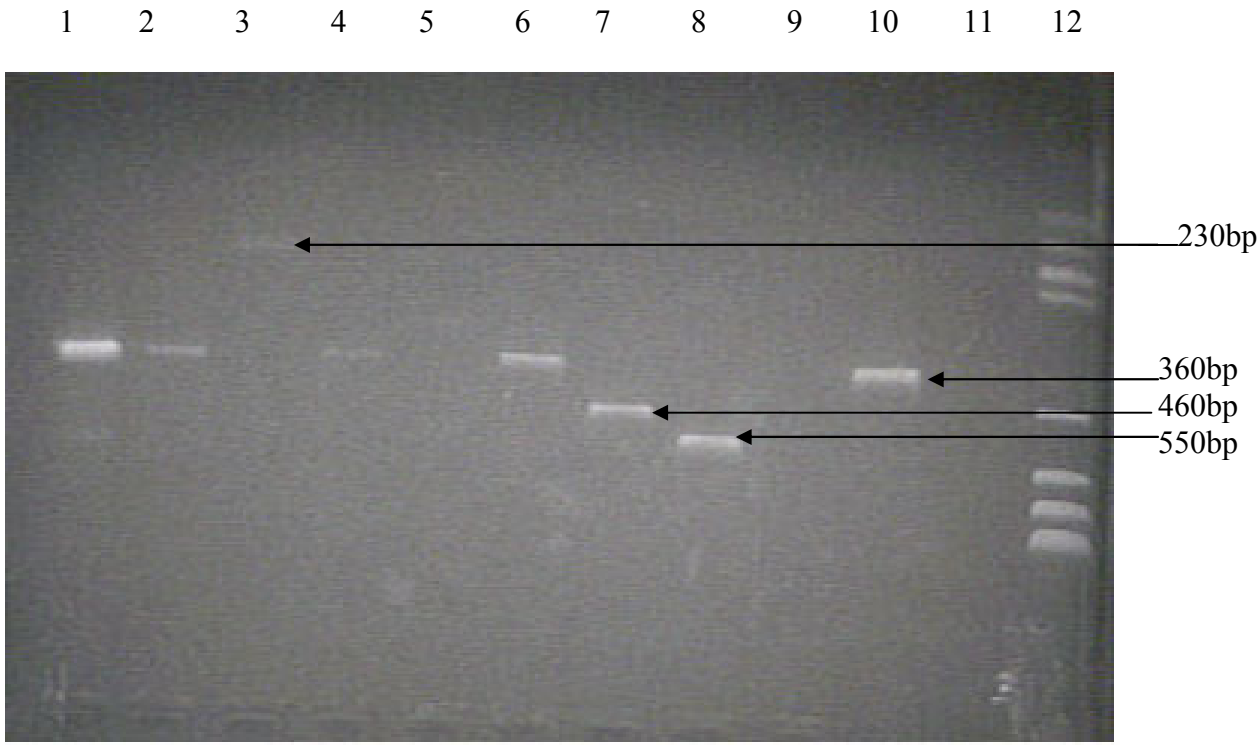


FIG. 3. Agarose gel electrophoresis showing results obtained using ITS-PCR for the detection of animal Trypanosomes from cattle in Sembabule district, Uganda.

Lane 1 = *T.brucei* from cow ; 2 = *T.brucei* from cow ; 3 = *T.vivax* from cow ; 4 = *T.brucei* from cow; Controls 5 = *T.simae*; 6 = *T.evansi*; 7 = *T.congo* kilifi; 8 = *T.congo* forest; 9 = *T.congo* savannah; 10 = *T.brucei*; 11 = Negative control; 12 = DNA Marker

ACKNOWLEDGEMENTS

Thanks to the Director of Livestock Health Research Institute (LIRI) Uganda, for logistic support and for allowing me to attend this meeting. I am gratefully indebted to IAEA for sponsorship the research activities. I thank all district and field staff and local council members for assistance in the research activities

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DETECTION OF T.B.RHODESIENSE TRYPANOSOMES IN HUMANS AND DOMESTIC ANIMALS IN SOUTH EAST UGANDA BY AMPLIFICATION OF SERUM RESISTANCE ASSOCIATED GENE

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Abstract

The human serum resistance associated (SRA) gene was identified in 28 (80%) of the 35 *T.b.rhodesiense* Trypanosomes from parasitologically confirmed sleeping sickness cases, using the primers designed by Radwanska and in 27 (77.2%) of the same 35 *T.b.rhodesiense* Trypanosomes using the primers designed by Gibson. However, about 20% of the 35 *T.b.rhodesiense* Trypanosomes could not be detected by SRA-PCR even when an aliquot of the first PCR was used in the second PCR, indicating that the gene may be absent in those Trypanosomes or the Trypanosomes could be having another variant of SRA not detectable by these primers since three variants of SRA genes have so far been identified or the amount of Trypanosomal DNA extracted from infected blood was too low to be detected. Since SRA genes resemble variable surface glycoprotein (VSG) genes, it may be that these isolates which are SRA-gene negative are indicative of some *T.b.rhodesiense* Trypanosomes with modified or lack SRA genes. The number of *T.b.rhodesiense* isolates which are SRA gene negative are too many to be accounted for as miss identification, but could be explained by simple loss of the SRA gene from the expression site in which it resides during antigenic variation. Analysis of Trypanosomes derived from domestic animals showed that, 79 (90.8%) of the 87 Trypanosomes isolated from cattle were positive by TBR-PCR, indicating that they are *Trypanozoon* while 8 (9.2%) were negative, suggesting that they could be *T.vivax, T.congolense* or *T.theileri*. When subjected to SRA-PCR, 10 (11.5%) of the 87 Trypanosomes isolates derived from cattle were positive, indicating that they could be *T.b.rhodesiense* circulating in cattle which is similar to the percentage of *T.b.rhodesiense* previously obtained in cattle in Serere, Soroti district. Some of the SRA-PCR negatives could as well be *T.b.rhodesiense* since this technique appears to miss some of the parasitologically confirmed cases of *T.b.rhodesiense* sleeping sickness which could be due to modified SRA genes or loss of the SRA genes from the expression sites in which they reside during the gene rearrangements associated with antigenic variation.

1. INTRODUCTION

The ability of only certain subspecies of *Trypanosoma brucei* Trypanosomes to infect humans has been one of the most important problems in the epidemiology of sleeping sickness in tropical Africa. This is a zoonotic disease with a wide variety of wild and domestic animals which act as reservoir hosts especially in rhodesiense sleeping sickness. Human pathogenic Trypanosomes of the subspecies *Trypanosoma brucei* are morphologically indistinguishable from those found only in other animals; and this has greatly hampered research on animal reservoirs. However, with the advent of biochemical and molecular markers some Trypanosome stocks circulating in animals were shown to be similar to those found in man [1]; [2]; [3]; [4]; [5]. The importance of both wild and domestic animals in the epidemiology of *T.b.rhodesiense* sleeping sickness in East Africa was recognised early (1958) by [6], but it was not until 1966 [7]; when it was conclusively shown that domestic cattle were reservoirs of *rhodesiense* sleeping sickness by direct human inoculation with cattle derived Trypanosomes. Since

then, the role played by domestic animals in the transmission of *T.b.rhodesiense* has been the subject of several investigations and is well documented [8]; [4]; [9]; [10].

However, the demonstration that human serum resistance associated (SRA) gene from a Ugandan *T.b.rhodesiense* isolate is expressed only in serum resistant associated variants of *Trypanosoma brucei rhodesiense* [11]. This opened the way to find out the Trypanolytic factor in human serum. This gene, which is related to variant surface glycoprotein (VSG) genes, was able to sufficiently confer human serum resistance on *T.b.brucei* by transfection [12]; [13]. It is now known that resistance to normal human serum is conferred by SRA gene that encodes a truncated form of the variant surface glycoprotein called serum resistance associated protein whose amino-terminal α–helix is responsible for resistance to normal human serum [14].The study therefore aimed at evaluating the SRA gene for the detection of *T.b.rhodesiense* Trypanosomes in humans and in domestic animals as well as to identify domestic animals which act as animal reservoirs of *T.b.rhodesiense* sleeping sickness in South Eastern Uganda.

2. MATERIALS AND METHODS

2.1. Sample size

Selection of villages in the districts was based on high prevalence of human and animal Trypanosomosis as per the respective district records of sleeping sickness cases in the health centres/hospitals. Villages with about 10% prevalence of animal Trypanosomosis were selected and domestic animals screened for the presence of Trypanosomes. In order to apply the normal approximation of the binomial distribution to calculate 95% confidence interval around observed proportion, we needed a sample size of about 100 infected animals. Assuming a 10% prevalence of animal Trypanosomosis, we needed to examine 1000 domestic animals in order to get 100 infected animals.

2.2. Parasitological test

A cross sectional study of *T.brucei* infections in domestic animals was conducted in the *T.b.rhodeisense* endemic districts of Kaberamaido, Soroti, and Tororo; and non endemic district of Sembabule**.** A team screened domestic animals in the selected areas using Microheamatocrit Centrifugation Technique (HCT). Each blood sample from parasitologically confirmed case was divided into two portions. One portion was inoculated intraperitoneally into *Mastomys nantalensis* rat for *T.b.rhodesiense* isolation or was stabilated using 10% glycerol in phosphate buffered saline glucose, pH 8.0 and preserved in liquid nitrogen. The second portion of the blood sample was used for DNA extraction for PCR technique for detecting *T.b.rhodesiense*.

2.3. Animal Trypanosomosis

A total of 87 Trypanosomal DNA samples from parasitologically positive cattle with Trypanosomes were prepared and analysed by TBR-PCR and SRA-PCR. All the positive cattle were treated with samorin free of charge.

2.4. Human Trypanosomosis (Sleeping sickness)

Two persons of 1,672 persons examined by the microhaematocrit centrifugation technique were positive for Trypanosomes in Kaberamaido district. The two diagnosed

sleeping sickness cases were referred to Serere Health Centre for treatment since Kaberamaido district did not have a sleeping sickness treatment centre at that time. All positive blood samples were divided into two portions; one portion stabilated using 10% glycerol in phosphate buffered saline glucose, pH 8.0 and preserved in liquid nitrogen. The second portion of the blood sample was inoculated into one mouse for propagation and isolation of Trypanosomes. Blood samples from previously diagnosed sleeping sickness cases in Soroti and Tororo districts were included in the analysis for the presence of SRA gene.

2.5. Extraction of DNA

Total DNA was extracted using Qiamp blood DNA extraction protocol from all the 87 infected blood samples from cattle and 35 infected blood samples from humans as described in [16]. The DNA in the eluent was precipitated using 20 µL of 3 *M* sodium acetate (pH 5.2) and 400 µL of pre-chilled 100% ethanol, mixed and centrifuged at 8,000 x g for 5 min. The pellet was washed once with 70% ethanol, centrifuged at 8,000 x g for 5 min and the DNA pellet re-suspended in 20 µL of double distilled water.

2.6. Polymerase chain reaction for *T.brucei* subspecies

The DNAs from Trypanosome isolates from domestic animals were amplified by TBR1-2 primers (TBR1 $^{5'}$-CGA ATG AAT ATT AAA CAA TGC GCA GT-$^{3'}$ and TBR2 $^{5'}$AGA ACC ATT TAT TAG CTT TGT TGC-$^{3'}$) for *T.brucei* subspecies in order to ascertain whether the Trypanosomes derived from domestic animals were. Indeed, *T.brucei* subgroup Trypanosomes before PCR involving TgsGP primers specific for *T.b.gambiense* was done. It also served as away of estimating the amount of DNA in the solution and hence what volume to use in the PCR. The PCR amplification was carried out as described by [15] Penchenier *et al* (1996) in 25 µL reaction mixture containing final concentrations of 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1% (v/v) Triton X-100, 200 µ*M* each of dATP, dCTP, dGTP, and dTTP, 0.1 µ*M* each of the 5' and 3' primers, 50 ng DNA template or 2 µL crude DNA preparation and 1 unit of Taq DNA polymerase. The amplification started with the initial denaturation step at 94^{o}C for 3 min and subjected to 35 cycles involving denaturation for 1 min at 94^{o}C, annealing at 56^{o}C for 1 min, and extension at 72^{o}C for 1 min and final elongation of 5 min at 72^{o}C. The absence of contaminants was routinely checked by inclusion of negative control samples in which the DNA sample was replaced with sterile water. Fifteen microlitres of each sample alongside control and marker DNA were electrophoresed in 2.0% w/v agarose gel containing 0.5 µg/mL ethidium bromide. The amplified products were observed by UV transillumination.

2.7. Polymerase Chain Reaction (PCR) using *T.b.rhodesiense* specific primers

PCR assay using primers(SRA-R $^{5'}$ ATA GTG ACA AGA TGC GTA CTC AAC GC $^{3'}$ and SRA-F $^{5'}$ AAT GTG TTC GAG TAC TTC GGT CAC GCT $^{3'}$) specific for *T.b.rhodesiense* serum resistance associated (SRA) gene was conducted to identify *T.b.rhodesiense* as described by [16]. PCR amplification was performed using 50 ng of DNA extracted from purified *T.b.rhodesiense* Trypanosomes as control DNA, or 10 µL of DNA extracted from domestic animal blood. The DNA amplifications was carried out in 25 µL reaction mixture containing final concentrations of 20 m*M* Tris-HCl, pH 8.7, 100 m*M* KCl, 50 m*M* $(NH_4)_2SO_4$, 1.5 m*M* $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP, 0.5 µ*M* each primers and 2.5 units of HotStar Taq DNA polymerase. SRA-PCR was performed using a GeneAmp PCR System 9700 from Applied

Biosystems with the initial incubation for 15 minutes at 95^{o}C, followed by 45 cycles involving denaturation for 1 min at 94^{o}C, annealing at 68^{o}C for 1 min, extension at 72^{o}C for 1 min; and a final extension for 10 min at 72^{o}C. The absence of contaminants was routinely checked by inclusion of negative control samples in which the DNA sample was replaced with sterile water. All samples were subjected to an initial PCR, followed by a second PCR using 1ul of the first PCR product in order to increase sensitivity. Twenty microlitres of each sample were electrophoresed in a 2% agarose containing 1 μg/mL ethidium bromide. The amplified products were observed by UV transillumination. Similarly, PCR assay using primers designed by [17]; for *T.b.rhodesiense* serum resistance associated (SRA) gene was performed using the same *T.b.rhodesiense* isolates in order to compare the results with those obtained in SRA-PCR designed in [16].

3. RESULTS

The human serum resistance associated (SRA) gene was identified in 28 (80%) of the 35 *T.b.rhodesiense* Trypanosomes from parasitologically confirmed sleeping sickness cases, using the primers designed in [16] and in 27 (77.2%) of the same 35 *T.b.rhodesiense* Trypanosomes using the primers designed by Gibson (Table I; Fig. 1). In the Serere area, 4 of the 11 *T.b.rhodesiense* isolates were SRA-PCR negative using primers [16]; and three of the same isolates were again SRA-PCR negative using [17]; primers (Table I). Similarly in Tororo area, 2 of the 22 *T.b.rhodesiense* isolates were SRA-PCR negative using [16]; primers and four including the two of the same isolates were again SRA-PCR negative using Gibson [17]; primers (Table I). However, about 20% of the 35 *T.b.rhodesiense* Trypanosomes could not be detected by SRA-PCR even when an aliquot of the first PCR (Table III) was used in the second PCR, indicating that the SRA gene may be absent in those Trypanosomes or the Trypanosomes could be having another variant of SRA not detectable by these primers since three variants of SRA genes have so far been identified or the amount of Trypanosomal DNA extracted from infected blood was too low to be detected. Since SRA genes resemble variable surface glycoprotein (VSG) genes, it may be that these isolates which are SRA-gene negative are indicative of some *T.b.rhodesiense* Trypanosomes with modified or lack SRA genes. The number of *T.b.rhodesiense* isolates which are SRA gene negative are too many to be accounted for as miss identification, but could be explained by simple loss of the SRA gene from the expression site in which it resides during antigenic variation.

Analysis of Trypanosomes derived from domestic animals showed that, 79 (90.8%) of the 87 Trypanosomes isolated from cattle (Table II) were positive by TBR-PCR, indicating that they are *Trypanozoon* while 8 (9.2%) were negative, suggesting that they could be *T.vivax, T.congolense* or *T.theileri*. When subjected to SRA-PCR, 10 (11.5%) of the 87 Trypanosomes isolates derived from cattle were positive, indicating that they could be *T.b.rhodesiense* circulating in cattle which is similar to the percentage of *T.b.rhodesiense* previously obtained in cattle in Serere, Soroti district [18]. Some of the SRA-PCR negatives could as well be *T.b.rhodesiense* since this technique appears to miss some of the parasitologically confirmed cases of *T.b.rhodesiense* sleeping sickness which could be due to modified SRA genes or loss of the SRA genes from the expression sites in which they reside during the gene rearrangements associated with antigenic variation.

TABLE I. RESULTS OF PCR AMPLIFICATION OF TRYPANOSOMAL DNA ISOLATED FROM INFECTED HUMANS FROM THREE DIFFERENT AREAS

Origin	Host	No. of isolates examined	Primers used in PCR					
			TBR 1 and 2		SRA(Radwanska)		SRA (Gibson)	
			+	-	+	-	+	-
Serere	Humans	11	11	0	7	4	8	3
Tororo	Humans	22	22	0	20	2	18	4
Kaberamaido	Humans	2	2	0	1	1	1	1
Total (%)		35	35 (100)	0 (0)	28 (80.0)	7 (20.0)	27(77.2)	8 (22.8)

TABLE II. RESULTS OF PCR AMPLIFICATION OF TRYPANOSOMAL DNA ISOLATED FROM INFECTED DOMESTIC ANIMALS FROM FOUR DIFFERENT AREAS

Origin	Host	No. of DNA tested	Primers used in PCR					
			TBR 1 and2		SRA(Radwanska)		SRA (Gibson)	
			+	-	+	-	+	-
Serere, N. E East Uganda	Domestic animals	46	39	7	6	40	7	39
Kaberamaido N.E. Uganda	Domestic animals	30	29	1	1	29	1	29
Sembabule in S. Uganda	Domestic animals	5	5	0	0	5	0	5
Tororo in S.E. Uganda	Domestic animals	6	6	0	3	3	2	4
Total (%)		87	79 (90.8)	8 (9.2)	10(11.5)	77 (88.5)	10(11.5)	77 (88.5)

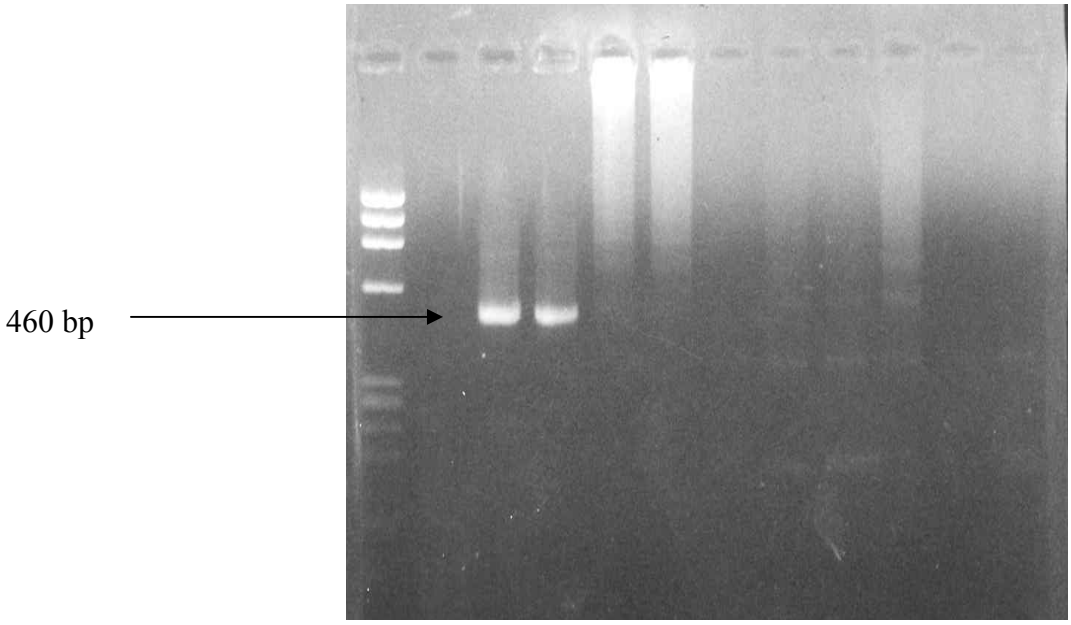

FIG. 1. Agarose gel electrophoresis showing results obtained SRA-PCR (Gibson et al.2002, primers).
1 = Marker; 2 = negative control; 3 = positive control SRA *T.b.rho*; 4 = positive SRA *T.brucei* from domestic animal; 5-12 = negative *T.brucei* from domestic animals

TABLE III. MOLECULAR DIFFERENTIATION OF *T.B.RHODESIENSE* TRYPANOSOMES FROM INFECTED CATTLE BLOOD SAMPLES FROM KABERAMAIDO, NEW FOCUS OF SLEEPING SICKNESS

Serial No	Code No	Inoculum	Origin		PCR results		
				Tryp Species	TBR	SRA (Radwanska) 1st 2nd	SRA(Gibson) 1st 2nd
1	Cow110	Blood	Kaberamaido	*T.brucei*	+++	- -	- -
2	326	Blood	Kaberamaido	*T.brucei*	+++	- -	- -
3	327	Blood	Kaberamaido	*T.brucei*	+++	- +	- +
4	328	Blood	Kaberamaido	*T.brucei*	+++	- -	- -
5	329	Blood	Kaberamaido	*T.brucei*	+++	- -	- -
6	330	Blood	Kaberamaido	*T.brucei*	+	- -	- -
7	334	Blood	Kaberamaido	*T.brucei*	+++	- -	- -
8	336	Blood	Kaberamaido	*T.brucei*	+++	- -	- -
9	337	Blood	Kaberamaido	*T.brucei*	+	- -	- -
10	338	Blood	Kaberamaido	*T.brucei*	+++	- -	- -
11	339	Blood	Kaberamaido	*T.brucei*	+++	- -	- -
12	340	Blood	Kaberamaido	*T.brucei*	++	- -	- -
13	341	Blood	Kaberamaido	*T.brucei*	++	- -	- -
14	342	Blood	Kaberamaido	*T.brucei*	+	- -	- -
15	343	Blood	Kaberamaido	*T.brucei*	+++	- -	- -
16	344	Blood	Kaberamaido	others	-	- -	- -
17	347	Blood	Kaberamaido	*T.brucei*	++	- -	- -
18	348	Blood	Kaberamaido	*T.brucei*	++	- -	- -
19	352	Blood	Kaberamaido	*T.brucei*	+	- -	- -
20	353	Blood	Kaberamaido	*T.brucei*	+	- -	- -
21	354	Blood	Kaberamaido	*T.brucei*	+	- -	- -
22	356	Blood	Kaberamaido	*T.brucei*	+++	- -	- -
23	377	Blood	Kaberamaido	*T.brucei*	++	- -	- -
24	378	Blood	Kaberamaido	*T.brucei*	++	- -	- -
25	380	Blood	Kaberamaido	*T.brucei*	+++	- -	- -
26	381	Blood	Kaberamaido	*T.brucei*	++	- -	- -
27	382	Blood	Kaberamaido	*T.brucei*	++	- -	- -
28	383	Blood	Kaberamaido	*T.brucei*	+++	- -	- -
29	384	Blood	Kaberamaido	*T.brucei*	+++	- -	- -
30	385	Blood	Kaberamaido	*T.brucei*	+++	- -	- -

4. DISCUSSION

Radwanska [16]; analysed 25 different *T.b.rhodesiense* strains from Uganda, Kenya and Rwanda and found out that 24 strains were SRA-PCR positive, but one strain STIB 884 was negative and was thought as a miss identification of the strain. In the present study 8 (20.0%) of the 35 *T.b.rhodesiense* from parasitologically confirmed sleeping sickness cases were SRA-PCR negative, indicating that there may be more than one factor involved in resistance to lysis by normal human serum (NHS). Furthermore, [16]; he also showed that strain TREU 927/4 used as the reference *T.brucei* strain for the Trypanosome genome sequencing project (TIGR database) was both SRA gene and SRA transcript negative, but resistant to lysis by NHS, again

indicating that resistance to NHS may be conferred by other factors not yet identified besides the SRA gene. Similar analysis using the same 35 *T.b.rhodesiense* strains and primers developed by Gibson [17]; showed that 7 (22.8%) were SRA-PCR negative (Table I). Since SRA gene resembles Variable Surface Glycoprotein (VSG) gene, it may be these *T.b.rhodesiense* isolates, which are SRA-gene negative, are true *T.b.rhodesiense* with modified SRA gene or lack SRA gene. The number of *T.b.rhodesiense* isolates which are SRA gene negative are too many to be accounted for as miss identification of the isolates, but the SRA-PCR negativity could be explained by simple loss of the SRA gene from the expression site in which it resides in the Ugandan *T.b.rhodesiense* [13]; during the gene rearrangements involved in antigenic variation. It is intended that DNA hybridization will be done on some of the *T.b.rhodesiense* isolates that are SRA gene negatives to confirm their status.

Analysis of Trypanosomes derived from domestic animals showed that 79 (90.8%) of the 87 Trypanosomes isolated from cattle (Table II) were positive by TBR-PCR, indicating that they were *Trypanozoon* while 8 (9.2%) were negative, suggesting that they could be *T.vivax*, *T.congolense* or *T.theileri*. When subjected to SRA-PCR, 10 (11.5%) of the 87 Trypanosomes isolates derived from cattle were positive, indicating that they could be *T.b.rhodesiense* circulating in cattle which is similar to the percentage of *T.b.rhodesiense* previously obtained in cattle in Serere, Soroti district [18].

On the other hand, some of the SRA-PCR negatives could as well be *T.b.rhodesiense* since this technique appears to miss some of the parasitologically confirmed cases of *T.b.rhodesiense* sleeping sickness which could be due to modified SRA gene or loss of the SRA genes from the expression sites in which they reside during gene rearrangements associated with antigenic variation. Alternatively, SRA gene negative Trypanosomes isolates could represent a group truly *T.b.brucei* which are non pathogenic to humans; and is similar to what was found in [17]; where the SRA gene was absent from West African *T.b.brucei*.

ACKNOWLEDGEMENTS

Thanks to the Director of LIRI, Uganda, for logistic support and for allowing me to attend this meeting. I thank all district and field staff and local council members for assistance in the research activities.

This investigation was supported by the IAEA.

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THE USE OF ITS1 RDNA PCR IN DETECTING PATHOGENIC AFRICAN TRYPANOSOMES

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Abstract

There are 11 different pathogenic Trypanosomes in Trypanosomiasis endemic regions of Africa. Their detection and characterization by molecular methods relies on species-specific primers, consequently several PCR tests have to be made on each sample. Primers ITS1 CF and ITS1 BR, previously designed to amplify the internal transcribed spacer (ITS1) of rDNA, have been evaluated for use in a universal diagnostic test for all pathogenic Trypanosomes. Blood was collected from 373 cattle and 185 camels. The primers gave constant PCR products with the stocks of each taxon tested. Members of subgenus Trypanozoon (*T.brucei, T.evansi, T.b.rhodesiense and T.b.gambiense*) gave a constant product of approximately 480 bp; *T.congolense savannah* 700 bp, *T.congolense kilifi* 620 bp and *T.congolense* forest 710 bp: *T.simiae* 400 bp, *T.simiae tsavo* 370 bp, *T. godfreyi* 300 bp and T.vivax 250 bp. The sensitivity of the test ranged from 10pg for Trypanozoon, T.congolense clade and *T.vivax* to 100 pg for *T.simiae* and *T.godfreyi*. The primers detect cases of multi-taxa samples, although the sensitivity was reduced with an increase in the combinations. A better detection rate of Trypanosome DNA was recorded with buffy coats than from direct blood. With the field samples, the diagnostic sensitivity was close to the sensitivity obtained using single reaction with species – specific primers for Trypanozoon 38/40 (95%) and *T.congolense savannah* 30/33 (90.9%) but was lower with *T.vivax* 25/31 (77.4%). The primers offer promise as a routine diagnostic tool through the use of a single PCR; however, further evaluation is recommended.

1. INTRODUCTION

Trypanosomiasis in animals (nagana) and humans (sleeping sickness) continues to cause immeasurable losses in tropical Africa. Accurate detection of Trypanosomes both in vectors and host blood relies heavily on molecular techniques (polymerase chain reaction, PCR). Such techniques have proved effective in the characterization of Trypanosomes [1]-[5]; typing new Trypanosomes [6] in the collection of epidemiological data [7] and in animal treatment. In Kenya, ten pathogenic Trypanosomes occur and overlap in most of the tsetse belt [8] such that multiple

infections of both host and vector are to be expected. This implies that several PCR tests have to be made to ascertain whether a collected sample is positive, negative or even of mixed infection. For instance a bovine sample from an endemic area in Kenya will require at least five species-specific PCR tests to cover the possibilities of Trypanosoma vivax, *T.congolense savannah*, *T.congolense kilifi, T.forest T.brucei* spp, three PCRs for suids (*T.simiae, T.godfreyi* and *T.simiae tsavo*) and eight for every tsetse sample collected.

This translates into an enormous cost which normally prohibits such studies. Efforts to combine already available primers (multiplex) in single PCR have been discouraging due to lower sensitivity compared to individual species-specific tests and the appearance of non-specific PCR products. The development of a universal single PCR based on ribosomal genes by [9] and further evaluated by [10]; detected combined Trypanosome infections. However, extensive evaluation of these primers (KIN2) was not done. The internal transcribed spacer (ITS) region of rDNA is a preferred target for a universal test because of its highly conserved flanking regions and size variability among Trypanosomes species and subgroups. This locus has 100-200 copies and each transcribed unit is composed of 18s, 5.8s and 28s rRNA genes separated by two ITS regions, [11]; [12]. New ITS-based primers were designed by Davila (unpublished) specifically to amplify ITS1. This study reports the evaluation of these new primers (ITS1 CF and ITS1 BR) both in the laboratory using Trypanosome reference DNA and in the field. Further, the test was compared with earlier ITS-based primers KIN1 and KIN2 [9]. It is anticipated that a successful universal PCR-based test would add value to the collection of epidemiological data on Trypanosomiasis, while easing the cost of running several PCRs, especially in the endemic zones of Africa.

2. MATERIAL AND METHODS

2.1. Experimental design

2.1.1. Field sample collection

Samples were collected from Trypanosomiasis endemic zones. Cattle samples (357) were taken from Nguruman in southern Kenya and 185 camel blood samples from northern Kenya. The animals were bled from the jugular vein (5 mL) into heparinized vacutainer tubes. Two capillaries were filled three-quarters full for parasite investigations using BCT [13]; and and HCT [14]; and determination of packed cell volume (PCV). Approximately 0.5 mL of blood was transferred into cryovials, mixed with phosphate buffered saline (PBS) and 10% glycerol, and stored in liquid nitrogen for DNA extraction. All parasitologically positive animals, and those judged infected using clinical symptoms by the field veterinarian, were treated with Trypanocides. Sixteen positive cattle samples from Lambwe valley were collected separately analysed during the sensitization and launching of a sterile insect technique project.

2.1.2. Trypanosomes DNA

Well-characterized reference DNA for the subgenera *Nannomonas* and *Duttonella* used in this study were obtained from Wendy Gibson, University of Bristol, while the members of the subgenus Trypanozoon, *T.lewisi* and *T.theileri*, were from well-characterized isolates from the KETRI cryobank. Further, characterized Trypanosome DNA from tsetse samples that had been analysed previously was included in this study [8].

2.1.3. Template preparation and PCR cycling

DNA from blood samples was extracted using commercially available QIA amp DNA mini kit (Qiagen, Australia). The purified DNA templates were stored at -20^{o}C for later use. For tsetse samples, DNA was initially extracted using the proteinase K method as detailed by [1] and later re-extracted using Qiagen kit for uniformity purposes. PCR was carried out in 25 µL mixtures containing 10X reaction buffer (670 m*M* Tris-HCI pH 8.8, 166 m*M* $(NH_4)_2$ SO_4, 4.5% Triton X-100, 2 mg/mL gelatin) (Fisher Biotech), 2 m*M* $MgCl_2$, 200 µ*M* of each of the four deoxynucleoside triphosphates (dNTPs), primers at 1 µM and 0.5 U of Taq DNA polymerase (Fisher Biotech). PCR cycles for ITS1 CF and BR primers were: initial step at 94^{o}C for 5 min, followed by 35 cycles of 94^{o}C for 40 s, 72^{o}C for 90 s, and final species-specific primers and their PCR conditions were those described by [1] for *Nannomonas*, [2]; for *Trypanozoon* and *Duttonella* by [15]; and [16].

KIN1 = $^{5'}$ GCGTTCAAAGATTGGGCAAAT $^{3'}$

KIN2 = $^{5'}$CGCCCGAAAGTTCCCAACC $^{3'}$ [9]

and those developed for this study:

ITS1 CF = $^{5'}$CCGGAAAAGTTCACCGATATTG $^{3'}$

ITS1 BR = $^{5'}$TTGCTGCGTTCTTCAACGAAA $^{3'}$ (Davilla, unpublished).

The ITS1 CF and BR primers were tested for sensitivity and ability to detect known spiked Trypanosome DNA. The amount of DNA template analysed for reference DNA was 50 ng and 2 µL of the template was used for field sample. Amplification products were resolved in 1.5% molecular grade agarose (Fisher Biotech) stained with ethidium bromide.

2.1.4. Sequencing

PCR products were purified from the agarose gel using a freeze-squeeze method. Briefly, the PCR band was cut from the gel and frozen at 20^{o}C for 4 h. The frozen gel slice was placed between parafilm, squeezed and approximately 50 µL of liquid collected. The collected volume was mixed with 1 µL of 3 *M* sodium acetate and 100 µL of 70% ethanol, and then left on ice for 20 min. The mixture was them centrifuged at 14,000 rpm for 10 min, the supernatant discarded and the resulting pellet vacuum-dried and resuspended in 10 µL PCR water. A total of 1 µL of this final sample was used in running the sequencing PCR reaction using either forward or reverse ITS1 primers. Sequencing was done in an AB1 automatic DNA sequencer (Applied Biosystems). For *T.godfreyi*, the resulting PCR product was cloned into a TOPO vector (Invitrogen) and sequenced (GenBank accession no. AY661891).

2.1.5. Alignment

The sequence obtained were analysed using the basic BLAST search tool.

(http://ww.ncbi.nlm.nih.gov/BLAST/) and alignment made with specific ribosomal products found in the GenBank using DNA man (Lynnon Biosoft, Canada).

3. RESULTS

3.1. Sensitivity

The analytical sensitivity of the ITS1 and BR primers was tested using serially diluted Trypanosome DNA. The resulting DNA dilutions were also analysed for analytical sensitivity using species-specific primers to check dilution accuracy.

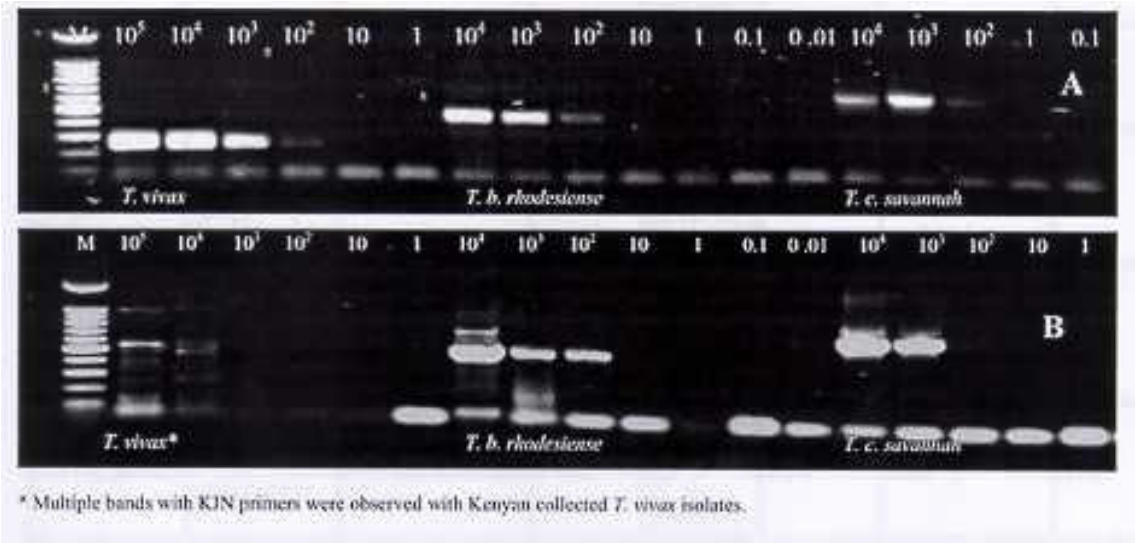
Sensitivities of between 10 pg and 100 pg were recorded with ITS1 CF and BR primers, while with species-specific ones, the sensitivities were equivalent to those already published by [1]; and [2] (*see* Table I and Fig. 1).

The ITS1 CF and BR based primers could detect all spiked DNA samples with double and triple combinations. However, the analytical sensitivity was very low in quadruple combinations. Use of >50 ng of reference DNA gave rise to non-specific PCR products while PCR reactions with ammonium sulphate buffers showed a higher sensitivity than those without.

TABLE I. RELATIVE ANALYTICAL SENSITIVITIES USING DIFFERENT PRIMERS

Isolate code/Trypanosome species	Origin	Species specific primers	Sensitivity	
			ITS1 CF×BR primers	KIN1 and KIN2 primers
KETRI 3267, *Trypanosoma evansi*	Samburu, Kenya	0.1 pg	10 pg	10 pg
ATCC 30025, *Trypanosoma b. gambiense*	London Hospital	0.1 pg	10 pg	10 pg
KETRI 22522,*Trypanosoma b.rhodesiense*	Lambwe valley, Kenya	0.1 pg	10 pg	10 pg
Gam 2, *Trypanosoma Congolense savannah*	Keneba, The Gambia	0.1 pg	10 pg	100 pg
TV030, *T.congolense forest*	The Gambia	0.1 pg	10 pg	100 pg
WG5, *T.congolense kilifi*	Kenya	0.1 pg	10 pg	100 pg
Ban 9, *Trypanosoma godfreyi*	Bansang, The Gambia	10 pg	100 pg	1 ng
Ken 2, *Trypanosoma simiae*	Keneba, The Gambia	1 pg	100 pg	1 ng
Y58 *Trypanosoma vivax*	Nigeria	1 pg	10 pg	1 ng

One Trypanosome=0.1 pg, ten Trypanosome=1 pg, etc. [1]; [12]. For the primers used for *Trypanozoon* see [2]. *Congolense* clade, *Trypanosoma simiae* and *T.godfreyi* [1], *T.vivax* [16]; *T.brucei brucei* and *T.simiae* tsavo were not done. Multiple bands with KIN primers were observed with Kenyan collected *T.vivax* isolates



∗ Multiple bands with KIN primers were observed with Kenyan collected *T.vivax* isolates

FIG. 1. Sensitivity of primers;ITS1 CF (A) and KIN1 and KIN2 (B) for Trypanosoma vivax (KETRI 22619), T.brucei rhodesiense (KETRI 2522) and T.congolense savannah (Gam 2).

3.2. PCR size products with ITS1 CF and BR

All members of the subgenus Trypanozoon gave an approximate band size of 480 bp; Nanomonas: *T.congolense savannah* 700 bp; *T.congolense kilifi* 620; *T.congolense* forest 700; *T.simiae* 400; *T.simiae tsavo* 370; *T.godfreyi* 300 and *Duttonella*; *T.vivax* 250 bp as shown in Table II and Fig. 2. Samples were re-extracted using Qiagen DNA mini kit.

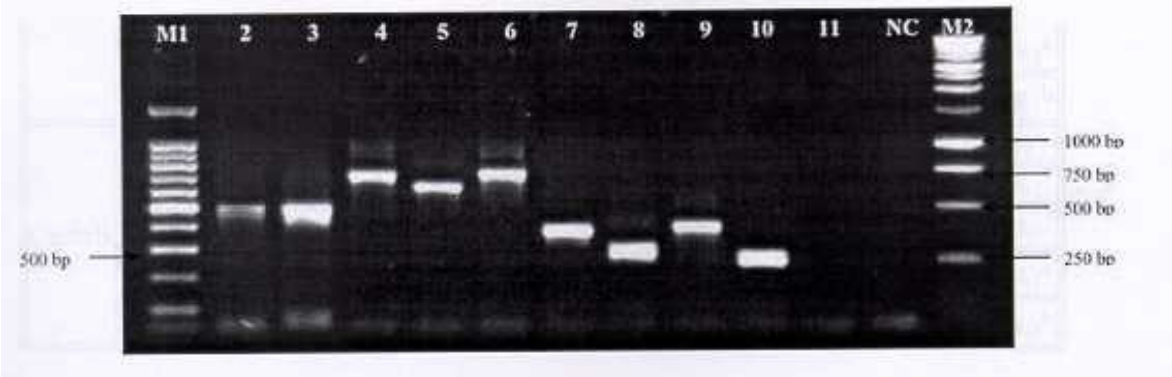


FIG. 2. Gel electrophoresis of reference DNA samples (50 ng) amplified with ITS1 CF and BR.
Lane *M1* 100 bp marker, lane 2 *T.brucei brucei*, lane 3 *T.evansi*, lane 4 *T.congolense savannah*, lane5 *T.congolense kilifi*, lane 6 *T.congolense forest,* lane 7 *T.simiae tsavo*, lane 8 *T.godfreyi*, lane 9 *T.simiae*, lane 10 *T.vivax* (Y58), lane 11 *T.lewisi,* NC negative control, *M2* 1 kb marker

TABLE II. PCR PRODUCT SIZE USING ITS1 CF AND BR SAMPLES FROM TSETSE AND COLLECTED IN 2002

Isolate	*Species*	Host/ origin	Year of isolation	Species-specific primers and ref	ITS (CF× BR)	Approximate PCR product size (gel)	Real size (sequence
KETRI 2533	*T.rhodesiense*	Cow, Kenya	1980	+++ [6]	+++	480	476
KETRI 2547	*T.rhodesiense*	Human Kenya	1981	+++ [6]	+++	480	476
KETRI 2479	*T.evansi*	Camel Kenya	1980	+++ [1]	+++	480	476
KETRI 3492	*T.evansi*	Camel Kenya	1992	+++ [1]	+++	480	476
KETRI 2710	*T.brucei brucei*	Cow Kenya	N/A	[2]	+++	480	476
KETRI 2901	*T.brucei brucei*	Cow Kenya	1970	+++ [2]	+++	480	476
ATCC30025	*T.brucei gambiense*	Human London	1921	+++ [2]	+++	480	476
KETRI 1829	*T.congolense savannah*	Cow Kenya	N/A	+++ [1]	+++	700	697
GAL 72	*T.congolense kilifi*	Tsetse Kenya	2002	+++ [1]	++	620	614
GAL 54	*T.congolense kilifi*	Tsetse Kenya	2002	+++ [1]	++	620	614
MWE 1	*T. congolense forest*	Tsetse Kenya	2002	+++ [1]	++	700	714
SH1 23	*T.simiae*	Tsetse Kenya	2002	+++ [1]	++	400	397
MWE 10	*T.simiae*	Tsetse Kenya	2002	+++ [1]	+	400	397
LAM 17	*T.godfreyi*	Tsetse Kenya	2002	+++ [1]	++	280	273
LAM 68	*T.godfreyi*	Tsetse Kenya	2002	+++ [1]	+	280	273
KETRI 1864	*T.simiae tsavo*	Tsetse Kenya	1974	+++ [1]	+++	370	369
KAK 23	*T.simiae tsavo*	Tsetse Kenya	2002	+++ [1]	++	370	369
Y58	*T.vivax*	Cow Nigeria	N/A	+++ [16]	+++	250	250
KETRI 2619	*T.vivax*	Cow Kenya	1951	+++ [1]	+++	250	246
GAL 34	*T.vivax*	Tsetse Kenya	2002	+++ [16]	++	250	250
NGU 3	*T.vivax*	Tsetse Kenya	2002	+++ [16]	++	250	248

+++ Bright, + weak but visible band

3.3. Field analyses

Analyses of field samples showed a Trypanosome prevalence of 86/357 (24.1%) and 48/185 (25.0%) in cattle and camels, respectively, using subgenus and species-specific primers. For bovine samples, 71 (27 *Trypanozoon, 33 Nannomonas* and 11 *T.vivax*), were of single infections and 15 double infections

(7 *T.b.rhodesiense/T.congolense* savannah, 4 *T.b rhodesiense/T.vivax* and 4 *T.congolense* savannah/*T.vivax*).

The ITS1 CF and BR primers detected 73 (84.9%), while KINI and KIN2 detected 58 (67.4%) of the samples positive using species-specific primers. The major differences between the KIN and ITS CF and BR primers were the inability of the KIN primers to detect *T.vivax* from field samples, although they detected reference *T.vivax* DNA (Y58) isolated in Nigeria, West Africa.

For camel samples, ITS1 CF and BR primers detected 35/48 (72.9%) and KIN primers 31/48 (64.5%) of Trypanosome DNA from direct blood isolation (Table III). However, with PCR done on 48 positive buffy coat, detection rates improved to 44/48 (91.6%) using ITS1 CF and BR and 43/48 (89.9%) using KIN primers while on the 35 bovine aparasitaemic samples that were species-specific positive, detection improved from 22/35 (62.9%) to 29/35 (82.9%) with ITS1 CF and BR and 17/35 (48.6%) to 26/35 (74.3%) with KIN primers.

Similar better results in detection and the characterization of mixed infections were also recorded with 16 microscopy-positive bovine samples from the Lambwe valley in Kenya (Fig. 3B)

TABLE III. COMPARISON BETWEEN MICROSCOPY, SPECIES-SPECIFIC PRIMERS, ITS1 CF AND BR PRIMERS

		Ssp specific primers	ITS1 CF and BR	KIN 1 and KIN2
		No.+ve	No. +ve	No. +ve
Cattle	MHCT positive	51	51 (l00%)	41 (80.4%)
	Aparasitaemic	35	22 (61.1%)	17 (48.6%)
	Buffy coat(aparasitaemic	35	29 (82.9%)	26 (74.3%)
	Total	86	73 (84.9%)[a]	58 (67.4%)
			80 (93%)[b]	67 (77.9%)
Camels	MHCT positive	19	19 (100%)	19 (100%)
	Aparasitaemic	29	16 (55.2%)	12 (41.4%)
	Buffy coat (aparasitaemic)	29	25 (86.2%)	24 (82.7%)
	Total	48	35 (72.9%)[a]	31 (64.5%)[a]
			44 (91.6%)[b]	43 (89.9%)[b]

[a]The total detection rates for MHCT positive and aparasitaemic samples
[b]Total detection rates for MHCT positive and buffy coats

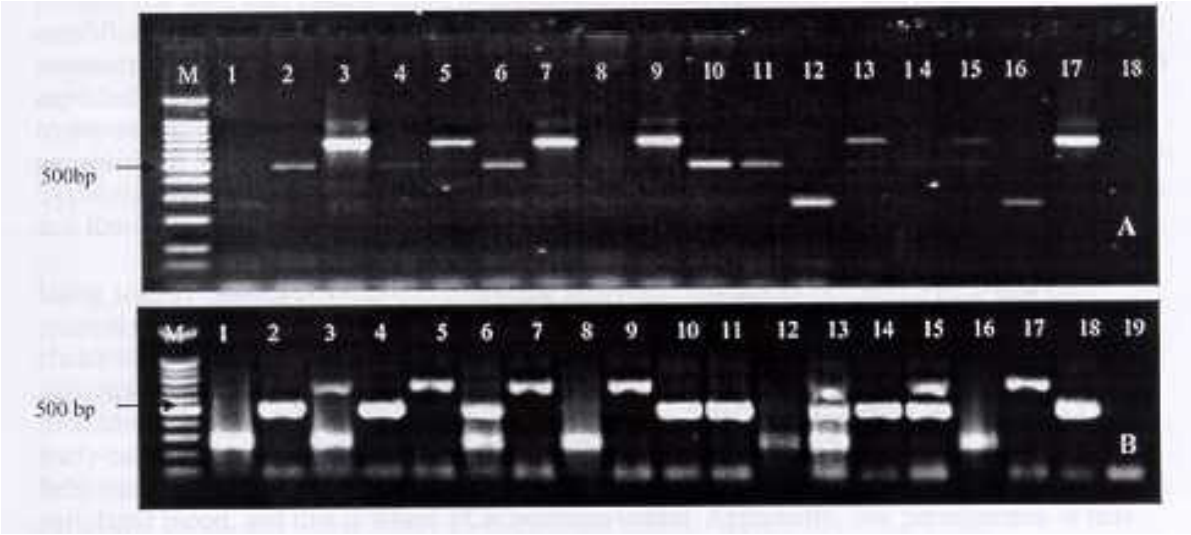


FIG. 3. Gel electrophoresis of PCR products from direct blood DNA isolation (A) and their respective buffy coats (B) from 16 MHCT positive bovine samples collected in Lambwe valley in Kenya.

Identification with species-specific primers. Lane 1. *T.vivax*; lane; 2. *T.brucei*; lane 3. *T.congolense savannah*/*T.vivax*; lane 4. *T.brucei*; lane 5. *T.congolense savannah;* lane 6. *T.brucei/T.vivax*; lane 7. *T.congolense savannah*; lane 8. *T.vivax*; lane 9. *T.congolense savannah*; lane 10. *T. brucei*; lane 11. *T. brucei*; lane 12. *T.vivax*; lane 13. *T.congolense savannah/T.brucei/T.vivax*; lane 14. *T.brucei*; lane 15. *T.brucei/T.congolense savannah*; lane 16. *T.vivax;* lane 17. *T.congolense savannah*-positive control; lane 18. *T.brucei*-positive control for B and negative control for print A; lane 19. negative control for B.

DISCUSSION AND CONCLUSION

The ITS1 and BR based primers showed a higher diagnostic sensitivity than previously described using the KIN primers. The major observation was the ability of ITS1 CF and ITS1 BR primers to detect more *T.vivax* samples than the KIN primers. Their specificity (no amplification with host and vector DNA) and capability of detecting all pathogenic Trypanosomes in a single PCR indicates a greater potential for this system as a universal test for pathogenic Trypanosomes. Furthermore, the primers did not amplify the non-pathogenic *T.lewisi* and *T.theileri* used in this study. ITS1 BR is designed from the conserved region of rDNA genes. Specifically ITS1 CF anneals to 18S and ITS1 BR in 5.8S regions of rDNA allowing the amplification of (ITS) 1 which varies in size within Trypanosomes species and in principle should differentiate Trypanosomes by their ITS1 sizes.

The ITS1 region of Trypanosomes varies in size [10]; [12]; except for members of the subgenus *Trypanozoon* which are thought to be closely relate, and their divergence may be a recent occurrence. Sequencing of PCR products in this study showed almost perfect homology with the respective sequences in GenBank. The observed single PCR products for each species were approximately the size as their sequenced product and were also in agreement with estimations from GenBank sequences. Lack of, amplification with non-pathogenic Trypanosomes was supported by *T.theileri* GenBank sequence AB007814 which shows only 25% homology with the ITS1 CF forward primer. This is expected because the primers were designed to improve homology with pathogenic Trypanosomes whilst minimizing homology with non-pathogenic Trypanosomes (Davila unpublished). Observed sequence differences in

ITS1 region for pathogenic and non-pathogenic Trypanosomes may indicate evolutionary divergence of the *Kinetoplastida* rDNA region [17].

Using serially diluted DNA in this study, the analytical sensitivity of the ITS1 CF and BR primers ranged between 10 pg (100 Trypanosomes) for Trypanozoon, *T.vivax* and *T.congolense clades* to 100 pg (1,000 Trypanosomes) for *T.simiae* and *T.godfryi*. The primers detected all parasitologically positive samples from direct blood isolation. With 36 aparasitaemic bovine field samples, sensitivity improved by 20% to 30% when the DNA was extracted from buffy coats (Table III). This is expected because centrifugation concentrates the parasites. Under field conditions, the most challenging cases are when parasites are not demonstrable in the peripheral blood, and this is where PCR becomes useful. Apparently, low parasitaemia in host blood is a characteristic of most Trypanosome infections, and especially *T.evansi* and *T.vivax* which may run chronic forms with no parasites demonstrable in the peripheral blood. In such cases, the concentration of Trypanosomes is important for improving the detection and the characterization of mixed infections (Fig. 3B).

ITS1 CF and BR primers show 100% homology with the available T.vivax sequence (accession no.U22316) while KIN1 and KIN2 show 75-90% homology, which may explain the observed difference in *T.vivax* detection rates between the two tests [10]; where differences were observed in *T.vivax* detection rates between the two tests. Due to the low detection rates of *T.vivax* with KIN primers it was suggested that other ITS primers should be designed to improve detection rates, an objective Davila (unpublished) took into account. Despite the improved detection rates of *T.vivax* with the new primers, we could only detect 25/31 (77.4%) of *T.vivax* from field samples in Kenya (all data not shown), a fact that may be related to low target DNA and /or existence of *T.vivax* genetic variants with changes in primer annealing regions. This fact is supported by the observation that some T.vivax from Kenya analysed in this study gave non-specific PCR products with KIN primers (Fig. 1B) and differing sizes of the ITS region (Table II*). T.vivax* genetic variants have been reported in the field [15]; and [5]. Further, this has been supported by the work of [17]; who noted that *T.vivax* 18 rDNA is evolving at 7 to 10 times the rate of non-salivarian Trypanosomes, and also significantly faster than all Trypanosomes.

The usefulness of the new test was demonstrated by its ability to detect mixed Trypanosome infections in cryopreserved samples and from field samples. In our analysis, we picked four isolates that had been previously categorized as single infections to be mixed infections. Direct sequencing of these products showed that KETRI 2729 contained a T.vivax [not the West African type because it was negative with TVW1 and TVW2, [1]; but positive with universal *T.vivax* primers [15]; [16]; and *T.congolense savannah*. Camel sample MG1 from a tsetse-infested area had *T.brucei* ssp, *T.vivax*, bovine samples GJ4 *T.brucei* ssp and *T.congolense savannah*, and GJ3 *T.brucei* ssp, *T.vivax* and *T.congolense savannah*. The test was not able to differentiate *T.congolense savannah* (697bp) and *T.congolense forest* (714bp) mixed infections based on the size of their PCR product in a normal agarose gel. However, for routine treatment purpose, the veterinarian would only be interested in knowing the infection status of the animal rather than infecting species, in which case the test fulfils the purpose. With mixed spiked DNA, the new test detected all of the expected double and triple combinations. However with the quadruple combination the sensitivity was low. Double infections in animals are a normal occurrence in animals, although common in tsetse flies [8]. The use of universal tests for pathogenic Trypanosomes would reduce

the cost of PCR three to five times as the number of reactions required per sample would be reduced to one [12]. Further this would be useful in picking out any new Trypanosome prevailing in the field. However the greatest challenge that will face ITS-based tests will be diagnostic sensitivity, because ITS1 has approximately 100-200 copies as compared to species-specific tests which target satellite DNA with over 10,000 copies [12]. In summary, our results show that the test has the ability to detect multiple species in a single PCR from field sample collected in Kenya. To further improve detection of Trypanosomes, DNA extraction from buffy coats if recommended.

ACKNOWLEDGEMENTS

This work received financial support from the International Atomic Energy Agency (IAEA) under grant no. KEN 11414 to J.K. Kinyua/Z.K. Njiru and supplementary funds from the Kenyan Government. We thank W. Gibson for supplying some reference DNA and the staff of Biochemistry Division, KETRI for technical support. The work reported here complies with the current laws of Kenya where the work was done. Nucleotide sequence data reported in this paper is available in the GenBank and DDBJ data bases under accession number AY661991.

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GENETIC DIVERSITY OF TRYPANOSOMA EVANSI IN THAILAND BASED ON A REPEATED DNA CODING SEQUENCE MARKER

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Abstract

Molecular characterization of Trypanozoon isolates is widely used for linkage analysis, individual identification or inter-population studies. We identified Trypanozoon subgenus through a single PCR based on internal transcribed spacer 1 (ITS1) of rDNA. *Trypanosoma evansi* was identified in all isolates distributed in Thailand. Therefore, we analysed genetic diversity through a single PCR based on a repeated DNA coding sequence marker within *T.evansi* isolates from various animals. Fifty-four isolates of *T.evansi* originated from 6 species of hosts in 20 provinces of Thailand were compared according to the genetic patterns of this marker. The results revealed that *T.evansi* isolates had a high degree of heterozygosity at this locus related to the repeated coding sequence. Therefore, 4 group-specific genotypes, namely group 1 (3/5), group 2 (3/6), group 3 (4/5) and group 4 (5/6), were demonstrated based on numbers of repeats in allele 1 and 2. Group 2 was found 25.9% (14/54) and shown the highest distributed in host species (5 species), whereas group 3 was the highest geographic spread (13 provinces) and defined as a predominant genotypes with 53.7% (29/54) of *T.evansi* isolates in Thailand. These results are very useful for study on the dynamics of parasite populations in animals.

1. INTRODUCTION

The Trypanozoon subgenus consists of five species and subspecies, *T.brucei brucei*, *T.b.rhodesiense, T.b.gambiense, T.evansi* and *T.equiperdum*. These parasites are morphologically identical and classified according to host, type of disease and geographical distribution. In Thailand, *T.evansi* had been recognized by the above criteria. *T.evansi* is a blood protozoan that causes Trypanosomiasis or surra in wide range of hosts in Thailand such as, elephants [14]; horses [3]; buffaloes [10]; [11]; [12], dairy cattle [17], beef cattle [5]; [19]; sows [15]; [16]; Samba and Rusa deer [8]; and hog deer [18].

Several DNA sequences have been used for the sensitive and specific PCR-based detection of livestock Trypanosome DNA in host blood and/or in insect vectors. Few attempts had been made to identify several Trypanosoma species through a unique PCR using one pair of primers designed from internal transcribed spacers (ITS) of the rDNA [6]; [13]. The ITS1 situated between 18S and 5.8S rRNA genes, the sequence of which is usually 300-800 bp in length. Recently, a single PCR using only one pair of primers had been used for detection and identification of Trypanosoma spp. of African livestock. Kin primers, reacted specifically with kinetoplastid species at the ITS1 and allowed the detection and discrimination of all Trypanozoon subgenus, species or types [13]. This primer did not react with apicomplexan species, or bacterial or mammalian DNA [7].

To characterize Trypanozoon isolates, the nucleotide sequence at an open reading frame 2 (ORF2) of the glucose transporter gene cluster located on chromosome X in *T.brucei* ssp. [1]; was designed primer as MORF2-REP marker [2]; (*see* Fig. 1). The ORF2 contains a variable number of 102 base pairs (34 amino acid) repeats, which constitute up to 2/3 of the total gene length [4]. Two alleles appear on agarose gel characterization with different sizes (repeats of the 102 bp unit) from 3 geographical clusters of *T.evansi* (South America, China, Africa) had been reported [2]. The size products are indicated in Table I.

In this report, we demonstrate that this method provided a rapid and simple method for the distinction of *T.evansi* from the other Trypanozoon subgenus. We analysed the length polymorphism of one repeated DNA coding sequence in 54 isolates of *T.evansi* distributed in Thailand (Table II).

2. MATERIALS AND METHODS

2.1. Parasite and nucleic acids isolation

DNA of Trypanosome isolates listed in Table II was extracted with a commercial DNA extraction kit (QAIGEN, Germany) from purified parasites by DEAE cellulose (DE-52) as previously described [9]. Some Trypanosome isolates cryo-preserved in glycerol since 1990, were extracted directly from Trypanosome infected mice bloods by the same DNA extraction kit.

2.2. Experimental design

2.1.1 Experiment I

Identification of Trypanozoon species was performed by a polymerase chain reaction (PCR). The extracted DNA was first tested individually with Kin primers in the same PCR mixture. Primer sequences were as follows:

Kin1 (antisense) = $^{5'}$ GCG TTC AAA GAT TGG GCA AT $^{3'}$

Kin2 (sense) = $^{5'}$ CGC CCG AAA GTT CAC C $^{3'}$ [7]

The PCR was performed in 50 µL reaction mixture containing 40.5 µL of sterile distilled water, 5 µL of 10 x optimized DyNazyme reaction buffer (FINNZYMES, Finland), 1 µL of 10 m*M* dNTP Mix, 1 µL of each primer (100 µ*M* stock), 0.5 µL of DyNAzyme (2 U/µL) and 1 µL of DNA template. PCR conditions were as followed: an initial step of 3 min at 94^{o}C, four cycles of amplification with 1 min denaturation at 94^{o}C, 1 min annealing at 58^{o}C, and 1 min elongation step at 72^{o}C; eight cycles of amplification with 1 min denaturation at 94^{o}C, 1 min annealing at 56^{o}C and 1 min elongation step at 72^{o}C; 23 cycles of amplification with 1 min denaturation at 94^{o}C, 1 min annealing at 54^{o}C and 1 min elongation step at 72^{o}C; and a final extension step of 5 min at 72^{o}C. PCR products were separated and analysed on 1.5% agarose gels and stained with 0.5 µg/mL ethidium bromide and visualized under ultraviolet light. The sizes of the products obtained were compared with the ITS1 size expected.

2.1.2. Experiment II

To study the DNA polymorphism, two specific primers (MORF2-REP) were designed for PCR analysis (Fig. 1).

Primer sequences were as follows:

MORF2-REP/P = $^{5'}$ TGC ATG GCA AAT AGC GAT GGG C $^{3'}$

MORF2-REP/M = $^{5'}$ATC GTC ACC TGG TGT ACT TCT C$^{3'}$ [2].

PCR amplifications were carried out with the Thermal cycler (BIORAD, USA) in 50 μL reaction mixtures containing 200 μ*M* of each dNTPs, 0.2 μ*M* of each primer, 3 m*M* $MgCl_2$, 1 unit of Taq DNA polymerase (QIAGEN, Germany) and 10 to 50 ng of purified genomic DNA of *T.evansi*. The samples were processed through 30 cycles consisting of 30 s at 95^{o}C, 30 s at 60^{o}C and 3 min at 72^{o}C followed by extension step of 10 min at 72^{o}C. PCR products were separated and analysed on 1.5% agarose gels and stained with 0.5 μg/mL ethidium bromide and visualized under ultraviolet light.

3. RESULTS

In this study, the size of the ITS1 products (540 bp) obtained with Kin primers was constant among 10 isolates of *T.evansi* tested (Fig. 2). DNA polymorphism of each isolate of *T.evansi* generated by MORF2-REP primers yielded 2 DNA fragments (alleles) ranging from 578 bp to 884 bp, corresponding to 3 to 6 repeats respectively, could be distinguished easily by agarose gel separation.

We analysed genetic diversity through a single PCR based on a repeated DNA coding sequence marker within *T.evansi* isolates from various animals. Fifty-four isolates of *T.evansi* originated from 6 species of hosts in 20 provinces of Thailand were compared as to the genetic patterns of this marker.

The results revealed that *T.evansi* isolates had a high degree of heterozygosity at this locus related to the repeated coding sequence. Therefore, 4 group-specific genotypes, namely group 1 (3/5), group 2 (3/6), group 3 (4/5) and group 4 (5/6), were demonstrated based on numbers of repeats in allele 1 and 2 (Fig. 3).

Group 2 showed 25.9% (14/54); the highest distributed in host species (5 species), whereas group 3 was the highest geographically spread (13 provinces) and defined as a predominant genotype with 53.7% (29/54) of *T.evansi* isolates in Thailand (Tables III and IV).

Group 3 was predominant for 10 years (1990-2000) whereas group 4 was found in 1995 (Table V.)

4. DISCUSSION AND CONCLUSION

Kin primers were used in the single PCR assay for the detection and discrimination of all *Trypanosoma* subgenus, species or types. The size of ITS1 products was demonstrated as followed: *T.vivax* (305 bp), *T.simiae* (435 bp), *T.theileri* (455 bp), Trypanozoon (540 bp), *T.congolense Kenya Coast* (680 bp), *T.congolense savannah* (750 bp), *T.congolense forest* (780 bp). All Trypanozoon tested (*T.equiperdum, T.evansi* and *T. brucei*) yielded the same size of PCR products [7]. In this study, we used the same primers and protocol to detect 10 isolates of Trypanosoma sp. The size of ITS1 products (540 bp) was the same size product of *T.equiperdum*, *T.brucei* and *T.evansi*.

To discriminate Trypanozoon species *(T.equiperdum, T.brucei and T.evansi*), we used the same MORF2-REP primers [2]; in a single PCR to discriminate 54 Thai isolates of Trypanosoma sp. The PCR products were ranging from 578 bp to 884 bp,

corresponding to 3 to 6 repeats respectively. The result was suggested that Trypanosoma species in all hosts was *T.evansi*, not *T.equiperdum* (6/7, 11/-), not *T.brucei brucei* (6/7, 9/11) and not African Trypanosoma species. The African Trypanosoma species showed DNA fragments ranging from 884 bp to 3842 bp, corresponding to 6 to 35 repeats, respectively (Table I). The Thai *T.evansi* clusters were not highly homogenous and showed genetic diversity. Only one genotype (5/6) in group 4 of Thai *T.evansi* isolates shared the specific genotype of the Chinese *T.evansi* cluster (Tables I and III).

The genotype of Thai *T.evansi* isolates seemed to be unique and different from South American and African isolates. However, group 4 was found in north-eastern part of Thailand (Khon Kaen, Nakhonratchasima, except Nakhonpathom) since 1995 was identical to the Chinese isolates of *T.evansi*. Therefore, further investigation in the north-eastern part will be useful to analyse the origin of this genotype because it shares the specific genotype with the Chinese *T.evansi* cluster.

In conclusion, a single PCR using only one pair of primers provided a rapid and simple method for the distinction of *T.evansi* isolates in Thailand. These results are very useful for study on the dynamics of parasite populations in animals.

ACKNOWLEDGEMENTS

We thank Pranee Rodtian and Wacharin Hin-on for the strain of Trypanosoma evansi in elephant (Friends of the Asian Elephant, FAE). This work was supported by government budget under the 2003 Project of Livestock Biodiversity.

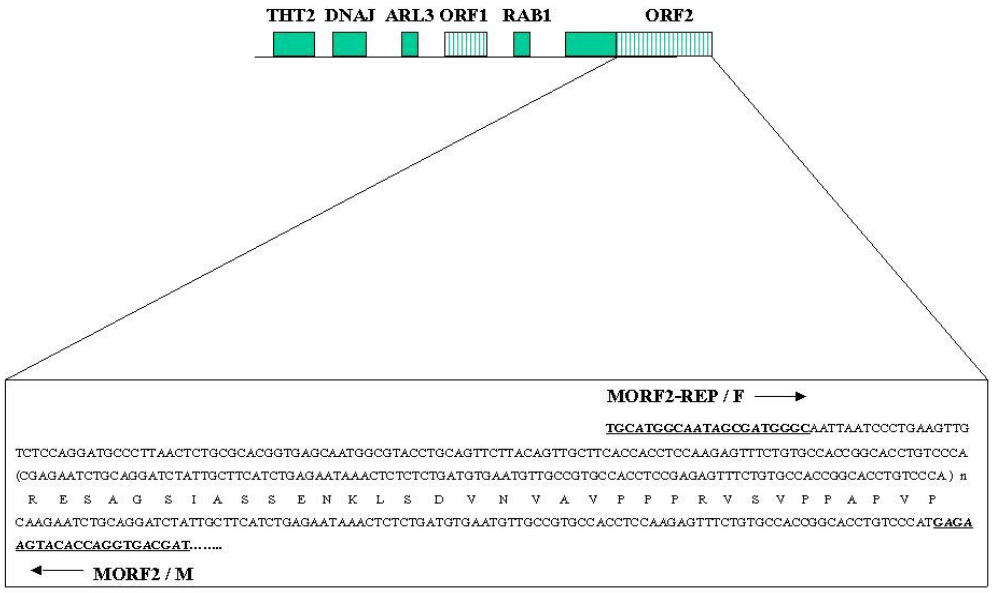

FIG. 1. Genomic organization of the downstream region of the glucose transporter gene cluster in T.brucei spp.
Nucleotide and corresponding amino acid sequences of the repeated coding sequence MORF2-REP.

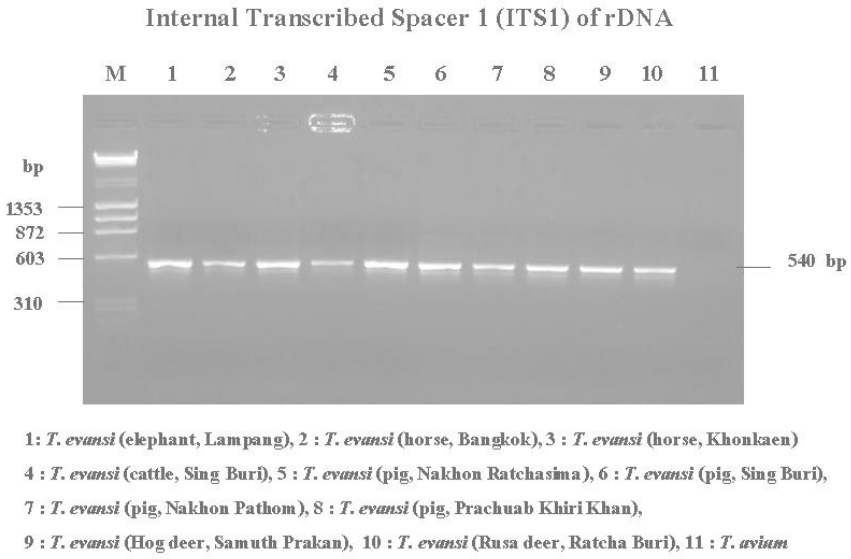

FIG. 2. PCR amplification of ITS1 gene of T.evansi from various animals in Thailand.

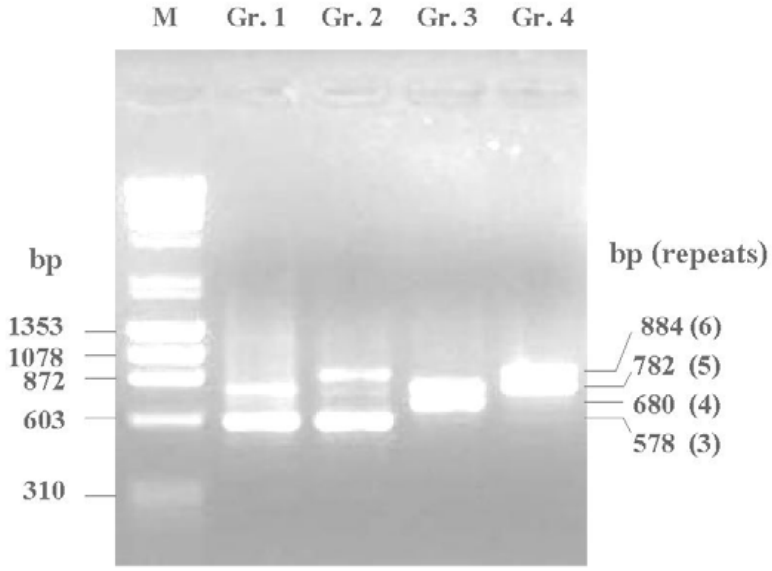

FIG. 3. PCR amplification and allele agarose gel characterization at the MOR2-REP locus from a range of different T.evansi isolates in Thailand.

M: a mix of λ DNA Hind III digest and ΦX 174 DNA Hae III digest marker ladder, 1: *T.evansi* Gr. 1 (3/5); 2: *T.evansi* Gr. 2 (3/6); 3: *T.evansi* Gr. 3 (4/5); 4: *T.evansi* Gr. 4 (5/6). In brackets, alleles identified directly from the agarose gel, Gr. are corresponding to Table III-IV.

TABLE I. SUMMARY OF INFORMATION ON THE ORIGIN OF THE TRYPANOSOME ISOLATES AND CHARACTERISTIC REPEATED CODING SEQUENCE PATTERNS [2].

Isolates Origin		Year	Host	Number of repeats in allele 1 and 2
Trypanosoma evansi				
	Columbia	1973	Horse	4 / 6
	Brazil	1989	Canine	4 / 6
	Brazil	1989	Canine	4 / 6
	China (Shangai)	1988	Bovine	5 / 6
	China (Zhejiang)	1985	Buffalo	5 / 6
	China (Nanjing)	1988	Bovine	5 / 6
	China (Guangxi)	1988	Mule	5 / 6
	China (Jiangxi)	1988	Buffalo	5 / 6
	Chad	1988	Camel	3 / 4
	Ethiopia	1988	Camel	6 / 7
Trypanosoma equiperdum				
	China (Beijing)	1979	Horse	6 / 7
	South Africa		Horse	11 / -
Trypanosoma brucei brucei				
	Ivory Coast	1997	Pig	6 / 7
	Uganda	1971	Tsetse fly	9 / 11
Trypanosoma brucei rhodesiense				
	Kenya	1961	Human	10 / 35
	Uganda	1960	Tsetse fly	10 / 35
	Tanzania	1991	Human	10 / 11
Trypanosoma brucei gambiense				
	Ivory Coast	1997	Human	7 / 10
	Cameroon	1974	Human	7 / 11
	Congo	1983	Pig	7 / 11
	Congo	1986	Sheep	7 / 13

TABLE II. LIST OF 54 ISOLATES OF *T.EVANSI* ACCORDING TO GEOGRAPHICAL ORIGINS KEPT AT NATIONAL INSTITUTE OF ANIMAL HEALTH, BANGKOK, THAILAND SINCE 1990

No.	Location	No. of isolate	Host	Year
1	Bangkok	1	Horse	2004
		1	Buffalo	1996
		1	Cattle	1994
2	Nonthaburi	1	Buffalo	1991
3	Samuthprakan	8	Hog deer	1997, 1998
4	Chachoengsao	8	Pig	1990, 1992
5	Prachinburi	1	Pig	1997
6	Chonburi	2	Pig	1993, 1994
7	Rayong	1	Cattle	1998
		1	Horse	1991
8	Nakhonpathom	1	Buffalo	2004
		1	Cattle	1999
		1	Pig	1992
9	Ratchaburi	1	Rusa deer	2001
		2	Pig	1997, 1999
10	Suphanburi	2	Pig	1991, 1999
11	Singburi	2	Pig	1998, 2002
		1	Cattle	2002
12	Saraburi	1	Cattle	1996
		1	Pig	1996
13	Lopburi	4	Cattle	1991
14	Nakhonratchasima	1	Buffalo	1999
		1	Pig	1997
15	Khon Kaen	1	Horse	2001
16	Phetchaboon	1	Cattle	1990
17	Phichit	2	Cattle	1999
18	Phetchaburi	1	Cattle	1991
19	Lampang	1	Elephant	2004
20	Prachuabkhirikhan	4	Pig	1999, 2000

TABLE III. SUMMARY OF INFORMATION ON THE ORIGIN OF T.EVANSI ISOLATES IN 6 HOSTS AND CHARACTERISTIC REPEATED CODING SEQUENCE PATTERNS

Host origin	Number of province	Number of isolates based on number of repeats in allele 1 and 2				Total
		3 / 5	3 / 6	4 / 5	5 / 6	
		Gr. 1	Gr. 2	Gr. 3	Gr. 4	
Elephant	1		1			1
Horse	3		1	1	1	3
Buffalo	3			2	2	4
Cattle	10	5	3	6		14
Pig	10	1	1	20	1	23
Deer	2	1	8			9
Total of host		3	5	4	3	
Total of isolate		7	14	29	4	54

TABLE IV. SUMMARY OF INFORMATION ON THE GEOGRAPHICAL DISTRIBUTION OF *T.EVANSI* ISOLATES AND CHARACTERISTIC REPEATED CODING SEQUENCE PATTERNS.

No	Province	Host	Number isolates based on repeats in allele 1 and 2				Total
			3/5	3/6	4/5	5/6	
1	Bangkok	B, C, H		1	2		3
2	Nonthaburi	B			1		1
3	Samuthprakan	D	1	7			8
4	Chachoengsao	P			8		8
5	Prachinburi	P			1		1
6	Chonburi	P	1		1		2
7	Rayong	C, H		1	1		2
8	Nakhonpathom	B, C, P	2			1	3
9	Ratchaburi	D, P		1	2		3
10	Suphanburi	P			2		2
11	Singburi	C, P			3		3
12	Saraburi	C, P		2			2
13	Lopburi	C	2	1	1		4
14	Nakhonratchasima	B, P				2	2
15	Khonkaen	H				1	1
16	Phetchaboon	C	1				1
17	Phichit	C			2		2
18	Phetchaburi	E		1			1
19	Lampang	P			4		4
20	Prachuabkhirikhan						
	Total of province		5	7	13	3	
	Total of isolate		7	14	29	4	54

B = Buffalo, C = Cattle, D = Deer, E = Elephant, H = Horse, P = Pig

TABLE V. SUMMARY OF INFORMATION ON THE GENOTYPE DISTRIBUTION OF *T.EVANSI* ISOLATES IN 5 YEARS INTERVAL SINCE 1990

Year	No. of isolates based on genotype				Total
	Gr. 1	Gr. 2	Gr. 3	Gr. 4	
1990 -1994	5	1	15	0	21
1995 - 1999	2	10	10	2	24
2000 - 2004	0	3	4	2	9
Total	7	14	29	4	54

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REAL-TIME PCR FOR DETECTION OF TRYPANOSOMA EVANSI IN BLOOD SAMPLES USING SYBR GREEN I FLUORESCENT DYE

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Abstract

A real-time PCR assay was developed for the detection of *Trypanosoma evansi* DNA in mouse blood samples. The PCR was performed with repetitive DNA primers targeting the 257-bp in *T.evansi* and with SYBR Green I fluorescent dye to monitor the amplicon accumulation. The minimal detection of purified *T.evansi* genomic DNA was 0.00338 pg per reaction. DNA template preparation was performed on *T.evansi* infected mouse blood samples using Chelex 100 resin. It was shown to be a simples and quantitative method as revealed by real-time PCR. The detection limit of the assay was as little as 0.039 Trypanosomes (0.0039 pg) per reaction corresponding to 39 Trypanosomes per mL of blood. DNA template of *T.evansi* from blood samples was amplified with as little as same amount of purified *T.evansi* genomic DNA. Similar efficiency between the real-time assay ensured quantitative results. No amplicon was obtained with samples from *Babesia bovis, B. bigemina*, *Theileria orientalis* and *Anaplasma marginale* infected cow blood. The results indicate that the real-time PCR assay described is a rapid and sensitive method suitable for the detection of *T.evansi* in blood samples in routine clinical laboratory practice.

1. INTRODUCTION

Trypanosoma evansi is a blood protozoan that causes typanosomiasis or surra in wide range of animals in Thailand including horses [3], cattle [5]; [24]; [26]; buffaloes [12]; [13]; [14]; sows [23]; Rusa deer [9]; hog deer [25]; and elephants [19]. Several diagnostic techniques for *T.evansi* infection rely on conventional Giemsa-stained thin blood-smear, hematocrit centrifugation technique (HCT) and mouse inoculation (MI) which were time-consuming and low sensitivity. Molecular tests for *T.evansi* could potentially improve diagnostic accuracy by using techniques such as PCR to detect *T.evansi* in blood [8]; [21]; [22]; [28]; [29]. The PCR coupled with ELISA technique (PCR-ELISA) to detect PCR product was highly sensitive and specific assay for *T.evansi* infection in animals and vector [6].

In this paper, we describe a more practicable technique for detection of *T.evansi* in blood samples using real-time PCR.

2. MATERIAL AND METHODS

2.1. Sources of *T.evansi*

One isolate of *T.evansi* obtained from dairy cow in Bangkok was used to infected mice for sensitivity test. Purified *T.evansi* isolated from mouse blood by DEAE cellulose 11) was used to extract genomic DNA by DNA extraction kit (FlexiGeneR, QIAGEN). DNA concentration was measured by spectrophotometer (SmartSpecTm 3000, BIO RAD). Ten isolates of *T.evansi* from infected mouse bloods originated from various hosts were also used to prepare DNA templates for specificity test (Table I).

2.2. DNA templates preparation

Chelex-100R was used to prepare *T.evansi* DNA template from mouse infected blood samples. Briefly, 50 µL of whole blood was lysed twice in 500 µL of 0.1 *M* ammonium chloride. The supernatant was discarded. The sediment was suspended in 50 uL of 0.002% SDS and 50 µL of 5% chelex-100® suspension in TE buffer (10 m*M* Tris-HCl, 0.1 m*M* EDTA, pH 8.0) was added. The mixture was gentle vortexed and heated at 70^{o}C for 8 min, then vortexed for 10 s following by heating to 100^{o}C for 4 min. The mixture was vortexed again and centrifuged at 10,000 rpm for 5 min at room temperature. Two microliters of supernatant containing parasite DNA was taken prior to PCR amplification.

2.3. Real-time PCR assay

For the real-time PCR assay, a final volume of 20 µL mixture contained 1x PlatinumR SYBRR Green qPCR Supermix-UDG (Invitrogen): 1.5 U PlatinumR Taq DNA polymerase, 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 3 m*M* MgCl2, 200 u*M* dGTP, 200 u*M* dATP, 200 µ*M* dCTP, 400 µ*M* dUTP, 1 U UDG, 1 µg BSA, 1 µ*M* each repetitive *T.evansi* DNA primer. PCR reactions were performed with 2 µL template and distilled water was used as a negative control. After activation of the PlatinumR Taq DNA polymerase for 2 min. at 50^{o}C and an initial denaturation for 2 min at 95^{o}C, PCR was performed for 50 and 70 cycles of denaturation at 94^{o}C for 5 s, annealing at 55^{o}C for 10 s and extension at 72^{o}C for 10 s with fluorescence monitoring. The amplification was carried out in a LightCycler Instrument (Roche Diagnostics). Melting curve analysis was performed during real-time PCR to analyze and verify the specificity of the reaction using melting curve program of Roche LightCyclerR version 4.0. Results were expressed by determination of crossing point (CP), which marked the cycle when the fluorescence of a sample exceeded the baseline signal.

2.4. Verification of sensitivity

To determine the detection limit of the real-time PCR assay and to establish a standard curve that could be used for quantification, a serial 10 fold dilution of purified genomic *T.evansi* DNA with a final concentration from 16.9 ng to 1.69 fg per assay was subjected to analysis. A Two-fold serial dilution of *T.evansi* in mouse blood samples containing from 5000 to 2.4 parasites per mL were performed under the same amplification in duplicate. The PCR products generated through the real-time assay were electrophoresed in an agarose gel and stained with ethidium bromide and SYBR Gold Nucleic Acid Gel Stain (Molecular Probes, Inc.)

2.5. Verification of specificity

Amplification of the 18S rRNA gene for each sample was performed with universal primers (Table II) to normalize the quality and quantity of DNA between samples. A panel of DNA samples from the related blood protozoa *Babesia bigemina, B.bovis, Theileria orientalis* and *ricketsia Anaplasma marginale* as well as *T.evansi* were tested by real-time PCR for primer specificity.

Another 10 isolates of *T.evansi* originated from various hosts were also tested by the real-time PCR for primer specificity.

3. RESULTS

3.1. Sensitivity of test

The minimal initial concentration of purified *T.evansi* DNA template was 0.00338 pg per reaction at crossing point of 53.6 cycles (Fig. 1B). The DNA templates, ranging from 338 pg to 0.00338 pg of purified *T.evansi* genomic DNA showed linearity (r = - 0.97) for the crossing point and DNA concentration over a 10000-fold range (Fig. 1C, 1D). The crossing point for real-time PCR amplification of DNA templates from *T.evansi* in mouse blood samples against the number of *T.evansi* per reaction (5 to 0.039 parasites per reaction corresponding to 5000 to 39 parasites per mL) showed linearity (Fig. 1E) with a significant coefficient of correlation (r = -0.96).

Melting curve analysis was performed to identify DNA products from the melting temperature (Tm) of each sample. The melting peak chart of purified *T.evansi* DNA and that from whole mouse blood, showed a homozygous sample contains one DNA sequence or one single peak, which each melt at an average Tm of 85.0^{o}C and 82.8^{o}C at 50 and 70 cycles of the amplification, respectively (Fig. 2A, 2B). No amplification or no primer dimers formation occurred without *T.evansi* DNA template.

The PCR product obtained from purified and infected mouse blood from *T.evansi* DNA amplification was 257 bp without primer dimers formation and non-specific band with no DNA template sample (Fig. 3).

3.2. Specificity test

DNA from all parasite samples included in the panel was amplified with the universal 18S rRNA primers, generating amplification curve (Fig. 4A) and confirming the quality and amount of DNA loaded per reaction. The repetitive *T.evansi* DNA primers reacted only with *T.evansi* DNA, generating amplification curve. No amplification of the other blood protozoa with the repetitive *T.evansi* DNA primer at crossing point of 55 cycles (Fig. 4B).

Melting curve analysis of the real-time PCR amplified ten isolates of *T.evansi* originated from various hosts showed identical Tm (Fig. 5A), and DNA fragment 257 bp in size (Fig. 5B).

4. DISCUSSION AND CONCLUSION

The minimal limit of detectable purified *T.evansi* genomic DNA in this study was 0.00338 pg per reaction. It was higher sensitive than previously published PCR assays which the minimal level of detection at 0.5 to 1 pg per reaction of *T.evansi* DNA was reported by using the same primers in this study [28]; [22]. The minimal detection of real-time PCR was also higher sensitive than PCR-ELISA which could detect *T.evansi* DNA at the level as less as 0.01 pg per reaction by using the same primers in this study [6].

The minimal detection of the real-time PCR in this study for detection of *T.evansi* using DNA templates derived from chelex-100® preparation was 38 parasites per mL of blood. It was higher sensitive than conventional parasitological techniques which microscopic examination from Giemsa stained thin blood film has a sensitivity of 10^5 Trypanosomes per mL of blood [16]. The diagnostic capability could be significantly improved by adopting simple, low-cost alternative, such as the hematocrit

centrifugation technique (HCT), which has a sensitive of 85 Trypanosomes per mL blood [17]. The mouse inoculation test (MI) is the most sensitive method for the isolation of *T.evansi* from infected animals. The MI test is able to detect *T.evansi* in bovine blood to a level of at least 25 parasites per mL of blood regardless of mouse strain used [18]. However, the real-time PCR assay on mouse blood in this study was higher in sensitivity than that shown recently [1]; for a real-time PCR assay for detection of *T.brucei* in human blood samples using Sybr Green I dye.

The DNA template derived from Chelex 100 resin preparation from blood sample on Whatman FTA cards. The detection limit of the assay was 100 Trypanosomes per mL blood. Furthermore, the assay in this study using whole blood shows proximately 2.5 times better sensitivity than using dried blood on Whatman FTA card.

The PCR product of purified *T.evansi* DNA analysed by agarose gel electrophoresis and stained with ethidium bromide could be seen as little as 0.5 pg per reaction [28]. This is equivalent to a genome content of 5 parasites, assuming that one Trypanosome parasite has a DNA content of 0.1 pg [4], but the amplicons of *T.evansi* amplified by the real-time PCR and analysed by agarose gel stained with SYBR gold could be seen as less as 0.00338 pg per reaction in this study.

Future studies should develop Real-time PCR assays for Trypanozoon (*T.evansi, T.equiperdum, T.b.brucei, T.b.rhodesiense, T.b.gambiense)* using detection by hydrolysis probes using ITS1 of rRNA primers [7]; and MORF2 primers [2]. Comparison of real-time PCR detection methods for Trypanozoon using ITS1 and MORF2 primers should be evaluated in term of diagnostic sensitivity in experimental parasite infected calves.

In conclusion, the real-time PCR assay is very sensitive and specificity assay that can be used to detect *T.evansi* in blood samples using *T.evansi* repetitive DNA primers and SYBR Green I fluorescent dye. The detection response was linear of purified T.evansi genomic DNA concentrations (r =-0.97) as well as parasite number (r =-0.96), and the assay was able to detect as little as 0.00338 pg of *T.evansi* genomic DNA and 39 parasites per mL of blood. The real-time PCR assay presents several important advantages over other methods of detection and quantification of *T.evansi* DNA. First, the real-time PCR is performed in a closed tube with no post-PCR manipulations, thereby reducing potential amplicon carryovers and post-PCR processing time. Second, the assay is quick; results can be confirmed within 1 h, thirdly, the assay response is sensitive and linear over a broad range of DNA concentrations.

ACNOWLEDGEMENTS

This research project number THA/11417-CRP D3.20.21 was supported under research contract No.11417/R3 of the International Atomic Energy Agency (IAEA) in the budget year 2004.

TABLE I. *T.EVANSI* ISOLATES ORIGINATED FROM VARIOUS NATURALLY INFECTED HOSTS WERE USED IN THIS STUDY

Host	Year	Place	Genotype(2,19)
Sow	1991	Suphanburi	4/5
Cattle	1991	Petchaburi	3/5
Cattle	1991	Lopburi	3/6
Hog deer	1997	Samuthprakan	3/5
Sow	1997	Nakhonratchasima	5/6
Cattle	1999	Pichit	4/5
Sow	2000	Prachuabkirikan	4/5
Horse	2003	Bangkok	3/6
Elephant	2004	Lampang	3/6
Cattle	2005	Bangkok	3/6

TABLE II. SEQUENCES OF PRIMERS USED IN THIS STUDY

Gene and primer	Sequence
Universal 18S rRNA (10)	
Sense primer	5’-CGGCTACCACATCTAAGG-3’
Antisense primer	5’-TATACGCTATTGGAGCTGG-3’
Repetitive *T.evansi* DNA	
Sense primer	5’-GCGCGGATTCTTTGCAGACGA-3’
Antisense primer	5’-TGCAGACACTGGAATGTTACT-3’

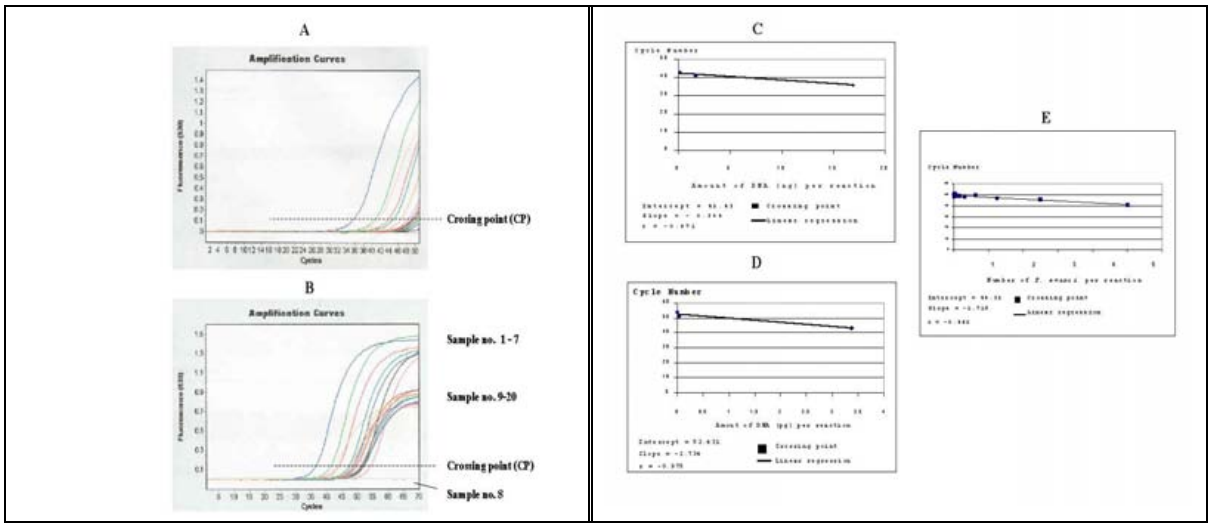

FIG. 1. Amplification curve of real-time PCR.

Amplified 10-fold serial dilutions (undiluted to 1:10,000,000) of purified *T.evansi* genomic DNA (16.9 ng, 1.69 ng, 0.169 ng, 1.69 pg, 0.169 pg, 16.9 fg,1.69 fg), (Sample no. 1-7,8:distilled water as negative control and 2-fold serial dilutions (undiluted to 1: 2,048) of *T.evansi* infected (5,000 to 2.44 parasites per mL) mouse blood (Sample no. 9-20) was performed with 2 µM each *T.evansi* repetitive DNA primer using Platinum SYBR ® Green qPCR SuperMix UDG supplemented with BSA. [A: 50 cycles, B: 70 cycles]

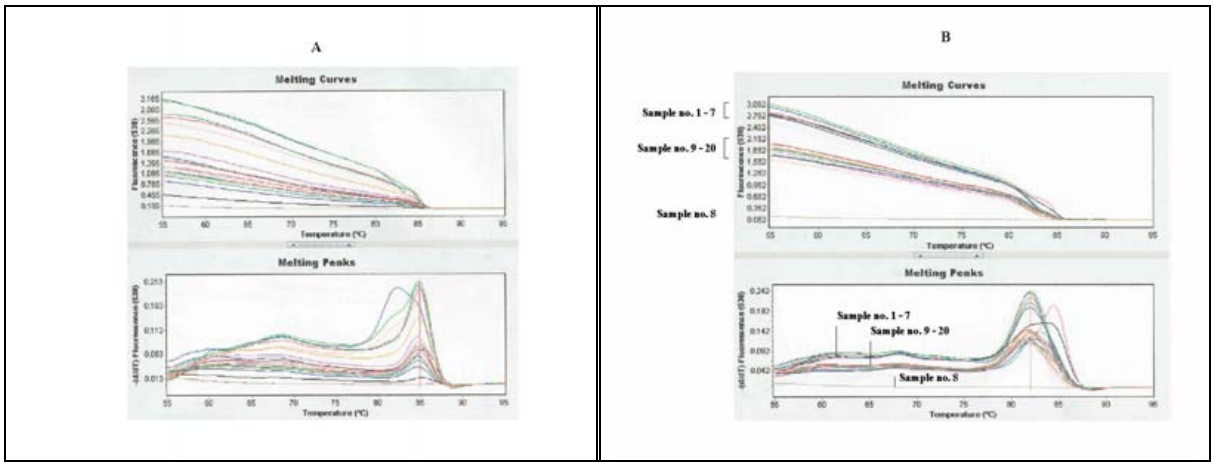

FIG. 2. Melting curve analysis of the amplicon obtained from real-time PCR.

Purified *T.evansi* genomic DNA and *T.evansi* infected mouse blood at 50 cycles (A) and 70 cycles (B) showed melting temperature (Tm) and fluorescence.

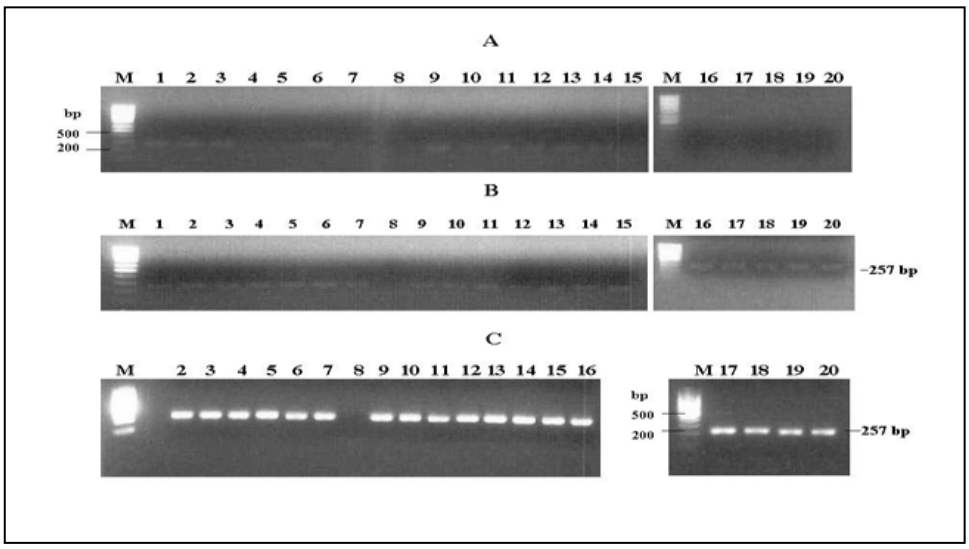

FIG. 3. Comparison of the amplicons using different methods.

The 257 bp stained with ethidium bromide (A, B) and SYBR Gold stain (C). Identical 10 microliter of the amplicons obtained from triplicated real-time PCR (A: 50 cycles, B, C: 70 cycles) were separated on 1.5% agarose minigels by electrophoresis (Mupid EX) at 100 volts for 30 min., and the image was photographed using gel documentation (BIO RAD).

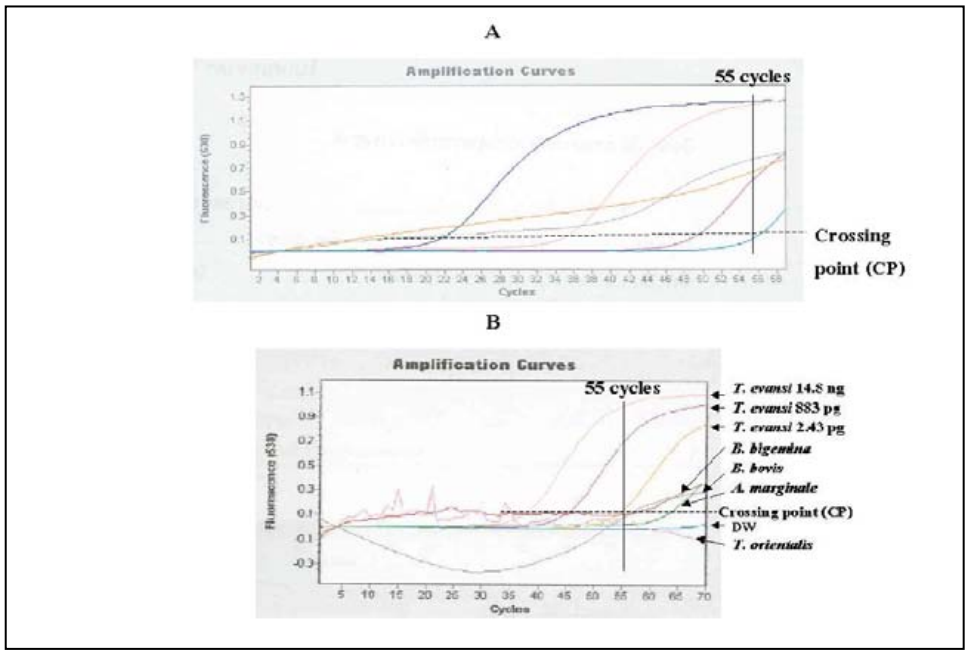

FIG. 4. Specificity test of real-time PCR.

Performed to amplify different purified *T.evansi* DNA concentration (14.8 ng to 2.43 pg) and other blood protozoan DNA with 18S rRNA primers (A) and 1 µM repetitive *T.evansi* DNA primers (B).

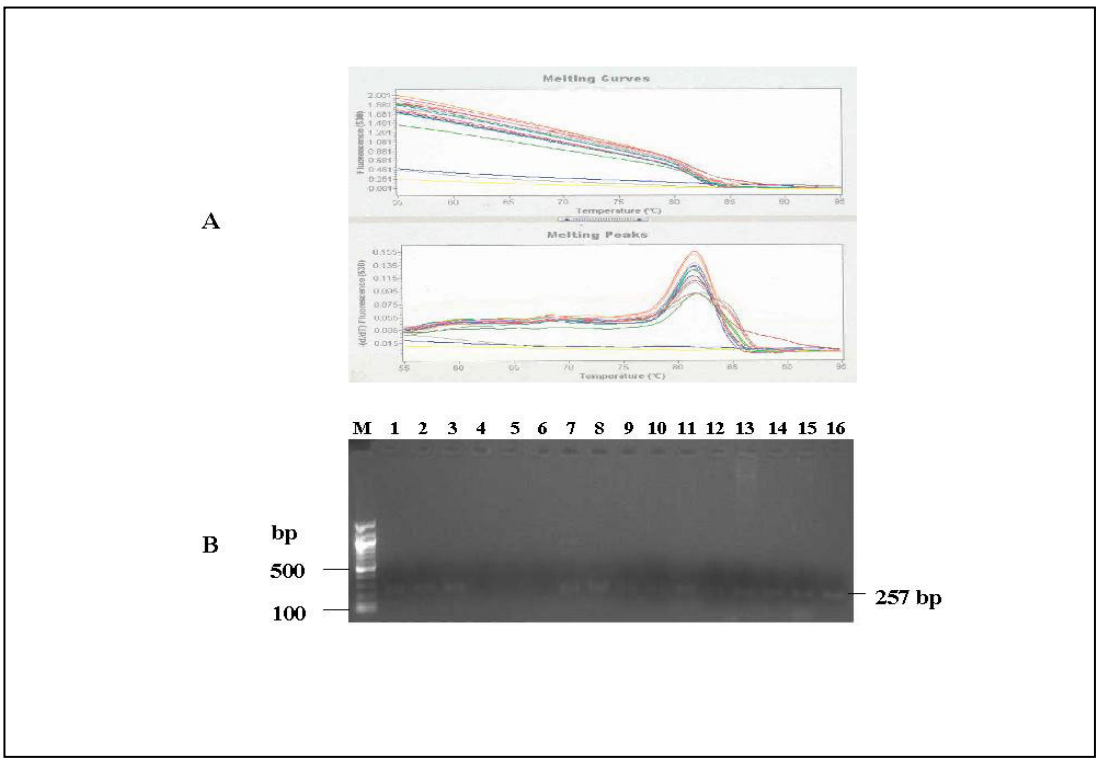

FIG. 5. Melting curve analysis of the amplicon obtained from real-time PCR.

Purified *T.evansi* genomic DNA and DNA obtained from 10 isolates of *T.evansi* infected mouse blood showed identical melting temperature without fluorescence from mouse, cow DNA templates, distilled water (A). Identical band(257 bp) of the amplicon (10 ul) was detected on 1.5% agarose gel stained with ethidium bromide (B).[M:100 bp DNA ladder, Lane 1:purified *T.evansi* DNA template(1.69 ng), Lane 2: purified *T.evansi* DNA template(0.169 ng), Lane 3:purified *T.evansi* DNA template(0.0169 ng), Lane 4:mouse DNA, Lane 5:Cow DNA, Lane 6:distilled water, Lane 7-16:*T.evansi* DNA templates from mouse infected bloods originated from various hosts according to Table I).

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APPLICATION OF DRIED BLOOD SAMPLE ON FTA® PAPER FOR DETECTION *OF TRYPANOSOMA EVANSI* BY PCR

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Abstract

A highly sensitive and specific polymerase chain reaction (PCR) assay for the detection of *Trypanosoma evansi* present in the dried blood on FTA® Paper was developed. A simple lysis method was used to remove of the red blood cells on a 1.2 x 1.2 mm paper in 0.2 mL PCR tube. The dried paper sample was placed directly into a 25 μL PCR reaction as a DNA template. The primer set was designed and synthesized to amplify a single band of 257 bp PCR product. The sensitivity limit of test was 2.2×10^{5} parasites/mL that was less than DNA templates derived from whole blood at 6.0×10^{-2} parasites/mL. The use of dried blood on FTA® paper was sensitive equivalent to the determination by using the conventional wet blood film (WBF) and less sensitive than microcentrifuge heamatocrit test (MHCT) as well as mouse inoculation test (MIT). This application is not only beneficial for detection of the parasite but also useful for epidemiological study and designing Trypanosomiasis control programme.

1. INTRODUCTION

Trypanosoma evansi is the blood protozoan that causes Trypanosomiasis or surra in wide range of hosts in Thailand such as horses [1]; buffaloes [2]; [3]; [4]; dairy cattle [5]; beef cattle [6]; [7]; sows [8]; Samba and Rusa deer [9]; and hog deer [10]. The clinical symptoms of *T.evansi* infection varied according to the animal species. Generally, the disease showed signs of depression, inappetite, fever, anemia, nervous signs and death in some cases. The abortion in the later stage of pregnancy and premature parturition were reported in infected buffaloes, cattle, pigs and hog deer. The diagnosis of *T.evansi* was basically relied on conventional Giemsa staining of thin blood films and mouse inoculation which were time-consuming and required experimental animals. The card agglutination test [11]; indirect fluorescent antibody test [12]; as well as the enzyme-linked immunosorbent assay [13]; were also used for the parasite detection. However, these tests showed the disadvantage in reproducibility due to the variation batch of antigen used and significant levels of false-negative and false-positive results. Moreover, these tests do not distinguish between current infections and residual antibody from previous treatment. A number of sensitive and specific techniques had been applied in the investigation of *T.evansi* infection such as DNA probes and DNA amplification [14]. Moreover, PCR amplification of crude blood on microscope slides in the diagnosis of *T.evansi* infection in dairy cattle had been reported [15]. In this study, we applied a simple technique for blood sample collection on Whatman® paper (FTA® Cards) and detection of *T.evansi* in dried blood samples using the highly specific and sensitive polymerase chain reaction (PCR).

2. MATERIALS AND METHODS

2.1. Parasites

One isolate of *T.evansi*, NIAH/43 strain was isolated from experimentally infected pigs (from horse origin) using mouse inoculation and cryopreserved in liquid

nitrogen. The parasite was sub-inoculated into a male BAL/C mouse until the parasitemia was 2.2 x 10^4 to 6.0 x 10^7 parasites/mL.

2.2. Experimental designs

One mL of heparinized blood was collected directly from the heart of the mouse. A series of 100-fold dilution of infected blood with normal mouse's blood was prepared and tested in conventional methods and in the PCR. The conventional methods for detection of *T.evansi* including, wet blood films (WBF), microhaematocrit centrifuge test (MHCT) and mouse inoculation test (MIT) was carried out in each dilution of blood sample. The blood volume in each test was 10 μL in WBF, 50 μL in MHCT and 500 μL in MIT. The PCR amplification was performed to detect *T.evansi* using DNA templates from each blood sample using two simple methods of DNA templates preparation. Duplication of each method was carried out to detect the parasite.

2.3. DNA templates preparation

Chelex-100® was used to prepare DNA template from whole blood samples. Briefly, 50 μL of whole blood was lysed twice in 500 μL of 0.1 *M* ammonium chloride. The supernatant was discarded. The sediment was suspended in 50 μL of 0.002% SDS and 50 μL of Chelex-100® suspension in TE buffer (10 m*M* Tris-HCl, 0.1 m*M* EDTA, pH 8.0) was added. The mixture was gentle vortexed and heated at 70°C for 8 min, then vortexed for 10 second following by heating at 100°C for 4 min. The mixture was vortexed again and centrifuged at 10,000 rpm for 5 min at room temperature. Five μL of supernatant containing parasite DNA was taken prior to PCR amplification.

Whatman® paper (FTA® Cards) was used to collect whole blood samples as manufacturer' guidance. The blood samples were dried and kept at room temperature for one week. A piece of 1.2 x 1.2 mm dried blood sample paper was placed into 0.2 mL PCR tube. The paper was washed twice with FTA Purification Reagent and rinsed twice with TE buffer (10 m*M* Tris-HCl, 0.1 m*M* EDTA, pH 8.0). The paper was dried up at 56°C for 10 min prior to use directly as DNA templates for PCR amplification.

2.4. Polymerase chain reaction (PCR)

Specific oligonucleotide primer used for the amplification of *T.evansi* DNA, was derived from repetitive nucleotide sequences [15]. The PCR amplification was performed using Thermal Cycler (Hybaid Sprint). Each reaction was carried out in 25 μL volume containing 1x PCR buffer (QAIGEN), 1.5 m*M* $MgCl_2$, 25 μ*M* of each primer, TR3 (5'-GCGCGGATTCTTTGCAGACGA-3') and TR4 (5'-TGCAGACAC TGGAATGTTACT-3') and 100 unit of Taq polymerase (QAIGEN). The PCR mixture was heated at 95°C for 5 min prior to monitoring the PCR cycle. One cycle of PCR was consisted of denaturation at 95°C for 45 s, annealing at 55°C for 45 s and polymerization at 72°C for 45 s. The cycle was proceeded for additional 29 cycles in the Thermal cycler. Ten microlitres of PCR products of approximately 257 bp in size was analysed by using 1.5% agarose gel electrophoresis, ethidium bromide staining and visualized under UV transilluminator.

3. RESULTS

To determine the minimum parasites/mL detected using PCR, DNA templates obtained from 2 different methods was prepared and tested. Results (Fig. 1) showed that PCR could detect parasite DNA at the level as less as 6.0 x10^{-2} parasites/mL which

DNA samples derived from the method of Chelex-100® preparation, whereas 2.2 x 10^5 parasites/mL which DNA samples derived from dried blood on the papers.

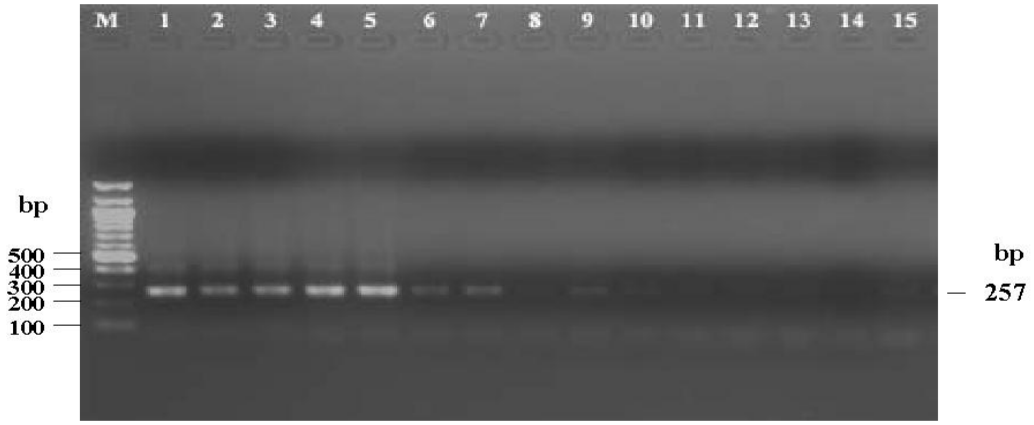

FIG. 1. Sensitivity of the each individual PCR.

Obtained using DNA templates derived from whole bloods (50 μL). Lane 1, Purified *T.evansi* DNA; lane 2-3, 6.0 x10^7 parasites/mL; lane 4-5, 6.0 x10^5 parasites/mL; lane 6-7, 6.0 x103 parasites/mL; lane 8-9, 6.0 x101 parasites/mL; lane 10-11, 6.0 x10^{-2} parasites/mL; lane 12-13, 6.0 x10^{-4} parasites/mL; lane 14-15, 6.0 x10^{-6} parasites/mL; lane M contains the molecular weight size markers consisting of fragment 100-1500 bp.

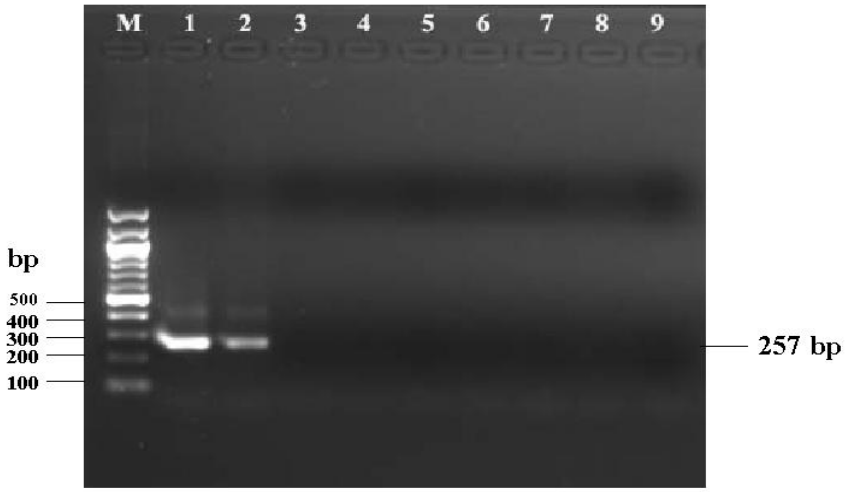

FIG. 2. Sensitivity of the each individual PCR.

DNA templates derived from dried blood on FTA papers: Lane 1, purified *T.evansi* DNA; lane 2, 2.2 $x10^{5}$ parasites/mL; lane 3, 2.2 x103 parasites/mL; lane 4, 2.2 $x10^{1}$ parasites/mL; lane 5, 2.2 $x10^{-2}$ parasites/mL; lane 6, 2.2 $x10^{-4}$ parasites/mL; lane 7, 2.2 $x10^{-6}$ parasites/mL; lane 8, 2.2 $x10^{-8}$ parasites/mL; lane 9, normal mouse blood; lane M contains the molecular weight size markers consisting of fragment 100-1500 bp.

Table I show that the determination by PCR using DNA templates derived from whole blood was more sensitive to the MHCT and MIT whereas using DNA templates derived from dried blood was equivalent to WBF.

TABLE I. SENSITIVITY OF *T.EVANSI* DETECTION IN DIFFERENT METHODS

	2.2 x 10^{5}	2.2x 10^{3}	2.2 x 10^{-1}	2.2 x 10^{-2}	2.2 x 10^{-4}	2.2 x 10^{-6}	2.2 x 10^{-7}	NC
WBF	+	-	-	-	-	-	-	-
MHCT	+	+	-	-	-	-	-	-
I. MIT	+	+	-	-	-	-	-	-
PCR (whole blood)	+	+	+	+	-	-	-	-
PCR (dried blood)	+							
	6.0 $x10^{5}$	6.0 $x10^{3}$	6.0 $x10^{1}$	6.0 x 10^{-2}	6.0 x 10^{-4}	6.0 x 10^{-6}	6.0 x 10^{-7}	
WBF	+	-	-	-	-	-	-	-
MHCT	+	+	-	-	-	-	-	-
II. MIT	+	+	+	-	-	-	-	-
PCR (whole blood.)	+	+	+	+		-	-	-
PCR (dried blood)	+	-	-	-	-	-	-	

4. DISCUSSION

The sensitivity of the PCR in this study for detection of *T.evansi* using DNA templates derived from Chelex-100® preparation was 0.022-0.06 parasite/mL which the volume of DNA templates was 5 μL (the equivalent of 100-300 parasites). It was higher than that of previously published PCR assays. The minimal level of detection at 1 pg of *T.evansi* DNA (corresponded to100 parasites/mL) was reported by using the same primers in this study [16]. Moreover, PCR-ELISA could detect *T.evansi* DNA at the level as less as 0.01 pg (corresponded to 1 parasite/mL) was also reported by using the same primers in this study [17]. It is likely that the sensitivity of the PCR depends on the volume of blood sample taken prior to DNA template preparation and reduction of PCR inhibitors such as haemoglobin and heavy metals. In this study, haemoglobin was removed by washing twice in 0.1 *M* Ammonium chloride. Heavy metals were absorbed by Chelex-100®. The result showed that PCR-based assay for the detection of *T.evansi* present in the dried blood on FTA® Paper was tested in parallel to the present of *T.evansi* in the whole blood. The sensitivity of this method was equivalent to the conventional WBF although the volume of dried blood on FTA® paper was less than 10 μL.

Haemoglobin, a PCR inhibitor was easily to remove from the papers by washing twice in FTA® Purification Reagent or 0.002% SDS. The sample papers in white colour (approximately 1/4 of paper absorbed 10 μL of blood) were dried up and directly placed into the PCR tube as DNA templates. The main advantage of using dried blood on papers as DNA templates is that the sample can be stored at room temperature and be convenient for shipment via mail. This method is suitable for convenient sample collection and shipment. That is beneficial for epidemiological survey. In addition, it is also useful for designing rational Trypanosomiasis control program in the endemic area.

ACKNOWLEDGEMENTS

This research project number THA/11417, CRP D3.20.21 was supported under Research contract No. 11417/R2 of the International Atomic Energy Agency (IAEA).

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DETERMINATION OF THE *TRYPANOSOMA CONGOLENSE* AND THE *TRYPANOSOMA EVANSI* ANTIBODIES DETECTION ELISA FOR THE DIAGNOSIS OF SURRA IN CATTLE IN THAILAND

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Abstract

An IAEA *Trypanosoma congolense* ELISA kit and *T.evansi* NIAH/ELISA were used for the detection of *T.evansi* antibodies in 850 dairy cattle in Thailand. The sensitivity of the *T.congolense* IAEA/ELISA and *T.evansi* NIAH/ELISA was high (100%), compared to parasitological test used. About 97% of the results obtained by *T.congolense* IAEA/ELISA matched to the results obtained by *T.evansi* NIAH/ELISA. The specificity of both ELISAs with respect to cross reactions with Babesia sp., Anaplasma sp. and Theileria were not established.

1. INTRODUCTION

Trypanosoma evansi is a global parasite. In Thailand it was first detected in mules in Rachaburi province in 1949. Now it is known to occur throughout Thailand not only in horses, but also in cattle, buffaloes, pigs, dogs cats, deer, hog deer and elephants with varying clinical manifestations [1]. The most severe clinical cases usually occurs in infected horses. Abortion at late stages of pregnancy or premature parturition have been reported in infected buffaloes, cattle and pigs [2]; [3]. Nervous signs caused by *T.evansi* invading the central nervous system have also been observed in infected cattle and hog deer [4]; [5].

The diagnosis of surra in Thailand relies principally on the detection of *T.evansi* in thin blood smears stained with Giemsa or by inoculation into rodents. Tuntasuvan [6] developed the indirect ELISA using crude somatic *T.evansi* antigen for the detection of antibody against *T.evansi* in pigs and in horses [7]. The test gave high specificity and sensitivity.

The objective of the study was to determine the use of the *T.congolense* IAEA/ELISA kit and *T.evansi* NIAH/ELISA for the detection of *T.evansi* antibodies in dairy cattle in Thailand.

2. MATERIALS AND METHODS

Blood samples were randomly collected from 850 dairy cattle on 39 farms in the Central and North East Thailand from January-May 2002. EDTA-blood samples were examined for *T.evansi* by thin blood smear test. Sera were separated from blood and assayed for *T.evansi* antibodies using *T.congolense* antibody detection IAEA/ELISA kit and *T.evansi* NIAH/ELISA.

2.1. *T.congolense* IAEA/ELISA

The Indirect ELISA kit is a product of the Animal Production Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria and the technique followed from the protocol supplied as in [8]. The crude *T.congolense,* CP81 antigen had been denatured with SDS and boiling (CP81#120299) and was supplied on precoated Immulon^R microplates (Dynex). The antigen coated plates were made ready for use by addition of 100 µL/well phosphate buffered saline (PBS, Medicago). After incubation at 37^{o}C for 1 h, they were washed with PBS containing Tween20 (PBS-T), 4 times. Test serum and control serum were diluted in PBS-T + 5% skimmed milk (PBS-TM) at 1:100 and 100 µL/well was added in duplicate and incubated at 37^{o}C for 1 h. The control sera composed of C++ (strong anti *T.congolense* positive serum), C+ (moderate anti *T.congolense* positive serum) and C- (negative anti *T.congolense* serum). The microplate was washed with PBS-T 4 times. The supplied anti-bovine IgG horseradish peroxidase conjugate (Sigma) was diluted in PBS-TM at 1:15000 and 100 µL/well added to the plate and incubated at 37^{o}C for 1 h. The microplate was washed with PBS-T 4 times. Tetramethyl benzidine hydrochloride, (TMB-pre prepared supplied through Kirkegaard & Perry Laboratories) was used as a chromogen/substrate at a concentration of 0.4 g/L and added 100 µL/well. After incubation at 37^{o}C for 15 min, 100 µL/well of 1M H_3PO_4 was added to stop the reaction and the plated were then read at 450 nm using ELISA reader (Biorad).

2.2. *T.evansi* NIAH/ELISA

The sonicated *T.evansi* NIAH41 antigen was diluted in carbonate-bicarbonate buffer (Sigma C-3041) at 1:100 and 100 µL/well added to MaxiSorp^R microplates (Nunc). After incubation overnight at 4^{o}C plates were washed with PBS-T (Sigma P-4711 + Tween20), 3 times. The positive control serum was from a cow infected with *T.evansi.* Test serum and the control serum was diluted in PBS-T at 1:50 and 100 µL/well was added in duplicate and incubated at 37^{o}C for 1 h. The microplate was washed with PBS-T 3 times. Protein G peroxidase conjugate (Zymed) was diluted in PBS-T at 1:5000 and 100 µL/well added to the plate and incubated at 37^{o}C for 1 h. TMB (Sigma T-3405) was used as substrate which was diluted in phosphate-citrate buffer with sodium perborate (Sigma P-4922) and added at 100 µ;/well. After incubation at 37^{o}C for 15 min, 50 µL/well of 1N sulphuric acid was added to stop the reaction. The OD was read at 450 nm using ELISA reader (Biorad). The OD values were calculated to percent positive value (%P) by setting the average OD value of C++ in IAEA/ELISA and the positive control serum in NIAH/ELISA as 100%.

3. RESULTS

3.1. Blood smear test

Using the blood smear test, *T.evansi* was detected on two farms (6/120 samples) whilst *Anaplasma* sp.*, Babesia bigemina* and *Theileria* sp. were also found in 5.0%, 0.87% and 36.67% of the cattle respectively (group 1). *T.evansi* was not detected on 37 farms (0/730 samples) and *Anaplasma* sp. and *Theileria* sp. were found in 5.07% and 8.63% of the cattle respectively (group 2).

3.2. *T.congolense* IAEA/ELISA kits

The average OD values and %P of the upper control limit (UCL) and lower control limit (LCL) of the control sera showed in Table I. If the %P decision value was double the mean %P of the pool negative control serum, the %P of the positive sample was equal or over 60 (30 x 2). The percent of positive samples were 78.9% (576/730) in group 1 and 98.3% (118/120) in group 2. Nevertheless 81 samples (67.5%) of group 2 had the OD values more than 2.0.

3.3. *T.evansi* NIAH/ELISA

The average %P of PBS, the negative control serum and the positive control serum were 20.6 (13–28), 38.0 (28–58) and 100 respectively. If the %P decision value was double the mean %P of the disease free group, the %P of the positive sample was equal or over 76 (38.0 x 2). Percent of positive sample was 99.2% (119/120) in group 1 and 79.3% (579/730) in group 2. Six positive samples by blood smear test were also positive by the IAEA-ELISA and the NIAH/ELISA. The range %P in the IAEA/ELISA and NIAH/ELISA were 90–200% and 97-164% respectively.

4. DISCUSSION

Since Luckins [9]; developed an indirect ELISA for the diagnosis of Trypanosomosis in cattle, various sensitive and specific ELISA techniques had been reported. Recently a simple ELISA test kit is required but *T.congolense* IAEA/ELISA kit was not. In the present study the sensitivity of *T.congolense* IAEA/ELISA kit and *T.evansi* NIAH/ELISA for the detection of *T.evansi* antibodies was 100% (6/6) because six positive samples by blood smear test were positive by both ELISA techniques (Table II). The percentage of the positive sample in group 2 was high (79.3%) using *T.evansi* NIAH/ELISA, though *T.evansi* was not detected on blood smear and the cattle showed no signs. It was suspected that the cattle on group 2 were infected with *T.evansi.* Because the samples were collected from January to May after high population of *Tabanus* sp. and other blood suckling flies [10].

The specificity of the NIAH/ELISA test required more cattle sera samples from a *T.evansi* free zone. Antibody cross-reaction between cattle infected with *T.evansi* and other blood parasites using NIAH/ELISA have to be studied also. About 97% of the positive and negative samples obtained from *T.congolense* IAEA/ELISA test matched to the result obtained from NIAH/ELISA test (Table III). It is understood that specificity studies using this kit have been made and that no cross reactions occur with antibodies against problem organisms (*Babesia* sp., *Anaplasma* sp. and *Theileria).*

In addition to this study, ten microplates of IAEA/ELISA kits, coated with denatured *T.vivax* were tested. The maximum and minimum OD values of ULC and LCL of the C++ were 0.652-0.150 and 0.627-0.138 where the recommended UCL-LCL was 1.568-0.688. Thus test sera could not be evaluated.

In conclusion the *T.congolense* IAEA/ELISA and *T.evansi* NIAH/ELISA are suitable for the diagnosis of surra in cattle in Thailand. The use of denatured antigens in the IAEA kit has been shown to bed suitable for diagnosis of Trypanosomes in general and this confirms the use for *T.evansi* in Thailand.

TABLE I. COMPARISON THE%P OF UCL AND LCL BETWEEN THE CONTROL SERA OBTAINED FROM *T.CONGOLENSE* IAEA/ELISA KIT AND IAEA REFERENCE VALUES

Control sera	Present study		IAEA reference	
	UCL	LCL	UCL	LCL
C++ (OD values)	1.312-0.827[a]	1.255-0.792[b]	1.845	0.508
C++ (%P)	104	96	103	97
C+ (%P)	63	58	54	35
C- (%P)	23	19	17	5
Cc (%P)	5	4	4	0

[a] = max. and min. OD values of the UCL on 18 plates
[b] = max.and min. OD values of the LCL on 18 plates

TABLE II. COMPARISON OF THE SENSITIVITY OF *T.CONGOLENSE* IAEA/ELISA AND *T.EVANSI* NIAH/ELISA FOR THE DETECTION OF *T.EVANSI* ANTIBODIES IN DAIRY CATTLE

	OD value (%P)	
Sample No. [c]	IAEA	NIAH
1	>2	0.745 (97%)
2	1.375 (90%)	0.822 (108%)
3	>2	1.075 (148%)
4	>2	0.999 (138%)
5	>2	1.054 (159%)
6	>2	1.089 (164%)

[c] = Serum sample was surra positive by blood smear test.

TABLE III. PERCENT POSITIVE SAMPLE BY IAEA/ELISA AND NIAH/ELISA

Diagnostic test		%positive samples
IAEA/ELISA (*T. congolense*)	Group 1	98.3 (576/730) [d]
	Group 2	78.9 (118/120)
NIAH/ELISA (*T.evansi*)	Group 1	99.2 (579/730)
	Group 2	79.3 (119/120)

[d] = number of reacted sample/total examined sample

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MOLECULAR DIAGNOSIS OF TRYPANOSOME SPECIES

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Abstract

Various parameters for handling samples, extracting nucleic acid and protocols for use of PCR to diagnose trypanosomes have been examined. The use of a commercial kit (Nucleon BACC 2, Amersham) was superior to other extraction methods. After comparisons, a touch-down thermocycling protocol was the procedure used. PCR testing protocols were successfully implemented using Kin primers that bind to an internal transcribed spacer region (ITS1) situated between the 18S and the 5.8S ribosomal subunit genes on nuclear DNA. Their sensitivity was lower than that of satellite DNA primers, particularly for *T.vivax*. A Kin primer-based PCR could detect and distinguish between a number of Trypanosome species in blood samples. The use of an ITS1 binding primer was 3–5 x cheaper than using classical species-specific primers since the number of PCR reactions per sample is reduced to one.

1. INTRODUCTION

1.1. Status of the Biotechnology Division, OVI

The Biotechnology Division has been actively involved with performing routine diagnostic PCR procedures for the detection of several veterinary pathogens. Well-equipped laboratories, staffed with experienced personnel are thus in place. In addition, this laboratory has been officially accredited according to ISO 17025 guidelines to perform PCR. The use of routine PCR diagnostic approaches for Trypanosome identification can therefore be readily implemented.

1.2. Methods of detecting and identifying Trypanosomes

There are several methods described for detecting tsetse-transmitted Trypanosomes according to OIE guidelines [1].

These:

(a) identify the agent

- Direct Examination
- Wet blood films
- Thick blood films
- Thin blood smear films
- Parasite concentration techniques
- Microhaematocrit centrifugation technique
- Dark-ground/phase-contrast buffy coat technique
- Anion exchange
- In vitro cultivation
- Animal inoculation
- Antigen detection (lack sensitivity and specificity)
- PCR

(b) Allows serological identification

- Indirect fluorescent antibody test
- ELISA
- Card agglutination test

These assays can't distinguish past from present infections.

1.3. Historical background

Although animal Trypanosomosis is considered to be one of the most important vector-borne animal diseases affecting sub-Saharan Africa, the incidence of disease in South Africa is limited to small discrete regions only. In Zambia, however, agricultural development is retarded by Trypanosomosis [2]. Trypanosomosis is also present in Mozambique, which borders northern KwaZulu Natal and Mpumalanga, South Africa.

No detailed information prior to 1920 exists on the incidence of Trypanosomosis in South Africa, except for the early discoveries of Bruce. The Rinderpest pandemic of the 1890s eliminated tsetse flies from previously infected areas viz. northern, north-western and eastern Transvaal and Zululand. Pockets of wild animals and tsetse flies survived and by 1905 nagana once again became a threat, with several outbreaks occurring in surrounding areas [3]. A serious outbreak occurred in Zululand in 1990 and emergency control measures were then implemented. *T congolense* and *T.vivax* were detected. The OVI has been involved in tsetse fly research by monitoring and developing control methods against G. brevipalpis and G. austeni. A field station was established for this purpose, situated in a nature conservation area next to Lake St Lucia, KwaZulu-Natal. There are 4 species of tsetse flies that have been recorded in South Africa [4]; viz. Glossina morsitans morsitans Westwood, the only species encountered in the most northerly parts of South Africa*, G. pallidipes Austen*, *G. brevipalpis Newstead* and *G.austeni Newstead* in Zululand. The three Trypanosomes most commonly found in northern KwaZulu-Zululand have been *T.brucei, T.congolense* and *T.vivax.*

1.4. The use of PCR for detecting Trypanosomes

The use of PCR for detecting Trypanosomes has been well described. It has been used as a tool to identify Trypanosome taxa [5]; and to detect organisms in blood [6]. It has been shown that PCR is more sensitive than buffy coat smear examinations [7]. PCR was also able to detect a single parasite in the vector [8]; and furthermore, a detection rate in blood was about twice that of direct parasitological techniques [9]. Infected animals can nevertheless remain negative with PCR because of the wide fluctuations in parasitaemia (10^6 to 10^{-3} parasites/ mL blood) [10]. PCR sensitivities can vary between 1 to 20 parasites/mL blood, depending on techniques used [10].

Species-specific primers are not available for all Trypanosoma spp. of veterinary interest e.g. *T.equiperdum*. Research is now directed towards amplification of the internal transcribed spacer of ribosomal DNA, which occurs as a multiple copy locus and is small in size but nevertheless varies in size between taxa. Primers binding to the internal ribosomal spacer region IRS1 have already been developed that can allow for the distinction of many different species.

2. MATERIALS AND METHODS

2.1. Blood samples

A number of *T.congolense* and *T.brucei* infected blood samples were received from the University of Natal, South Africa and were as controls.

A large panel of blood samples were provided by a visiting research fellow from KETRI in Kenya.

The Trypanosome species supplied included: *T.vivax*, *T.congolense savannah*, *T.congolense* forest, *T.congolense* Kilifi, *T.brucei gambiense*, *T.brucei rhodesiense*, *T. lewesi, T.simiae* Tsavo, *T.theileri*, *T.evansi* and *T. godfreyi.*

Several blood samples derived from roan antelope from a game farm were obtained from the Parasitology Division, OVI. These had been submitted for serological testing and were shown to be CATT positive.

2.2. DNA Extraction

DNA extraction for PCR was performed according to several different methods in order to determine the optimal procedure: viz. Nucleon Bacc 2, a Brucella extraction method, the Chomczynski method, QIAamp ® DNA Blood Mini Kit extraction method (Qiagen), and MDS and FTA matrix-based procedures.

2.2.1. Nucleon BACC 2 extraction (Amersham Life Sciences)

TABLE I. PROTOCOL FOR BACC 2 EXTRACTION

Reagent/procedure	Time/Volume
Blood	300 µL
Reagent 1	1.2 mL
Vortex	10 s
Centrifuge 13,000 rpm	3 min
Reagent 2 to pellet	400 µL
Vortex	10 s
Reagent 3	50 µL
Vortex	10 s
Incubate 65ºC	5 min
Chloroform	200 µL
Vortex	10 s
Centrifuge 13,000 rpm	1 min
Add Biophenol to supernatant	Equal volume
Vortex	10 s
Centrifuge 13,000 rpm	1 min
Remove supernatant and repeat	
96% EtOH and mix	1mL
Centrifuge 13,000 rpm	2 min
70% EtOH to pellet	500 µL
Vortex	10 s
Centrifuge 13,000 rpm	1 min
Air dry pellet	10 –15 min
dd Water	50 µL

* Biophenol: phenol:chloroform:isoamyl alcohol (25:24:1)

2.2.2. Chomczynski method

This method, [11]; although routinely used for RNA extraction from tissues, was also tested for extracting Trypanosome DNA from infected blood.

TABLE II. PROTOCOL FOR CHOMCZYNSKI METHOD

Reagent/procedure	Time/Volume
Mix blood with Solution D*	Equal volume
Add 2 *M* sodium acetate, pH 6	100 μL
Water saturated phenol then mix	1 mL
Chloroform-isoamyl alcohol (49:1)	200 μL
Mix	10 s
On ice	15 min
Centrifuge 10,000 g	20 min
Transfer aqueous phase	
Add isopropanol	1 mL
- 20 º C	1 h
Centrifuge 10,000 g	10 min
Dissolve pellet in 75% ethanol	
Centrifuge	
Air dry	15 min
Dissolve pellet in 0.5% SDS	50 μL

* 4M guanidinium thiocyanate, 25 m*M* sodium citrate, pH 7; 0.5% sarcosyl; 0.1 *M* 2-mercaptoethanol.

2.2.3. Brucella extraction method (Romero et al. 1995 [12])

TABLE III. PROTOCOL FOR BRUCELLA EXTRACTION METHOD

Reagent/procedure	Time/Volume
Blood	500 µL
Add Buffer 1*	500 µL
On ice	30 min
Centrifuge 12,000 rpm	15 min
1x SSC to pellet then vortex	500 µL
Centrifuge 12,000 rpm	15 min
Add Buffer 2** to pellet then vortex	500 µL
Add proteinase K 20 mg/mL	20 µL
Incubate 50 ºC	2 h
Add Biophenol*** then vortex	500 µL
Centrifuge 12,000 rpm	10 s
Add 7.5 *M* ammonium acetate	1/3 volume
Add absolute ethanol	2 volumes
Centrifuge 12000 rpm	10 s
Wash pellet in 70 ethanol	
Centrifuge 12000 rpm	10 min
Air dry	
Dissolve pellet in dd H_2O	50 µL

* Buffer 1: 20 m*M* NaCl; 20 m*M* EDTA; 20 m*M* Tris HCl, pH 7.5; 0.5% Triton X-100; ** Buffer 2: 10 m*M* NaCl; 50 m*M* EDTA; 50 m*M* Tris-HCl, pH 7.5; 1% SDS; *** Biophenol: Phenol:chloroform:isoamyl alcohol (25:24:1)

2.2.4. MDS method (Prof. Denis York, Molecular Diagnostic Systems PTY (LTD), Durban, South Africa

TABLE IV. PROTOCOL FOR MDS METHOD

Reagent/procedure	Time/Volume
Blood	300 µL
Reagent 1	1.2 mL
Vortex	10 s
Centrifuge 13,000 rpm	3 min
Reagent 2 to pellet	400 µL
Vortex	10 s
Reagent 3	50 µL
Vortex	10s
Incubate 65ºC	5 min
Chloroform	200 µL
Vortex	10 s
Centrifuge 13,000 rpm	1 min
Biophenol to supernatant	Equal volume
Vortex	10 s
Centrifuge 13,000 rpm	1 min
Remove supernatant and repeat	
96% ethanol and mix	1mL
Centrifuge 13,000 rpm	2 min
70% ethanol to pellet	500 µL
Vortex	10 s
Centrifuge 13,000 rpm	1 min
Air dry pellet	10-15 min
dd Water	50 µL

2.2.5. FTA® matrix (Whatman BioScience)

TABLE V. PROTOCOL FOR FTA MATRIX METHOD

Reagent/procedure	Time/Volume
Pipette blood onto matrix	25 μL
Air dry	~ 1 h
Outline each sample with pencil	
Remove plastic and place card in tray	
Add FTA Purification Reagent	
Shake	5 min
Decant	
Repeat 2 x	
Submerge card in TE, shake	5 min
Decant	
Repeat TE steps	
Allow to air dry	2 h
Remove 2mm punch from area	
Transfer to tube	
Add PCR mix to tube	~ 20-35 μL

2.2.6. QIAamp ® DNA Blood Mini Kit extraction method (Qiagen)

TABLE VI. DNA BLOOD MINI KIT EXTRACTION METHOD (QIAGEN)

Reagent/procedure	Time/Volume
Proteinase K	20 µL
Add blood	200 µL
Add Buffer AL (contains chaotropic salt)	200 µL
Incubate 56 ºC	10 min
Add absolute ethanol	200 µL
Vortex, centrifuge	
Add to QIAamp spin column	
Centrifuge 6000 g	1 min
Add Buffer AW1 (wash)	500 µL
Centrifuge 20,000 g	3 s
Place column in 2 mL collection tube, discard tube with filtrate	
Centrifuge full speed	1 min
Place column in clean Eppendorf tube discard tube with filtrate	
Add Buffer AE (elution) to column	200 µL
Incubate at room temperature	10 min
Centrifuge 6000 g	1 min
Perform another AE elution step	
Collect eluates	

2.3. PCR Primers [13]; 14]

Kin 1 5'-GCG TTC AAA GAT TGG GCA ATG-3'

Kin 2 5'-CGC CCG AAA GTT CAC C-3'

2.3.1. Primers according to A. Dávila (pers. comm.)

ITS1CF 5'-CCG GAA GTT CAC CGA TAT TG-3'

ITS1AF 5'-CTC TGC GGG ATT CCT TGC-3'

ITS1BR 5-TTG CTG CGT TCT TCA ACG AA-3'

Set A: ITS1A-F + ITS1B-R

Set B: ITS1C-F + ITS1B-R

2.4. PCR Reagents

2.4.1. DNA polymerase enzymes

HotStarTaq™ (Qiagen)

HotStarTaq™ with Q-solution (Qiagen)

FastStart Taq (Roche)

2.4.2. PCR reagents for Kin method

TABLE VII. PROTOCOL FOR KIN METHOD

Reagent	Amount
Water	14.3 μL
Fast Start buffer	2.5 μL
Fast Start $MgCl_2$	0.5 μL
dNTPs	0.5 μL
Kin 1 primer	1.0 μL
Kin 2 primer	1.0 μL
Fast Start Taq 1 unit	0.2 μL
Template DNA (~10 ng)	5.0 μL
Total	25 μL

2.4.3. PCR Reagents according to A. Dávila

TABLE VIII. PROTOCOL FOR DAVILA METHOD

Reagent	Amount
Water	x
DMSO	5%
10 x Buffer*	
dNTPs	0.49 m*M*
Primer 1	0.2m*M*
Primer 2	0.2m*M*
Fast Start Taq	0.75 U
Template DNA (~10 ng)	~ 10 ng
Total	25 μL

* 100 m*M* TrisHCl, pH 8.3; 35 m*M* $MgCl_2$, 750 m*M* KCl

2.5. PCR Thermocycling protocol

2.5.1. Touchdown procedure

TABLE IX. PROTOCOL FOR TOUCHDOWN PROCEDURE

Temperature	Duration	Cycles
94ºC	5 min	1 cycle
94ºC	30 s	4 cycles
58ºC	30 s	
72ºC	1 min	
94ºC	30 s	8 cycles
56ºC	30 s	
72ºC	1 min	
94ºC	30 sec	30 cycles
54ºC	30 sec	
72ºC	1 min	

2.5.2. PCR Method according to A. Dávila

TABLE X. PROTOCOL FOR TOUCHDOWN PROCEDURE

Temperature	Duration	Cycles
95ºC	5 min	1 cycle
95ºC	1 min	35-40 cycles
60ºC	1 min	
72ºC	1 min	
72ºC	10 min	1 cycle

2.6. Analysis

A 2% agarose gel was routinely used for analysis. Electrophoresis was usually performed at 120 V for approximately 1 h. A commercial molecular weight marker (100 bp ladder; Promega) was used for size estimation. A photo documentation system (Syngene GeneGenius; Bio Imaging System) was used for recording gel data.

According to [14] the following conditions are described for suitable size separation: 2.5% agarose gel electrophoresis, at 130 V for 120 min. These conditions will be followed in future for species determination in diagnostic samples.

2.7. CATT: Card agglutination Trypanosome test

A total of 84 serum samples from camels and horses were supplied by a visiting research fellow from Mongolia. These were evaluated at OVI for presence of antibodies to *Trypanosoma* using CATT (Free University of Brussels and the Institute of Tropical Medicine, Belgium). The basis for selecting these serum samples was not indicated. A few were also selected for PCR analysis.

The CATT assay: 25 μL test serum (1/4 or 1/8 dilution) was added to 45 μL of antigen (variable surface antigen VSA common to all *T.evansi* stocks) deposited on one of the ten the circular test areas on the supplied plastic card. Stirrer rods were used to mix and spread out mixture in the circle. The positive control was a goat antiserum. The

negative control was BSA solution. The plastic card was rotated for 5 min at low speed (60–70 rpm) with an electrical card rotator. Reading and interpretation was done immediately thereafter. Blue clumping/granules, was indicative of a positive result.

TABLE XI. PROTOCOL FOR CATT TEST

Reagent/Procedure	Time/ Amount
T.evansi antigen	45 μL
Add test serum	5 μL
Rotate card slowly	5 min
Read and interpret	

3. RESULTS AND DISCUSSION

The Kin primer-based PCR we implemented could detect and distinguish between a number of Trypanosome species in blood samples which we had obtained from various different sources. The sizes of amplicons obtained helped in determining the subtypes present, although sufficient electrophoretic separation was needed to do so. The use of a commercial kit (Nucleon BACC 2, Amersham) was found in our hands to be superior to other extraction methods. Preparations for extraction using Chelex® [15] and DNAzol BD-polyacryl carrier ® (Molecular Research Centre Inc.) have also been described as being effective and economical. The use of an ITS1 binding primer is regarded as being 3-5 x cheaper than using classical species-specific primers since the number of PCR reactions per sample is reduced to one. Their sensitivity is; however, lower than that of satellite DNA primers because of limited repetitiveness and partial homologies. Satellite DNA is repeated 10,000-20,000 times in contrast to ITS1 sequences (100-200 fold) [16].

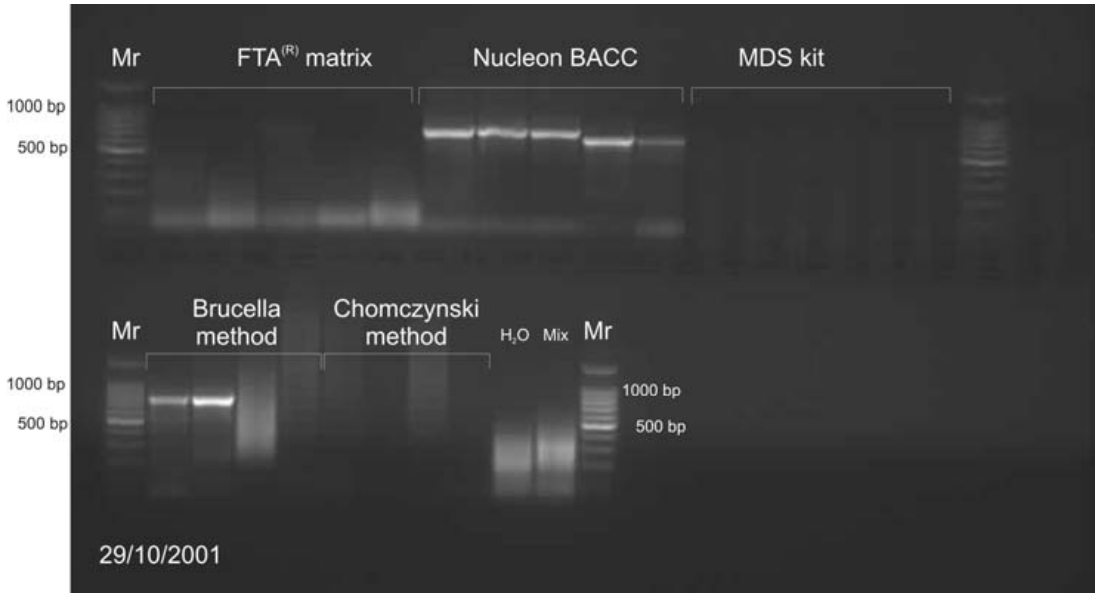

FIG. 1. Agarose gel showing PCR amplicons from samples processed by different DNA extraction methods.

Blood samples contained either *T.congolense savannah* (750 bp) or *T.congolense forest* (780 bp).

3.1. Optimization reactions A

Several blood samples containing either *T.congolense forest* or *T.congolense savannah* were tested in parallel using different extraction methods. Kin primers only were used. A commercial extraction procedure (Nucleon BACC 2, Amersham) yielded better results than a FTA matrix and Brucella extraction methods (Fig. 1). We failed to obtain results using MDS, QIAamp and Chomczynski-extraction methods. The latter method is usually used for RNA extraction and was therefore possibly not a suitable choice. The Nucleon BACC 2 method was thus the extraction method used for all subsequent procedures described.

3.2. Optimization reactions B

Two different polymerase enzymes were also evaluated (Fig. 2 and 3). FastStart proved superior to HotStar Taq polymerase as regards to the number of positive results obtained. The Q solution was also evaluated with HotStar as it alters the melting temperatures of nucleic acids. No significant improvements were noted. A 15 min activation temperature improved efficacy of both enzymes.

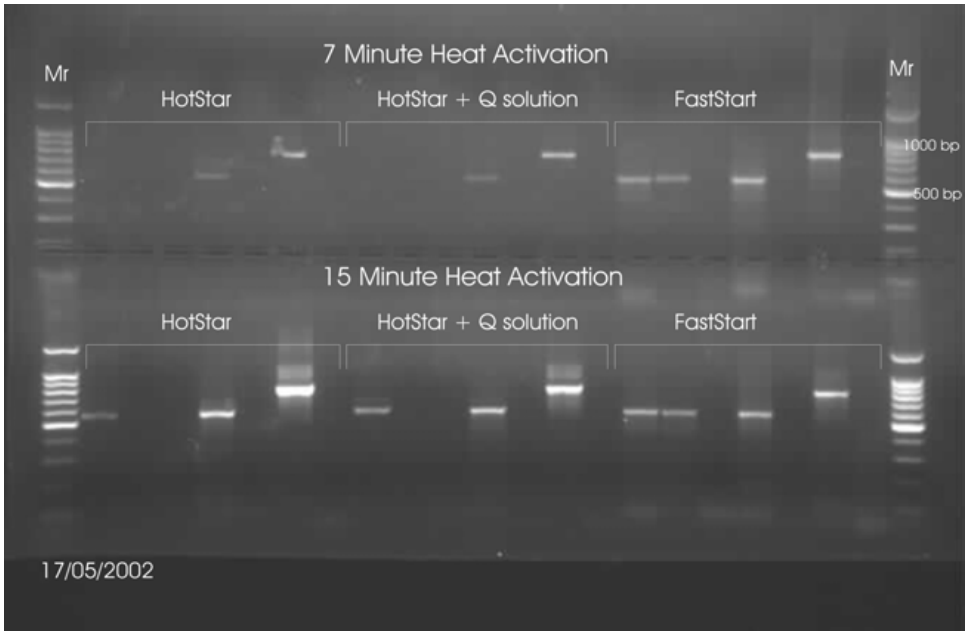

FIG. 2. Comparison of different polymerases.

Agarose gels in Fig. 2 show PCR amplicons derived from parallel sets of five Trypanosome blood samples containing either *T.congolense* savannah (780 bp) or *T.brucei* (540 bp). The 6th samples are negative water controls. PCRs were performed using HotStar, HotStar and Q solution and FastStart Taq polymerase enzymes. 7 and 15 min heat activation step were also compared. Mr: 100 bp molecular size marker.

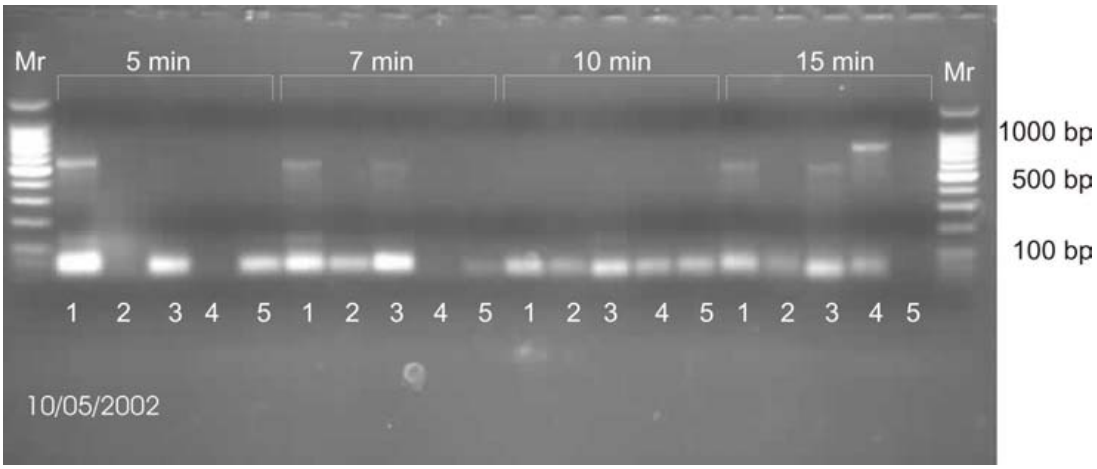

FIG. 3. PCR comparison using different heat activation times.

Using HotStar Taq polymerase. Four different blood samples (*T.congolense* and *T.brucei*) were tested in parallel. Lanes 5: water controls. Mr: 100 bp molecular size marker.

3.3. Thermocycling protocol used

A touch-down thermocycling protocol which, when initially shown to be successful, was the procedure used for all subsequent PCR reactions. It differed somewhat from the method as indicated below (Desquesnes *et al*., 2001) [14].

TABLE XII. PROTOCOL FOR THERMCYCLING PROCEDURE

Temperature	Time	Cycles
94ºC	3 min	1
94ºC	1 min	4
58ºC	1 min	
72ºC	1 min	
94ºC	1 min	8
56ºC	1 min	
72ºC	1 min	
94ºC	1 min	23
54ºC	1 min	
72ºC	1 min	
72ºC	5 min	1

3.4. Detection of various Trypanosome species

All the PCR reactions described made use of the Kin primers which bind to the internal transcribed spacer region ITS1. We successfully implemented PCR testing protocols, using the Kin primers described in [14]. These primers bind to an internal transcribed spacer region (ITS1) situated between the 18S and the 5.8S ribosomal subunit genes on nuclear DNA [13].

The length of ITS1 is between 300 and 800 bp which varies between Kinetoplastida species, but is presumed to be constant within a species. Taxa, which can be distinguished by size include: *T.vivax, T.theileri, T.simiae, T.congolense* savannah, *T.congolense* forest, *T.congolense* Kilifi). In the case of the Trypanozoon species (*T.equiperdum*; *T.brucei*; *T.evansi*) species-specific differentiation is not possible [16].

The sensitivity of the Kin primers is regarded to be low for detecting *T.vivax* because of only a 75 to 90% sequence homology to the forward and reverse Kin primers [14]. The predicted amplicon sizes [14] for various Trypanosome species are tabulated as follows:

TABLE XIII. PREDICTED AMPLICON SIZES

Species	Amplicon size
T.vivax	305 bp
T. simiae	435 bp
T.theileri	455 bp
Trypanozoon spp.	540 bp
T. brucei	540 bp
T.evansi	540 bp
T.equiperdum	540 bp
T.congolense Kenya	680 bp
T.congolense savannah	750 bp
T.congolense forest	780 bp

Samples of various species gave amplicon sizes in the expected ranges (Figs 4, 5 and 6) with the exception of several specimens that didn't always yield any results or gave unexpected sized products. The former poor test repeatability was probably due to operator inexperience as several students were being trained.

T.vivax has a predicted size of 305 bp, but amplicons seen were invariably larger in size (Fig. 4, lane 40; Fig. 5, lanes 7, 15; Fig. 6, lane 14) and comparatively weaker in strength.

Similarly, the *T.theileri* samples either failed to yield products of the expected size and/or were barely visible (Fig. 4: lane 3; Fig. 5: lane 4, 24; Fig. 6: lane 11). *T. lewesi*-containing blood specimens, gave relatively weak products usually in the range expected for *T.theileri* (455 bp). *T.theileri* is regarded as a usually apathogenic organism of cattle. *T. lewesi* is a rodent Trypanosome. Samples containing *T.congolense* forest and savannah gave amplicons in the region expected for each viz. 780 bp and 750 bp respectively (Fig. 4: lanes 21, 39, 41; Fig. 5: lane 5; Fig. 6: lane 17).

More effective size determination with suitable control samples would be required to give greater certainty.

Three samples gave products in the size expected for *T.congolense* Kenya (680 bp) cf Fig. 7, lane Kenyan samples 1, 2, 10. Samples containing all *T.brucei* subspecies yielded amplicons of the expected size (540 bp) cf Fig. 1 lane 10, 12, 13, 15, 16 etc. Kin primers cannot distinguish Trypanozoon species (*T. brucei, T.evansi, T.equiperdum*) since all yield product sizes of 540 bp.

Species-specific primers are available for the latter two species and would be required for identification.

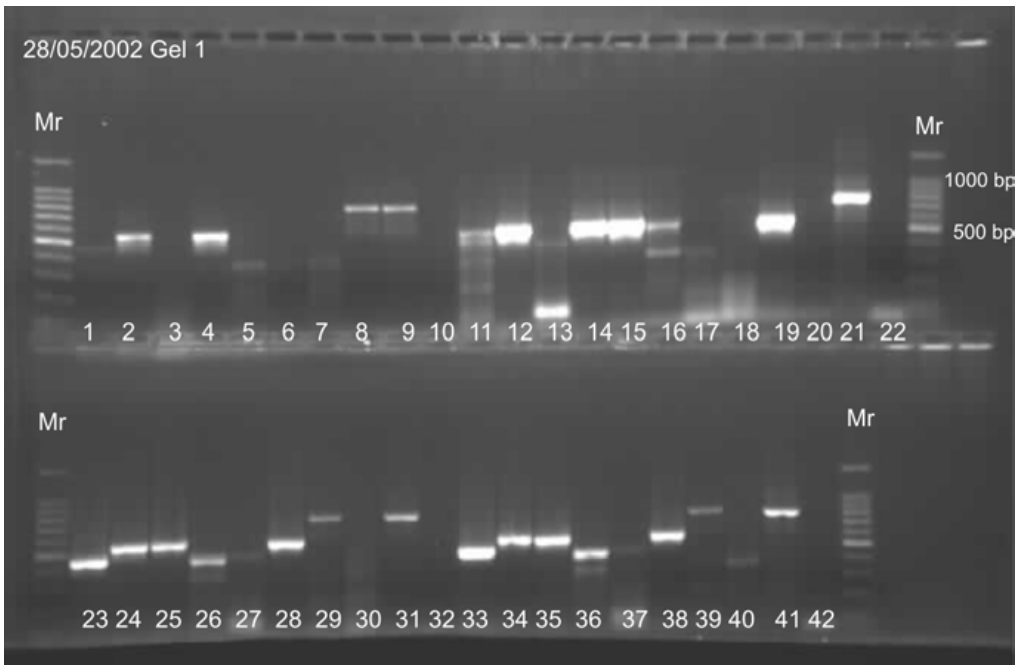

FIG. 4. Agarose gel (1) showing PCR amplicons from various Trypanosome-infected blood specimens.

Mr: 100 bp molecular size marker. *T. lewisi*: Lanes 1, 13, 27, 37; *T.brucei gambiense*: Lanes 12; *T.brucei rhodesiense*: Lanes 2, 15, 24, 34; *T.brucei brucei*: Lanes 7, 14, 16, 17, 19, 20, 28, 38; *T.theileri*: Lane 3; *T. simiae*: Lanes: 5, 23, 33, 26, 36; *T. congolense*: 8, 9, 18, 21, 29, 31, 39, 41; *T.vivax*: Lane 30, 40, Water controls: Lanes: 10, 22, 32, 42.

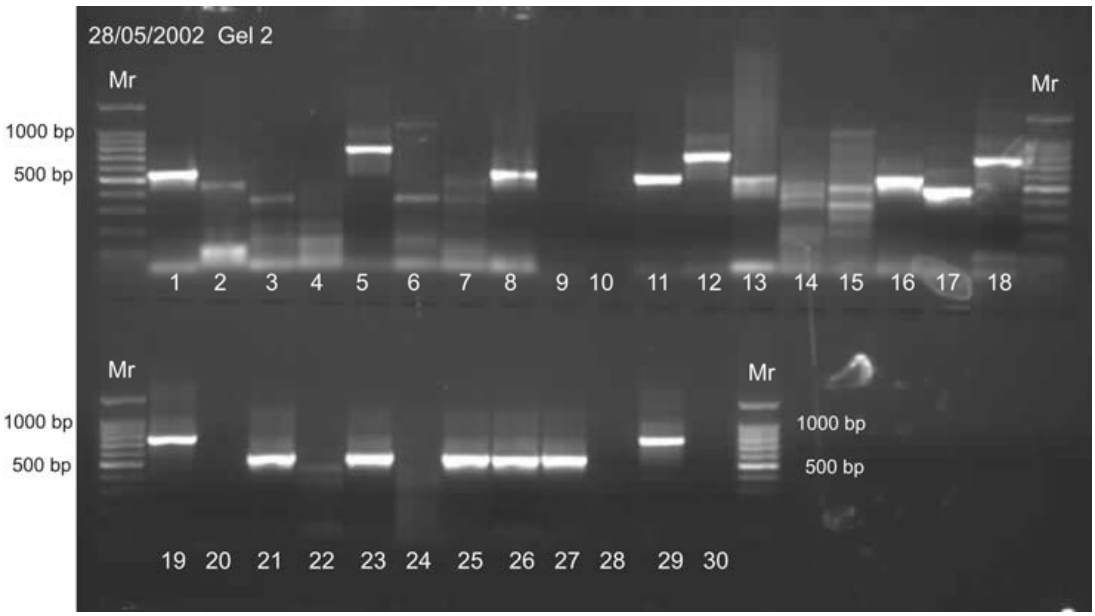

FIG. 5. Agarose gel (2) showing PCR amplicons from various Trypanosome-infected blood specimens.

Mr: 100 bp molecular size marker. *T. lewisi*: Lanes 2, 14, 22; *T.brucei gambiense*: Lanes 1, 11; *T.brucei rhodesiense*: Lanes 8, 13, 23; *T.brucei brucei*: Lanes 3, 16, 21, 25, 26, 27; *T.theileri*: Lane 4, 24; *T. simiae*: Lanes 6, 17; *T. congolense*: Lanes 5, 9, 12, 18, 19, 29; *T.vivax*: Lane: 7, 15, 28. Water controls: Lane: 10, 20, 30.

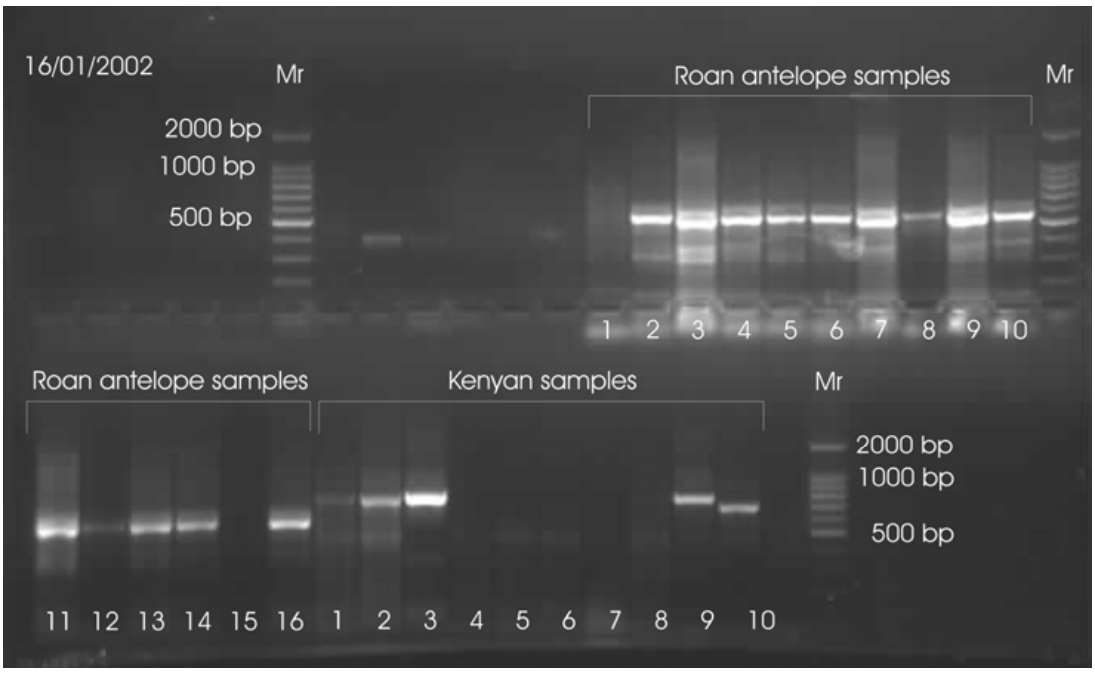

Fig. 6. Agarose gel) showing PCR amplicons from various Trypanosome-infected blood specimens.

Mr: 100 bp molecular size marker. *T. lewisi*: Lanes 9; *T.brucei rhodesiense*: Lanes 13; *T.brucei brucei*: Lanes 10, 15, 16; *T.theileri*: Lane 11; *T. congolense*: Lane 17; *T.vivax*: Lane: 14. Water controls: Lane: 8, 18.

Blood samples derived from roan antelope were also tested using PCR. These had been shown to be positive serologically using CATT. The PCR products obtained (Fig. 7) suggested presence of Trypanozoon species (540 bp), although a previous gel had also given products suggestive of *T.theileri* (450 bp). The latter is a stercarian tabanid-transmitted parasite that occurs widely in cattle and is usually regarded as apathogenic.

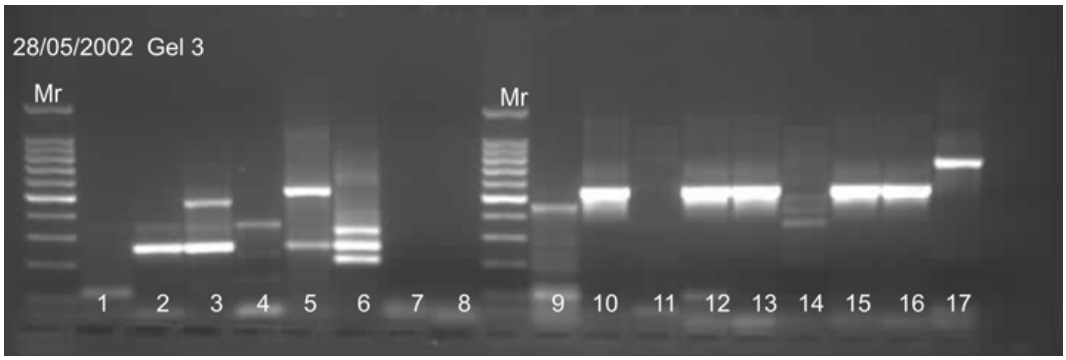

FIG. 7. Agarose gel showing PCR amplicons obtained from roan antelope.

Blood samples (1 to 16) that were all CATT positive. Kenyan samples 1 to 10 were confirmed Trypanosome positive samples supplied by KETRI. Lane 1 and 2: *T congolense savannah*. Lane 3 supposedly *T.vivax,* but not according to gel. Samples 9 and 10 were controls viz. *T.*congolense savannah (750 bp) and *T.congolense Kenya coast* (680 bp).

3.5. ICATT

Sera from camels and horses from Mongolia were evaluated at OVI for presence of antibodies to *Trypanosoma* using ICATT. The basis for selecting these serum samples was not indicated. In total 96% of horse sera and 37% camel sera tested positive against *T.evansi* variable surface antigen using CATT. It is known that antibodies to other salivarian Trypanosomes can also react to such antigens.

TABLE XIV. RESULTS ANALYSING SERA FROM CAMELS AND HORSES USING ICATT

	Positive	Negative	Uncertain	Total
Horse	23 96%	1 4%	0	24
Camel	22 37%	32 53%	6 10%	60

Five of these CAT positive sera samples were also tested using PCR. The resultant amplicons from two samples (Fig. 8), although of strong intensity, were smaller than any predicted sizes. Faint bands of a higher size are, however, visible in sample 2. It is known that a recent dourine outbreak occurred in China, Russia and Ethiopia [15]. Desquesnes states that no laboratory has a recent isolate definitely identified as *T.equiperdum*. Also many older isolates originally classified as *T.equiperdum* were confused with *T.evansi*. These aspects should be kept in consideration should these samples undergo further evaluation.

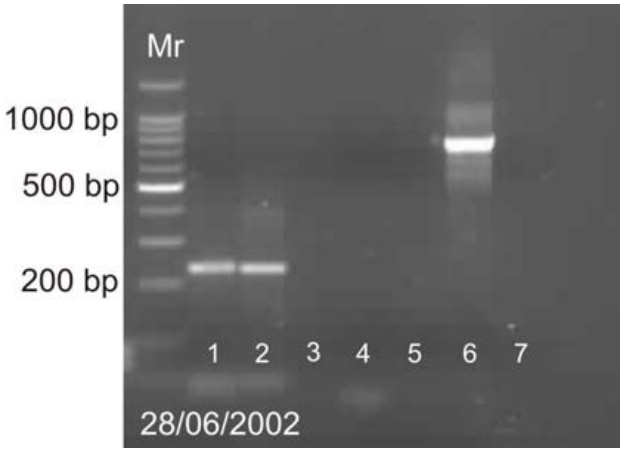

FIG. 8. Agarose gel showing PCR amplicons from camel and horse sera testing.

CATT positive Mr: molecular size marker; Lane 6 *T.congolense* control; Lane 7 water control.

4. FUTURE

We have made contact with researchers in several African countries e.g. Kenya, Burkina Fasso, Nigeria with whom further collaboration can now take place. This will include being a diagnostic referral centre for testing blood samples for presence of Trypanosomes. Other Trypanosome-binding primers will still be evaluated. An additional PCR method according to Dávila (pers comm), using other primers and thermocycling parameters will still be examined. In addition, species-specific primers will be used in selected cases viz. those described in [8] *T.congolense savannah* TCS1 and TCS2; *T.congolense forest*: TCF1 and TCF2; *T.congolense Kenya Coast* YCK1 and TCK2; *T.vivax: TV* [9].

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USING PCR FOR UNRAVELING THE CRYPTIC EPIZOOTIOLOGY OF LIVESTOCK TRYPANOSOMOSIS IN THE PANTANAL, BRAZIL

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Abstract

Trypanosoma vivax and *Trypanosoma evansi* are livestock parasites of economic importance in Africa, Asia and South America. In the Pantanal, Brazil, they cause economic losses in both cattle and equines. Little is know of their maintenance and spread in nature, particularly in terms of reservoirs and means of mechanical transmission. Here we report for the first time the use of PCR for the detection of *T.vivax* and *T.evansi* in bovines, buffaloes and sheep. Whereas parasitological diagnosis detected only 2 T.vivax infections, one in buffalo and another in a cow, PCR detected infections in 34.8% buffaloes, 44.7% bovines and 37.3% sheep. Trypanozoon primers detected 41.8% infections in buffaloes and 8.1% in cattle. PCR revealed 6.9% mixed infections in buffaloes and 5.3% in cattle. The potential role of cattle and buffaloes as hosts and reservoirs of T.vivax is discussed, as well as the implications of possible extravascular foci in the maintenance of livestock Trypanosomosis.

1. INTRODUCTION

Trypanosoma vivax (sub-genus *Dutonella*) and *Trypanosoma evansi* (sub-genus *Trypanozoon*) are species of the *Trypanosomatidae* family that affect a wide range of wild and domestic animals in Africa, Asia and Latin America. They were most likely introduced into Latin America during European colonization, either with infected cattle from Senegal in 1830 or with Spanish settlers by the 15th century [2]. Since then both parasites have established and spread throughout the region. In the New World, *T.vivax* affects mainly cattle and buffaloes, and *T.evansi* affects mainly horses and dogs. For *T.evansi* a number of reservoirs have been suggested: capybaras (*Hydrochaeris hydrochaeris*), coatis (*Nasua nasua*), and vampire bats (*Desmodus rotundus*) among others reviewed in [3]; [5]. The latter species is particularly important as it acts as both reservoir and vector [6]. Little is known about reservoirs for *T.vivax*. In reviewing the scanty literature, [4]; [7]; [8] it is suggested that ungulates and deer may be the reservoirs of *T.vivax* in Latin America, as wild Bovidae are in Africa ([9]

T.vivax and *T.evansi* represent a potential risk for nearly 300 million cattle, 1.8 million buffaloes and 16 million horses in South America [5]. The first official report of T.vivax in the region was in French Guyana in 1919 [10]. In Brazil, it was reported for the first time by Shaw and Lainson [11] in the northern region (Pará State) and subsequently in the Amazon region [12] and Amapá state, Serra Freire [13]. Fifteen years later in 1996 reports were made [8] of outbreaks due to T.vivax in the Northern Pantanal in Zebu crossbred animals. Low Packed Cell Volume (PCV) values and anemia are a common characteristic in hosts affected by these *Trypanosoma* species, however, fever, anemia, abortion, progressive weakness, loss of appetite, lethargy, substantial weight loss in a short time, and emaciation have also been reported during outbreaks in the lowlands of Brazil and Bolivia [14].

Molecular diagnostic techniques such as PCR have proven to be very specific and sensitive tools, capable of detecting parasitemias as low as 1 Trypanosome per mL of blood ([15] or even 1pg of Trypanosome DNA in the presence of host DNA [16]. PCR has also been able to detect clearance of Trypanosome DNA in blood of cattle after 3-4 days of drug administration ([17]. PCR has also been proposed as a tool for retrospective studies to detect Trypanosome DNA directly in serum [18]. Despite its sensitivity and widespread use, PCR has not yet been used for epizootiological studies of livestock Trypanosomosis in Brazil.

The Pantanal is a large wetland located in the center of South America divided into 11 sub-regions bordering Bolivia, between 16° and 21° S and 55° and 58° W. It covers an area of 140,000 km^2, ranging in altitude from 80 to 130 m above sea level [19]. The Brazilian Pantanal is the largest freshwater wetland in the world and is the home for at least 650 species of birds, 260 species of fish, 80 species of mammals, and 50 species of reptiles [20]. It is also one of the most important beef cattle breeding regions in Brazil. Extensive cattle ranching has been the dominant economic activity in the region for the past two centuries. About 80% (118,000 km^2) of the land is used for cattle ranching with nearly 4 million heads of cattle (evaluated at about US$ 1 billion), 140,000 horses and 5,000 buffaloes [20].

Here we present new data using PCR for estimating prevalence of both parasites in cattle, sheep and buffaloes in Southern Pantanal, discussing their distribution and potential strategies for maintenance and spread in nature.

2. MATERIALS AND METHODS

2.1. Study area and animals

Ranches from the Pantanal of Paraguay (R7) and from the Pantanal of Nhecolândia (R1, R2, R3, R4, R5, R6, R8, R9) sub-regions of Southern Pantanal (Fig. 1.) were chosen based on two criteria: a) previous parasitological findings of *T.vivax* and b) no previous *T.vivax* studies. Details concerning the ranches are provided in Table I. Altogether, 8 calves and 355 adult crossbreed bovines (*Bos taurus* X *Bos indicus*), 43 water buffaloes (*Bubalus bubalis*) and 83 sheep (*Ovis aries*) were randomly sampled.

2.2. Parasitological diagnosis

Eppendorf tubes (500 µL) containing one confetti of filter paper (Whatman 4®) were prepared in the Laboratório de Biologia Molecular de Tripanosomatídeos (LBMT-Oswaldo Cruz Institute, Rio de Janeiro, Brazil) for the field studies. All sampled animals were bled from the jugular vein using a vacuum system (Vacuum II®, Labnew, Campinas, Brazil) containing EDTA. Two capillary tubes were taken for each blood sample and centrifuged to obtain the hematocrit. The first was examined by parasitological diagnosis [22] at ranch R9 (Nhecolandia sub-region of the Pantanal). The buffy coat from the second tube was collected on filter paper inside the 500 µL Eppendorf tubes, and allowed to dry. These samples were processed later at the LBMT.

2.3. DNA extraction

DNA extraction was performed with Chelex-100® 1% based on [23] and [24] with minor modifications, briefly: 500 µL of Milli-Q water was used for the initial wash of the confetti during 30 min. After 15 min the tubes were inverted 2-3 times, then, after 10 min centrifugation at 12,000 rpm, 450 µL of supernatants were discarded and 100 µL Chelex-100® 1% added. The tubes were inverted several times, incubated at 56ºC during 30 min, boiled during 8 min and vortexed for 2 min at maximum speed. After a final 5 min centrifugation at 12,000 rpm, 80 µL of supernatants were placed in a fresh tube and stored at –20ºC.

2.4. PCR diagnosis

PCR was performed in Perkin Elmer 9600® PCR machines. All reactions were done in a volume of 10 µL using 1 µL of DNA as template. Specific primers targeting the satellite DNA of *Trypanosoma vivax* (TVW1: CTG AGT GCT CCA TGT GCC AC; TVW2: CCA CCA GAA CAC CAA CCT GA) and *Trypanozoon* (TBR1 GAA TAT TAA ACA ATG CGC AG ; TBR2 CCA TTT ATT AGC TTT GTT GC) described in [25] were used. The PCR cocktail was modified based on the same authors: 1.5 m*M* $MgCl_2$, 1 µ*M* of each primer, 200 µ*M* of each dNTP, 0.5 unit Taq polymerase, and 5% DMSO. The PCR conditions were: an initial step of 5 min at 95ºC, followed by 35 cycles of 1 min at 95ºC, 1 min at 55ºC and 1 min at 72ºC, with a final extension step of 10 min at 72ºC. When weak products were obtained, samples were submitted to 40 cycles.

Purified *T.vivax* DNA (ILdat2160) originating from isolate Y486, obtained from a naturally-infected Zebu cow from Yakawada, Nigeria [26] was kindly provided by Noel Murphy (ILRI) and used as a positive control. The *T.evansi* positive control was kindly provided by Marc Desquesnes. In every batch of PCR, non-infected cattle DNA and double distilled water were used as template for negative control. Negative controls were included in every gel run.

PCR products were resolved in 2% agarose gels. Gels were stained with ethidium bromide and photographed under ultraviolet light.

2.5. Statistical analyses

Chi-square (X^2) test was used to evaluate a possible correlation between PCR positive bovines and PCV values.

3. RESULTS

3.1. Parasitological results and clinical signs

Only one herd of bovines from R5 sampled in March 2000 presented poor conditions, and from that herd just one cow was parasitologically positive for *T.vivax* with a PCV of 34%. Among buffaloes one young animal (3 y old) from R5 was parasitologically positive for *T.vivax* with a PCV of 31%. Just 2 Trypanosomes were observed by parasitological examination either in the infected buffalo or bovine. Blood smears of buffy coats were prepared and stained with Panotico kit, and the morphology of the few parasites available was compatible with *T.vivax*.

The remaining animals did not present either clinical signs or parasites detectable parasitologically in the blood. PCV values ranged from 23-59% in bovines, from 22-44% in buffaloes and from 17-42% in sheep. PCV mean values ranged from 32.4% (SD 4.52) to 42.6% (SD 1.52) in PCR positive animals and, from 31.0% (SD 3.15) to 40.6% (SD 1.75) in PCR negative animals. There was no statistically significant difference in the mean PCV values of PCR positive and PCR negative animals (P<0.001).

3.2. PCR diagnosis and prevalence

Positive and negative PCR controls always produced the expected results. Among bovines, *T.vivax* prevalence obtained by PCR varied from 2.5% (1/39) at ranch R3 (July 2000) to 77.5% (31/40) in R1 and R5 (March 2000). In buffaloes, prevalence ranged from 14.2% (4/28) at R6 (April 2001) to 73.3% (11/15) at R5 (March 2000). Prevalence in sheep ranged from 7.5% (3/40) to 65.1% (28/43) at R5, in July and February 2000, respectively.

Trypanozoon primers gave positive results in 41.8% (18/43) of buffaloes and 8.2% (29/355) of cattle. Sheep and calves were negative with these primers.

Mixed infections (*Trypanozoon* + *T.vivax*) detected by PCR were: 6.9% (3/43) in buffaloes and 5.3% in cattle (19/355). Details about PCR prevalence are shown in Table II.

The monthly mean precipitation in the Nhecolandia sub-region was 347.7 mm^3 (March 2000), 9 mm^3 (July 2000) and 113.6 mm^3 (April 2001) (Balbina Soriano, pers. Commun.). A very high *T.vivax* prevalence was recorded in the rainy season in March 2000 (48.75% bovines, 73.3% buffaloes and 65.1% ovines). PCR prevalence for *T.vivax* was lower in the following rainy season (April 2001) (34% in bovines and 14.2% in buffaloes), nevertheless it was higher than during the dry season (5.4% in bovines and 7.5% in sheep) in July 2000 (Fig. 2). Most PCR-positive bovines for *T.vivax* (98.7%) were distributed between 25%-59% PCV values. Only 0.84% of bovines (3/355) presented PCV below 25% and from those just 2 animals were PCR positive for *T.vivax*.

4. DISCUSSION

Trypanozoon primers can detect *T.evansi*, *T.brucei spp*. and *T.equiperdum*. *T.brucei spp*. is not present in Latin America. Little is known about *T.equiperdum* in the region; it affects exclusively equines and is sexually transmitted [27] [5]. Consequently, we believe the positive results obtained with *Trypanozoon* primers are due to *T.evansi*. This species is widespread in several continents and affects many different domestic animals, with buffaloes being major hosts in Asia [28]. In Indonesia, cattle and buffaloes present mild or asymptomatic infections, suggesting enzootic stability ([29], with occasional occurrence of epizootics with high mortality [30]. In the Pantanal *T.evansi* has been reported in horses, dogs, coatis, capybaras and small rodents (reviewed by [5]. Cattle, goats [31]; [32] capybaras and coatis [33]; [34]; [35] have been considered to act as reservoirs of infection. Our findings support the report of [36] on 2.3% and 4.2% *T.evansi* seroprevalence in bovines from the northern Pantanal using Ag-Elisa and Ab-ELISA, respectively. Although we were unable to demonstrate the parasite on blood films, presumably due to low parasitemias, to our knowledge, this is the first report of *T.evansi* in buffaloes in Latin America. Although we only obtained

negative results for *T.evansi* in sheep, more studies including goats and a larger number of samples from different sub-regions should be performed to elucidate the role of these species in the epizootiology of *T.evansi* in the Pantanal.

No *Tabanidae* and *Stomoxyinae* were observed feeding on cattle during our field studies, which took place following periods of abnormally low precipitation. Similarly, just one bovine presented a *T.vivax* infection detected by parasitological examination. Biting flies are typically associated with the rainy season in the Pantanal and can reach high density resulting in high parasite prevalence and outbreaks [9]; [37]. A different correlation has been reported in French Guyana with higher *T.vivax* prevalence associated with the dry season when animals are more susceptible to stress [38]; and also tabanids are more visible. Interestingly, we also found a correlation among higher PCR prevalence and the rainy season in the present study (Fig. 2). Consequently in all cases, the high prevalence of *T.vivax* was related to the season of high tabanid density.

Our inability to detect parasites microscopically in the blood suggested very low parasitemias and/or extravascular foci, as demonstrated in goats [39]. This raises the question of why *T.vivax* is well-distributed in bovines, buffaloes and sheep, when mechanical transmission is typically unsuccessful at low biting fly density and low parasitemia.

We hypothesize that bovines, buffaloes and sheep may be acting as cryptic reservoirs of infection for *T.vivax*. In general, African wild ungulates are more Trypanotolerant than the N'Damma cattle (known to be Trypanotolerant) [40]. Moreover, [41]; showed that the African buffalo and Boran cattle have a high reservoir potential to transmit *T.vivax* to *G. m. centralis*. The African buffalo has been reported to be highly resistant to *T.vivax* experimental infections, showing a higher ability to control parasitemia than cattle and goats [42]. However, the Brazilian buffalo may sometimes become sick with natural infections and need treatment [43]. More recently [44]; showed that *Trypanosoma sp.* prevalence estimated using the microhematocrit method varied from 3.8% (3/78) to 6.9% (4/23) among two herds of water buffaloes (*B. bubalis*) in Venezuela. Although those authors were unable to determine the Trypanosome species based on morphology and serology, they suggested the infections were due to *T.vivax*, because only ovines, and not rodents, became experimentally infected, developing high parasitemias and anaemia. *T.evansi* normally grows in laboratory rodents and despite a serological report by [45]; it has not been reported to naturally infect sheep in Latin America. It is almost certain then that the infections in buffaloes reported by [44]; were due to *T.vivax*. These results provide further evidence for the role of buffaloes as cryptic reservoirs of *T.vivax* in South America, since the PCV mean values recorded (32.2% to 35.1%) were similar to the ones reported in the present study (PCV mean 32.8%, SD 4.6).

The hypothesis that bovines themselves could be acting as reservoirs for *T.vivax* is supported by our findings showing no significant differences in PCV between PCR positive and negative animals. The PCV values we found in PCR positive animals for *T.vivax*, could be considered as normal, since mean PCV values in healthy cattle was reported to vary from 24.0% to 46.0% with a 35% average [46]. Normally, anaemia is related to parasitaemia in the initial stage of the infection, then, if parasitaemia is low, one would expect normal PCV values. High prevalence and low parasitaemia found in our studies provide strong indication that animals can be infected and carry *T.vivax* whilst presenting normal PCV values and no clinical signs.

No simple explanation for a successful mechanical transmission in low biting flies density and low parasitaemia is available, indicating a multi-factorial way of transmission. According to [47]; the remaining blood in the mouth parts of tabanids after feeding is around 10 μL, which means that a parasitaemia of 10^5 parasites/mL would allow mechanical transmission of Trypanosomes. The detection limit of the parasitological technique we used has been reported to be 500 Trypanosomes/mL, [48]. If these data could be extrapolated to the Pantanal region, mechanical transmission would be unlikely during the period of our sampling, even with one bovine and one buffalo presenting a parasitaemia that, based on [48]; was estimated to be about 10^3-10^4 Trypanosomes/mL. *T.vivax* parasitaemia are highly fluctuating [4]; then an enough level of parasitaemia allowing mechanical transmission can occur anytime in the field. On the other hand, the frequency and role of congenital transmission [49]; has not been widely studied in nature, then it may add further to the parasite transmission in the absence of successful mechanical transmission by hematophagous insects.

There are several other particular conditions in the Pantanal that can potentially contribute to the transmission of *T.vivax* and *T.evansi*. According to [50]; during extensive and prolonged annual flooding (5 to 6 m) in the Pantanal, there is a reduction in the area available for grazing. Thus, all animals (domestic and wild) seek refuge in limited areas of dry land forest. As a result disease transmission by biting flies may become intense.

In an experimental infection in goats, Whitelaw [39]; clearly showed that cyclically transmitted *T.vivax* invades and survives in the cerebrospinal fluid (CSF), causing choroiditis, meningitis and meningoencephalitis in the central nervous system. Moreover, they also observed the parasite in the lymph and aqueous humor, presenting in this latter a markedly different VAT (variable antigenic type) from those parasites found in CSF, lymph and blood. Extravascular foci are not an exclusive characteristic of *T.vivax*. The invasion of central nervous system in horses [51]; leading sometimes to meningitis [52]; as well as the aqueous humor (sometimes by large numbers of Trypanosomes) has been reported for *T.evansi*. Considering those studies, the possibility of extra vascular foci for mechanically transmitted *T.vivax* should not be excluded to occur in naturally infected bovines, mainly in lesser important tissues than CSF, where the parasite may stay for long time without killing immediately the host.

According to [53]; Trypanosome sub-populations expressing different VATs may compete among themselves within microenvironments and niches represented by the different tissues of a given infected host. However, the invasion of tissues other than blood could also represent a parasite strategy to hide until an opportunity arises to grow in peripheral blood. Once enough parasites (or high parasitaemia of a given sub-population) are available on the peripheral blood, mechanical transmission would be allowed in the presence of hematophagous insects. Extravascular foci and/or competition among VATs sub-populations could explain why *T.vivax* apparently disappear or become cryptic between two outbreaks in enzootic regions [38]. Moreover, according to [54]; in an enzootic situation the disease can become cryptic, but still produce measurable production losses (e.g. decreased milk production and loss of weight).

The present study has provided evidence for the existence of widespread *T.vivax* infections at very low parasitaemia in the absence of clinical signs in bovines, ovines and buffaloes in the Southern Pantanal. The high PCR prevalence found in the rainy

season is an indication of the potential for seasonal mechanical transmission in the region. Additionally, the relevance of congenital transmission in nature and the possibility of extravascular foci need to be investigated since it is of relevance for control measures based on chemotherapy, representing a potential source for relapse infections.

ACKNOWLEDGEMENTS

We thank C. da Gama for valuable technical assistance. Embrapa/Centro de Pesquisa Agropecuaria do Pantanal and M. Dantas for field support. FAPERJ, CNPq, IOC and IAEA (Developing, validating and standardising methodologies for the use of PCR and PCR-ELISA in the diagnosis and monitoring of control and eradication programmes for Trypanosomosis) for financial support. N. Murphy and M. Desquesnes for gifts of purified DNA. Our special thanks to S. Mihok and M. Desquesnes for critical reading of the manuscript. To R. Melon for the assistance with statistical analyses. We also thank B. Soriano (Embrapa-Pantanal) for kindly providing her unpublished data on rainfalls in the Pantanal.

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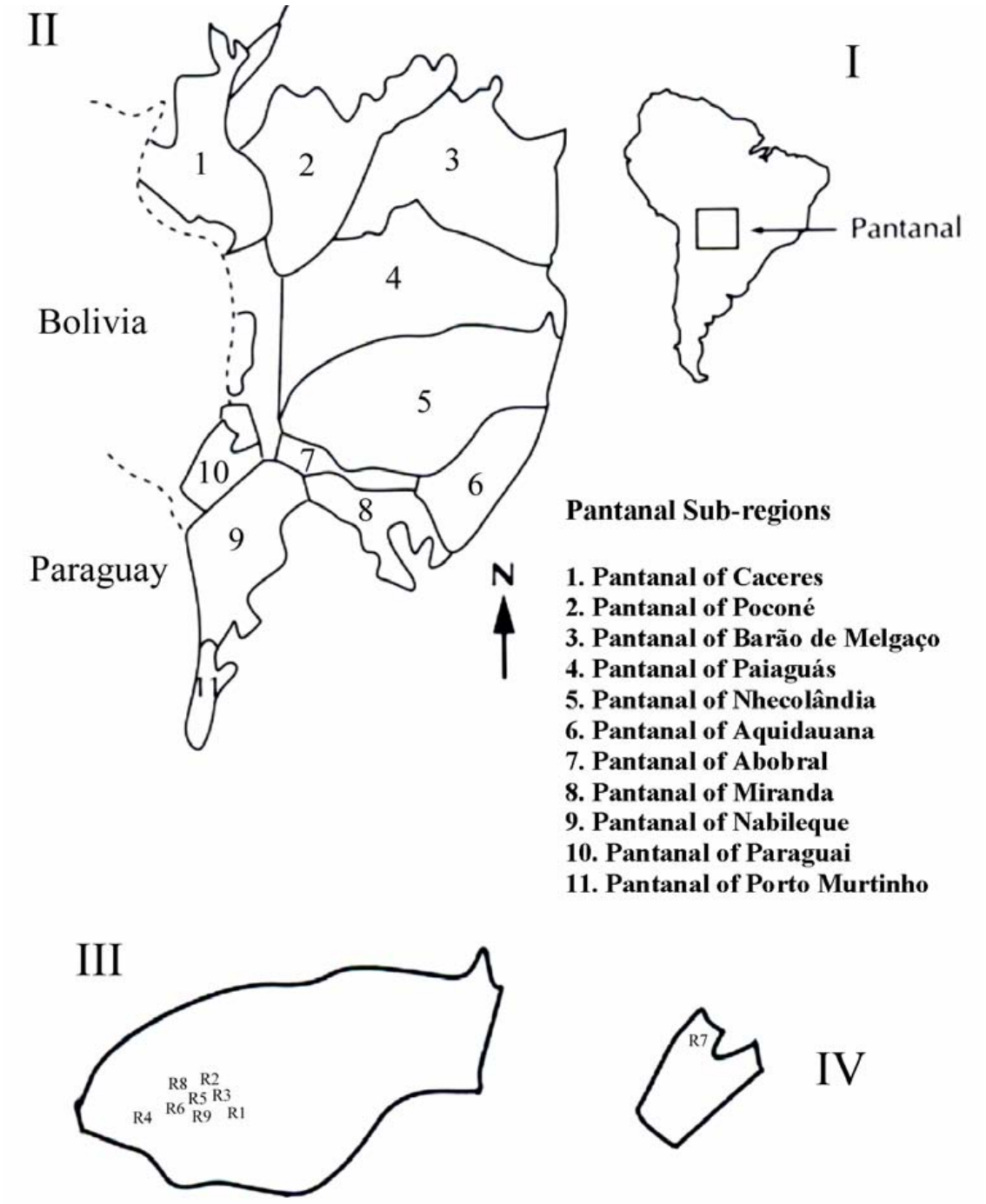

FIG. 1. Location of the Pantanal and ranches sampled.

I: The Pantanal in South America. II: The Pantanal and its 11 sub-regions. III: The Pantanal of Nhecolândia sub-region and ranches sampled (R1, R2, R3, R4, R5, R6, R8, R9). IV: The Pantanal of Paraguai sub-region and ranch sampled (R7).

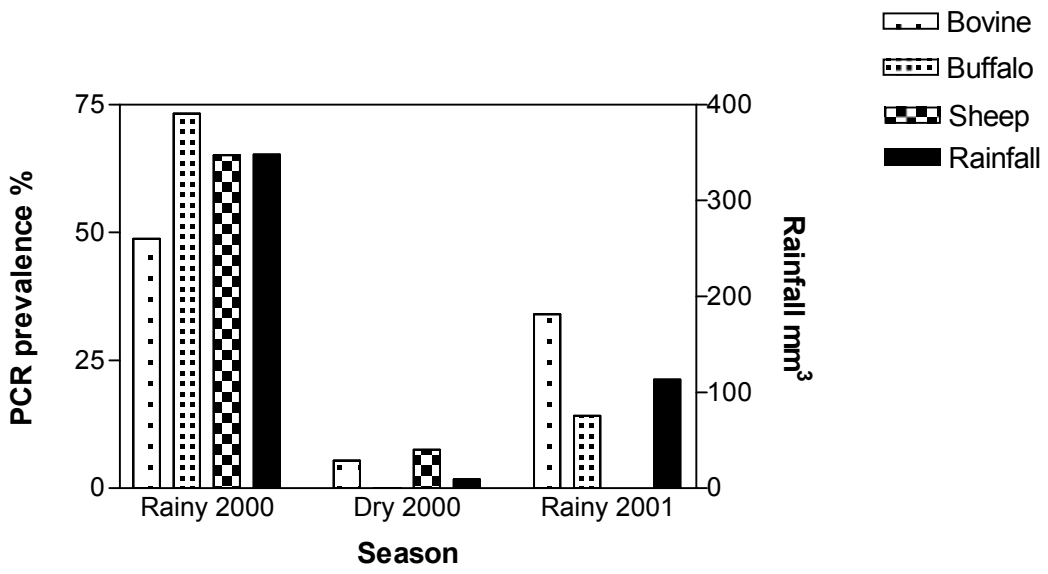

FIG. 2. Seasonal prevalence of Trypanosoma vivax.

TABLE I. DETAILS ABOUT RANCHES, SEASON AND ANIMALS SAMPLED

Ranch	Previous parasitological reports	Host species	N	Pantanal Sub-region	Sampling date	Season
R1	None	Bovine	40	Nhecolândia	03/2000	Rainy
R2	None	Bovine	18	Nhecolândia	04/2001	Rainy
R3	None	Bovine	39	Nhecolândia	07/2000	Dry
		Bovine	40	Nhecolândia	03/2000	Rainy
R4	None	Bovine	47	Nhecolândia	04/2001	Rainy
R5	None	Bovine	40	Nhecolândia	03/2000	Rainy
		Buffalo	15	Nhecolândia	03/2000	Rainy
		Ovine	40	Nhecolândia	07/2000	Dry
		Ovine	43	Nhecolândia	02/2000	Rainy
		Bovine	35	Nhecolândia	04/2001	Rainy
		Bovine	35	Nhecolândia	07/2000	Dry
R6	None	Buffalo	28	Nhecolândia	04/2001	Rainy
R7	*T.vivax*	Calves	7	Paraguai	03/2000	Rainy
		Bovine	23	Paraguai	03/2000	Rainy
R8	None	Bovine	26	Nhecolândia	05/2001	Dry
		Bovine	11	Nhecolândia	03/2000	Rainy
R9	None	Bovine	1	Nhecolândia	03/2000	Rainy
			489			

TABLE II. PCR AND PARASITOLOGICAL PREVALENCE ACCORDING TO HOST SPECIES AND RANCHES

Number	Region	T.vivax + PCR%	T.vivax -PCR	Tryp + PCR%	Tryp - PCR	T.vivax PCR%	T.vivax parasite	Parasite	Date
		Buffalo							
28	N	4 (14.28)	24	18 (64.28)	10	3 (10.71)	0 (0)	0	28-Apr-01
15	N	11 (73.33)	4	0 (0)	15	0 (0)	1 (6.66)	0	01-Mar-00
43									
		Cow							
40	N	31 (77.5)	9	0 (0)	40	0 (0)	0 (0)	0	29-Feb-00
18	N	1 (5.55)	17	5 (27.77)	13	1 (5.55)	0 (0)	0	27-Apr-01
40	N	20 (50)	20	0 (0)	40	0 (0)	0 (0)	0	29-Feb-00
39	N	1 (2.56)	38	0 (0)	39	0 (0)	0 (0)	0	21-Jul-00
47	N	25 (53.19)	22	11 (23.40)	36	11 (23.40)	0 (0)	0	24-Apr-01
40	N	31 (77.5)	9	0 (0)	40	0 (0)	1 (2.5)	0	02-Mar-00
35	N	3 (8.57)	32	0 (0)	35	0 (0)	0 (0)	0	24-Jul-00
35	N	12 (34.28)	23	12 34.28)	23	6 (17.14)	0 (0)		29-Apr-01
23	P	12 (52.17)	11	0 (0)	23	0 (0)	0 (0)	0	21-Feb-00
11	N	11 (100)	0	0 (0)	11	0 (0)	0 (0)	0	26-Feb-00
26	N	11 (42.30)	15	1 (3.84)	25	1 (3.84)	0 (0)	0	25-Apr-01
1	N	1 (100)	0	0 (0)	0	0 (0)	0 (0)	0	29-Feb-00
355									
		Calf							
7	N	2 (28.57)	5	0 (0)	7	0 (0)	0 (0)	0	21-Feb-00
1	N	1 (100)	0	0 (0)	0	0 (0)	0 (0)	0	29-Feb-00
8	N								
		Sheep							
43	N	28 (65.11)	15	0 (0)	43	0 (0)	0	0	25-Feb-00
40	N	3 (7.5)	37	0 (0)	40	0 (0)	0	0	19-Jul-00
83									
489				47 (9.61)		22 (4.49)	2 (0.40)	0	

EVALUATION OF A POLYMERASE CHAIN REACTION ASSAY FOR THE DIAGNOSIS OF BOVINE TRYPANOSOMIASIS AND EPIDEMIOLOGICAL SURVEILLANCE IN BOLIVIA

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Abstract

Sporadic outbreaks of bovine Trypanosomiasis have been reported in Bolivia since 1996 when *T.vivax* and *T.evansi* were identified for the first time by parasitological means. However, comprehensive epidemiological information concerning *T.vivax* and *T.evansi* in the country is lacking. Current parasitological and serological diagnostic methods for Trypanosomiasis have important limitations either in their sensitivity or specificity, which can result in unreliable data when applied in epidemiological studies. PCR assays are a recently developed procedure that might help to overcome the constraints of parasitological and serological assays. Therefore, the objective of this study was to evaluate PCR assays as a diagnostic tool for epidemiological studies in Bolivia. PCR assays for diagnosis of Trypanosome infection in cattle were evaluated for their ability to detect Trypanosome DNA in blood spots samples collected from cattle in four different provinces from the Bolivian lowlands and the results compared with those obtained with standard parasitological (Micro Haematocrit Centrifugation Technique (HCT) and stained smears) and serological methods (Card Agglutination Test for *T.evansi* (CATT), and Antibody ELISAs for *T.vivax* and *T. congolense*). Kappa agreement analysis showed a significant agreement between PCR assays and results from parasitological methods but there was no agreement when PCR was compared with serological assays. Some samples from *T.vivax* smear positive animals were negative by PCR, therefore modifications to the PCR assay conditions were undertaken to try to improve agreement between PCR and parasitological assays. Changes in the template DNA concentration or the use of an alternative primer sets resulted in improvements in the PCR detection rate, but not all the parasitologically positive samples were detected by PCR. Results from PCR assays for *T.vivax* and *T.evansi* were combined with results from parasitological and serological assays to provide information on prevalence rates for the four provinces from where the samples were obtained. The present study established evidence of the usefulness of PCR as diagnostic tool for epidemiological studies and confirmed that cattle Trypanosomiasis appears to be endemic in several regions of the Bolivian lowlands.

1. INTRODUCTION

Cases of *T.vivax* Trypanosomiasis in Bolivia have been reported since 1996 when commercial cattle transactions between the Brazilian and Bolivian borders were considered to be the source of introduction of Trypanosomiasis into the Bolivian lowlands from the Brazilian Pantanal [1]. During the first outbreaks of disease in cattle in Bolivia, both *T.vivax* and *T.evansi* were identified by parasitological observations. These outbreaks took place in the province of Chiquitos, Santa Cruz department,

Bolivia. Later, further outbreaks of disease were confirmed in other provinces of Santa Cruz lowlands and the Department of Beni [1]. Since the first case of disease in Chiquitos, the spread has been attributed to cattle movement within the Santa Cruz department, and the presence of high numbers of the vector (tabanids) [2]; [3] has facilitated sporadic outbreaks on the few last years. At present, there is no information about the prevalence and distribution of *T.vivax* and *T.evansi* in the department. Currently the only data available are those relating to the outbreak, when the parasite identification was made using parasitological methods.

A common pattern of the Trypanosome infections is an intermittent parasitaemia that, in *T.vivax* and particularly *T.evansi* infections in cattle, is often very low. This feature complicates the diagnosis of infected animals, which in Bolivia was traditionally based on parasite identification in blood films. The effectiveness of parasitological methods as a diagnostic tool is limited by their low sensitivity, as they cannot identify animals with chronic infections and low. In order to try to overcome this problem, serological diagnostic techniques were developed but they did not succeed in completely resolving this problem. Serological diagnosis based on antibody detection lacks specificity and cannot differentiate current from past infection [4]; [5]. Antigen detection assays are able to identify current infections but it has been proved that this kind of tests also lacks sensitivity [6]; [7]. Recently, molecular diagnostic techniques particularly Polymerase Chain Reaction (PCR) assays appear to be a promising technique for the diagnosis of Trypanosome infection based on the detection of Trypanosome DNA. Primer sets for the specific diagnosis of *T.vivax* or *T.evansi* have been developed [8]-[13], however, limitations of sensitivity and specificity have been also reported for this DNA based diagnostic assays [14]; and most of these PCR assays have not been tested under field conditions for the diagnosis of natural infection.

There is a need for accurate information about the prevalence and distribution of bovine Trypanosome infection in Bolivia in order to establish risk factors and develop control strategies. Several diagnostic assays are in use for epidemiological studies in the country, but most of them have limitation either in their sensitivity or their specificity. Currently, PCR assays appear to offer an opportunity to overcome some of these problems in the diagnosis of Trypanosome infection. The objective of this study was to evaluate PCR assays applied to field samples from Bolivia as a diagnostic tool for epidemiological studies in this country.

2. MATERIAL AND METHODS

2.1. Sampling and parasitological analysis

The sample collection was made between June and September 2001 from the provinces of Guarayos (n = 252), Sara (n = 248), Velazco (n = 150) and Yacuma (n = 250). The sample areas are located in Santa Cruz department (first 3 provinces) and Beni department (Fig. 2). Both departments are located in the Bolivian Lowlands. Each locality was divided into four geographical strata to provide even geographical covering of the sampling area. The farms were randomly selected with each stratum, based on a frame supplied by the local farmer's society. Finally, the sampled animals were randomly selected within each farm. The number of samples by farm was decided proportional to the total number of animals in the farm, or according to availability

The Micro Haematocrit Centrifugation Technique (MHCT) for parasitological diagnosis was carried out in situ, as part of the sampling. Blood for preparing thin blood films and for the MHCT diagnosis was collected from cattle's ear veins. Additional blood samples were collected from cattle's tail vein into vacutainer tubes with heparin

for blood spots preparation and without heparin for serological test. The thin blood films and the serum samples were prepared for laboratory diagnosis at LIDIVET (Santa Cruz, Bolivia). Blood spots on filter papers and then immersed in acetone were prepared for PCR assays at Centre for Tropical Veterinary Medicine (CTVM), university of Edinburgh.

2.2. Serological analysis

All the serum samples were tested at LIDIVET using the following serological techniques: Cart agglutination test for Trypanosomiasis/*Trypanosoma evansi* (CATT/*T.evansi*) supplied by ILRAD, Nairobi and Belgian University of Brussels, Institute of molecular biology and carried out according to supplier's instructions. Results discrimination was based on the degree of agglutination observed. No agglutination reaction was considered as negative, while reactions of +, ++, or +++; were considered as positive. Two antibody ELISA Kits using pre-coated plates either with *T.congolense* or *T.vivax* denatured antigens, were supplied by the Join FAO/IAEA programme. Both tests were carried out according to the kit's bench protocol and serum results were expressed as the percentage positive value (PP) of the strong positive serum control supplied with the kit. Only results of samples from ELISA plates that met at least the first level of Internal Quality Control (IQC) evaluations were used and their positive/negative discrimination was based on PP cut off points recommended by IAEA and already evaluated with Bolivian samples by [15].

2.3. PCR analysis

2.3.1. Samples for PCR analysis

With some samples missing, almost all blood spots samples from Guarayos (n = 251) and Sara (n = 241) provinces were tested by PCR. For economical reasons only 127 samples from Yacuma and 48 from Velasco randomly selected were also tested by this method.

2.3.2. DNA extraction from blood spots

The DNA extraction was carried out as described by [16]. A 6 mm diameter disc was punched from the blood spot preparations and placed into a 500 μL microcentrifuge tube, 200 μL of sterile distilled water was added and the tube incubated at 37°C in a thermocycler for 30 min, then centrifuged at 10 000 rpm for 10 min and the resulting supernatant (cold eluate) was removed. The disk was resuspended in 100 μL of sterile distilled water and incubated at 99°C for 30 min. After centrifugation the supernatant ('hot eluate') was removed and transferred to a clean tube. The resulting hot eluate was stored at 4°C and processed within 24 h of isolation.

2.3.3. Controls preparation

T.vivax and *T.evansi* DNA positive controls were extracted from whole blood of infected animals following the blood and body fluid spin protocol of Qiagen (QIAamp DNA mini kit).

A clean filter paper disk was included in every extraction as negative control to ensure the absence of cross contamination in the amplification process.

2.3.4. DNA amplification on blood spots

The following oligonucleotide primers were used:

T.vivax specific primers giving amplification product of 400 bp [11].

TWJ1 = 5' CAG CTC GGC GAA GGC CAC TTG GCT GGG 3'

TWJ2 = 5' TCG CTA CCA CAG TCG CAA TCG TCG TCT CAA GG 3'

T.brucei primers used for *T.evansi*.- amplification product of 177 bp [8].

TBR1= 5' CGA ATG AAT AAT AAA CAA TGC GCA GT 3'

TBR2 = 5' AGA ACC ATT TAT TAG CTT TGT TGC 3'

The PCR assay conditions used were standard conditions optimized at the Centre of Tropical Veterinary Medicine (CTVM), Edinburgh, UK. All the samples were processed using TWJ1/2 and TBR1/2 primer sets. The PCR amplifications were conducted in 25 μL reaction mixtures containing as final concentrations: 10 m*M* Tris-HCL, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 μ*M* each of the four deoxynucleoside triphosphates (dNTPs) and 1 unit of Taq DNA polymerase (REDTaq SuperPak DNA Polymerase, Sigma). These concentrations were the same for both primer sets. Primers were supplied to a final concentration of 2 μ*M* (TWJ1/2) and 0.4 μ*M* (TBR1/2).

DNA eluate preparations as well as negative control accounted for 7.5 μL of the total PCR reaction volume. One μL of positive control was used in the final 25 μL PCR reaction mixture.

Amplifications were carried out using Peltier Thermal Cycler (DNA engine DYAD). For TWJ1/2, cycles consisted of an initial denaturing step at 94°C for 3 min followed by 30 cycles of denaturing step at 94°C for 1 min, and annealing step at 55°C for 2 min, and an extension step at 72°C for 2 min. Each amplification was completed with a 72°C incubation for 5 min to ensure the completion of the extension reactions. For TBR1/2, cycles consisted of an initial denaturing step at 94°C for 3 min followed by 30 cycles of denaturing step at 94°C for 45 s, and annealing step at 60°C for 1 min, and an extension step at 72°C for 30 sec. A terminal extension at 72°C for 5 min.

2.4. Modification of PCR assay

Twelve parasitologically positive samples were reassessed under the modifications applied in this process. Ten of these samples, 6 from Sara and 4 from Guarayos, were *T.vivax* positives, identified by smear observations. The remaining 2 samples were MHCT positives from Guarayos. 6 of the 10 samples positives by smears were also positive by MHCT and 1 of these samples was already detected TePCR positive.

A total of 38 samples negative by parasitology, but with PCR produced bands that were too weak to be classified as PCR positive or the molecular size of the band was not clear, were also reassessed under the modifications applied in this process. The following modifications were carried out to the PCR assays:

Modification of the template DNA concentration

The PCR procedure was modified by changes in the volume and/or dilution of the blood spot eluate in the final reaction volume 25 µL. Each sample was tested using the following blood spot eluate volumes of: a) 10 µL, b) 7.5 µL and c) 10 µL of diluted eluate in distilled water 1/10. The reaction conditions remained the same for TWJ1/2 and TBR1/2.

Use of alternative primer sets for T.vivax

T.vivax specific primers , amplification product of 175 bp [10].

TVWA = 5' GTG CTC CAT GTG CCA CGT TG

TVWB= 5' CAT ATG GTC TGG GAG CGG GT

The amplification reaction was conducted at the same reagent concentrations as for TWJ and TBR. The primers were supplied to a final concentration of 1µM. Cycles consisted of an initial denaturing step at 94°C for 3 min followed by 30 cycles of denaturing step at 94°C for 30 sec, and annealing step at 60°C for 1 min, and an extension step at 72°C for 30 sec. A terminal extension at 72°C for 5 min.

2.5. Gel electrophoresis

The total 25 µL of amplification mixture were loaded onto a 1.5% agarose gel. Electrophoresis was processed for 40 min at 100 V. The gel was stained with Ethidium bromide and the samples were examined under UV light.

2.6. Data analysis

Data analyses were carried out based on the PCR assay results and the results obtained of the parasitological and serological diagnosis from LIDIVET. The agreement of test results was based on the analysis of the Kappa statistics using a computer programme Win Episcope 2.0 Prevalence rates and comparison of proportions were carried out based on X^2 or Fisher exact test using the programme Epi Info version 6.

3. RESULTS

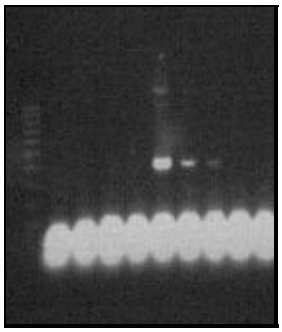
3.1. Optimization of the PCR Assay

PCR products from the *T.vivax* DNA control preparation were only obtained with the primers TWJ1/2 resulting in a PCR amplification product of 400 bp. This single band was seen when the control preparation sample was diluted up to 1/10 000. No PCR product was produced when the TBR1/2 primers were tested with the *T.vivax* control preparation (Fig. 1).

The TBR1/2 primer set produced a ladder pattern with a principal band of 177 bp amplification product, when amplified with the *T.evansi* control preparation. The lowest dilution at which a PCR product was obtained was 1/10 000. No amplification product was obtained when this control sample was tested with the TWJ1/2 primer set (Fig. 2).

Once the conditions for both PCR assays had been optimized, they were validated against DNA extracted from a blood spot from Bolivia which was obtained from a known positive animal for *T.vivax* by blood smears, and Trypanosomiasis positive by MHCT and the 3 serological tests used. A 400 bp band with the TWJ1/2 primers was observed and an ~177bp band with the TBR1/2 primer set was also obtained.

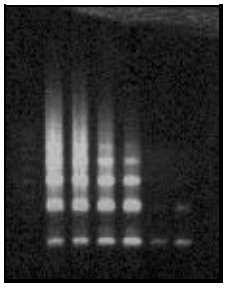
M a_1 a_2 a_3 a_4 b_1 b_2 b_3 b_4



M	Marker
a_1-a_4	*T.evansi* controls
b_1-b_4	*T.vivax* controls
b_1	1:100
b_2	1:1000
b_3	1:10 000
b_4	1:100 000

FIG. 1. T.vivax and T.evansi positive controls tested with the TvPCR assay at different dilution.

M a b c d e f g



M	Marker
a	1:5
b	1:10
c	1:50
d	1:100
e	1:1000
f	1:10 000
g	1:100 000

FIG. 2. T.evansi control tested in diferent dilutions with the TePCR assay.

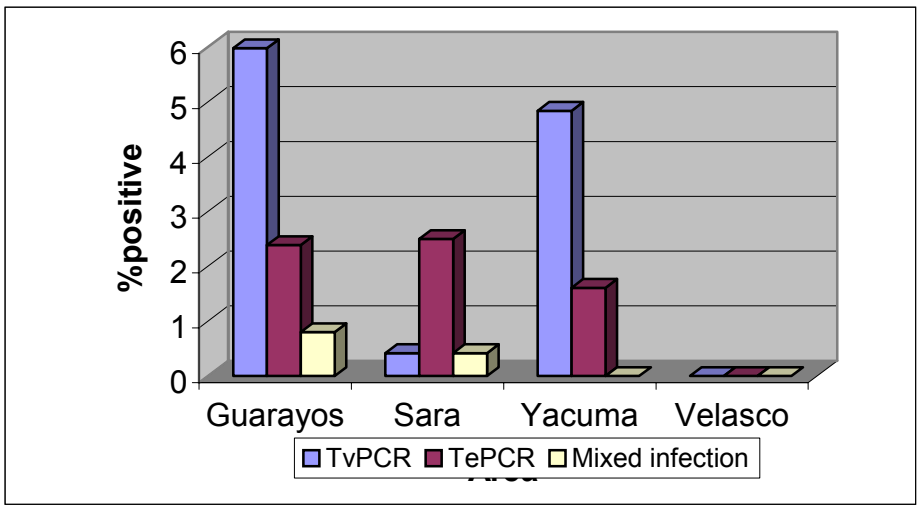
3.2. Application of PCR to blood spots from Bolivia

PCR products produced by one or both of the primer sets were found in blood spots collected from animals in three of the four provinces studied: Guarayos, Sara and Yacuma. No PCR product was amplified from any blood spots from Velazco.

Samples positives by TWJ1/2 or TBR1/2 were found in each of the three areas. *T.vivax* PCR product was found in the following proportions: 5.98% in Guarayos, 0.42% in Sara and 4.84% in Yacuma. While the proportions of positives found for *T.evansi* PCR were: 2.39% in Guarayos, 2.50% in Sara and 1.61% in Yacuma. Mixed infections with both parasites were only found in Guarayos and in Sara. The proportion of positives for *T.vivax* in relation to *T.evansi* was higher in Guarayos and Yacuma, while in Sara the proportion of *T.evansi* was higher in relation to that of *T.vivax* positives. The highest proportion of *T.vivax* PCR positives was found in Guarayos and the lowest in Sara. The proportion of *T.evansi* positives was similar between Sara and Guarayos with the lowest proportion in Yacuma (Fig. 3).

3.3. Comparison of results from PCR Assays with results of parasitological assays

Trypanosome positive results by parasitological methods were obtained only in two of the four provinces studied; Guarayos and Sara. Table II shows the agreement between the results obtained with PCR assays with those obtained by parasitological assays. Differences were seen in the level of agreement between Guarayos and Sara.



TvPCR: PCR assay for *T.vivax*
TePCR: PCR assay for *T.evansi*

FIG. 3. Proportion of positive results obtained by province.

PCR assays showed a high degree of agreement with the parasitological methods in Guarayos, however, no agreement was found between these tests in Sara.

3.4. TvPCR (TWJ1/2)

In Guarayos, 13 samples were diagnosed positive for *T.vivax* by stained smear microscopy. Nine of these samples were positive by both smears and TvPCR. A significant agreement was found between the results obtained by thin smears and TvPCR (Kappa = 0.622). In Sara, none of the TvPCR positive results agreed with the positives by smears. Furthermore, TvPCR produced one positive result compared with 6 *T.vivax* positives diagnosed by smear observations. No significant agreement was found between smears and TvPCR in Sara (Kappa = -0.007) (Tables I and II)

Nine samples in Guarayos and 4 in Sara were found positives for Trypanosomes by MHCT. In Guarayos an agreement between TvPCR and MHCT was seen with 5 positive samples, but in Sara none of the TvPCR positive samples agreed with the positives by smears. Differences were seen in the level of agreement between Guarayos and Sara. While an agreement between MHCT with TvPCR was found in Guarayos (Kappa = 0.389), no statistical agreement was found in Sara (Kappa = -0.007) (Tables I and II).

Statistically significant agreement was found between TvPCR with both smears and MHCT only in Guarayos. However, a higher degree of agreement was observed between TvPCR and smears (Kappa = 0.622) than TvPCR and MHCT (Kappa = 0.389). In Guarayos TvPCR detected an additional 6 positives, which were negative by parasitological methods. However, TvPCR failed to detect 6 parasitologically positive samples (four of which were *T.vivax* positive by thin smear). Similar results were observed in Sara, with TvPCR unable to detect 6 parasitologically positive samples (Table III), but only one TvPCR positive was detected in this area, which was negative by parasitological methods.

3.5. Te PCR (TBR1/2)

T.evansi was not detected in smears from any of the regions sampled. However, TePCR did detect positives in Guarayos, Sara and Yacuma (Fig. 3). No agreement analysis could be done, therefore, in relation to TePCR and smears observations.

MHCT detected Trypanosomes only in Guarayos and Sara. A significant agreement between these tests was found only in Guarayos (kappa = 0.245) (Table II), where 2 MHCT positive results were also positives by TePCR. In Sara, however, there was no agreement between MHCT and TePCR. In Guarayos seven of the MHCT positives were TePCR negatives while in Sara all 4 MHCT positives were negatives by TePCR Table III).

TABLE I. NUMBER OF POSITIVE SAMPLES DETECTED BY PARASITOLOGICAL METHODS

Province	Test			
	Thin smears		MHCT*	Total
	T.vivax	*T.evansi*		
Guarayos	13	0	9	252
Sara	6	0	4	248

* Micro haematocrit centrifugation technique

TABLE II. DIAGNOSTIC AGREEMENT BETWEEN PCR ASSAYS AND PARASITOLOGICAL METHODS

Province	Area					
	Guarayos			Sara		
	Concordant results		Kappa values+	Concordant results		Kappa values
	Positives	Negatives		Positives	Negatives	
*Tv*PCR vs Thin smears	9	232	0.622	0	233	-0.007
*Tv*PCR vs MHCT	5	233	0.389	0	235	-0.007
*Te*PCR vs MHCT*	2	238	0.245	0	230	-0.020

* There is no agreement analysis between TePCR and thin smears because *T.evansi* was not diagnosed in the smears observations.

+ Arbitrary bench marks for evaluating kappa values are : >0.81: almost perfect agreement; 0.61-0.80: substantial agreement; 0.41-0.60: moderate agreement; 0.21-0.40: fair agreement; 0-0.20: slight agreement; 0: poor agreement and negative results: no agreement. Kappa rages from 1 (complete agreement) to 0 (agreement is equal to that expected by chance). PCR shows a statistical agreement with parasitological techniques only in Guarayos. There was no agreement between PCR and the parasitological methods in Sara.

3.6. MODIFICATION OF PCR ASSAY

3.6.1. Modification of the template DNA concentration-evaluation of parasitologically positive samples found PCR negatives using the original assay conditions (TWJ1/2 primer set)

Modifications in the template DNA preparation concentrations used in the PCR assays improved the assay detection rate with an improved agreement obtained between PCR assays and parasitological methods

Changing the volume of the DNA template preparation (blood spot eluate) from 7.5 µL to 10 µL did not improve the detection rate of the parasitologically positive samples tested. However, improvements were obtained when the blood spot eluate was

diluted 1/10 in distilled water and then used in a volume of 10 μL in the total 25 μL PCR reaction mixture. TvPCR produced PCR products in 2 out of 6 samples from Guarayos, which had been classed as PCR negative under the original assay conditions. However, four samples from Guarayos, and all the 6 samples from Sara remained PCR negative under the new assay conditions. Similar modifications to the TePCR assay did not result in improved agreement with the MHCT. With all the TePCR negative samples remaining negative. Reassessment of PCR doubtful samples by the original conditions, but negatives by parasitology.

No differences were seen in the quality of band resolution when using eluate at a volume of 7.5 μL or 10 μL in both cases; the TWJ1/2 primer set confirmed five out the 38 samples. Seven samples and additional 2 samples, however, were classified as PCR positive when the DNA template preparation (blood spot eluate) was diluted 1/10. The assay modifications did not result in improvement in band resolution or PCR detection rate when carried out with TePCR assay.

3.6.2. Alternative primer sets for T.vivax

Improved results in relation to the PCR detection rate were observed when retesting both parasitologically positive samples but PCR negatives (Table III) and TvPCR doubtful samples, with the TVWA/B primer set. Evaluation of parasitologically positive samples but PCR negatives by the original conditions (TWJ1/2). Using TVWA/B primer set, 4 out of 6 samples from Guarayos and 1 out of 6 samples from Sara were amplified at all the eluate volume and dilutions tested. TVWA/B primer set detected three more positive samples than the number of samples detected by TWJ1/2 primer set when the eluate was diluted 1/10. Furthermore, TVWA/B primer set was the only one that amplified DNA from samples from Sara (Fig. 4).

TABLE III. RETESTED SAMPLES POSITIVE BY PARASITOLOGY AND NEGATIVES BY PCR

No	Province	Smears*	MHCT	TWJ	TWJ1/10+	TVW	TBR
11	Guarayos	Pos	N	N	N	N	N
198	Guarayos	Pos	Pos	N	N	Pos	N
200	Guarayos	Pos	Pos	N	Pos	Pos	Pos$
222	Guarayos	N	Pos	N	N	Pos	N
224	Guarayos	N	Pos	N	N	N	N
252	Guarayos	Pos	N	N	Pos	Pos	N
263	Sara	Pos	Pos	N	N	Pos	N
267	Sara	Pos	Pos	N	N	N	N
344	Sara	Pos	Pos	N	N	N	N
348	Sara	Pos	N	N	N	N	N
442	Sara	Pos	Pos	N	N	N	N
446	Sara	Pos	N	N	N	N	N

*All the thin smears positives were confirmed as *T.vivax*. No *T.evansi* positive was reported by parasitology.

+ TWJ1/2 used with DNA template diluted 1/10.

$ TePCR positive sample detected when the test was applied to all the blood spots and later when modifications in the DNA template concentrations were applied.

N = negative result.

Pos = Positive result.

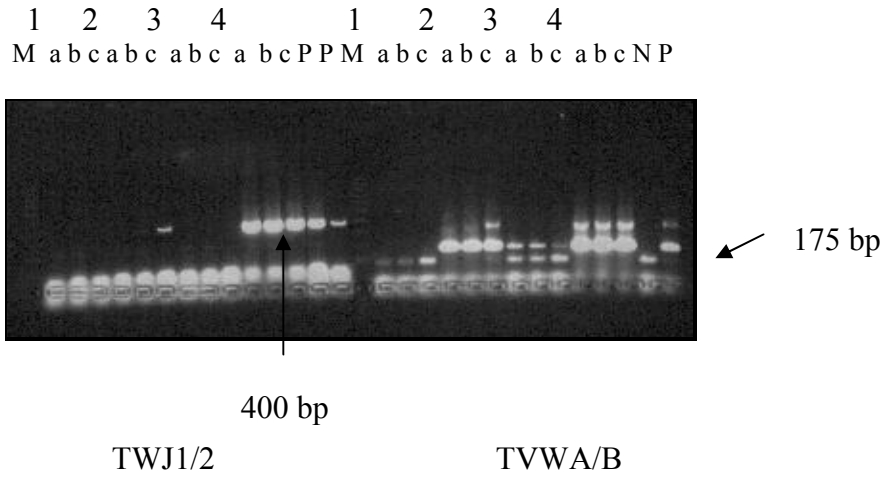


FIG. 4. Parasitologically positive samples tested with modification in the DNA concentration and with a new set of primers.

M	Molecular marker.
a	Template DNA used at a volume of 10 μL.
b	Template DNA used at a volume of 7.5 μL.
c	Template DNA diluted 1/10.
1	Known negative sample used as control.
2	Parasitologically positive sample from Guarayos.
3	Parasitologically positive sample from Sara.
4	Known Parasitologically positive sample from Guaray used for the first time when optimizing the PCR assay now was used as one of the controls.

3.6.3. Reassessment of PCR doubtful samples by the original conditions, but negatives by parasitology

PCR products were obtained with samples at all three template DNA concentrations. TVWA/B primer set detected 8 positive samples. One more than the number of positive samples detected by TWJ1/2 with elution preparation diluted 1/10. And 3 further more positives samples than the number of samples detected by TWJ1/2 without modifications to the DNA template preparation.

3.7. Comparison between TWJ1/2 and TVWA/B primers

Tables IV and Fig. 5 summarize the results obtained under different assay conditions with TWJ1/2 and TVWA/B primer sets. Improved PCR detection rate was obtained with the TVWA/B primer set when compared with the TWJ1/2 primers. Changes in the DNA template concentration by dilution of the blood spot eluate preparation improved the detection rate of the TWJ1/2 primer set. However, TVWA/B detected the highest number of TvPCR positives irrespective of changes in assay conditions.

3.8. Comparison of PCR Assay Results with Serological Assays

High proportion of positive results was obtained with each of the serological tests. However, in most cases there was no significant agreement between the results from these tests and those from PCR assays (Tables V and VI).

TABLE IV. RESULTS OBTAINED WITH MODIFIED DNA TEMPLATE CONCENTRATIONS AND A DIFFERENT PRIMER SET

Samples	Total tested	TWJ		TWJ 1/10		TVW	
		Positive	Negative	Positive	Negative	Positive	Negative
Parasitologically positives	12	0	12	2	10	5	7
PCR doubtful	38	5	33	7	31	8	30
Total	50	5	45	9	41	13	37

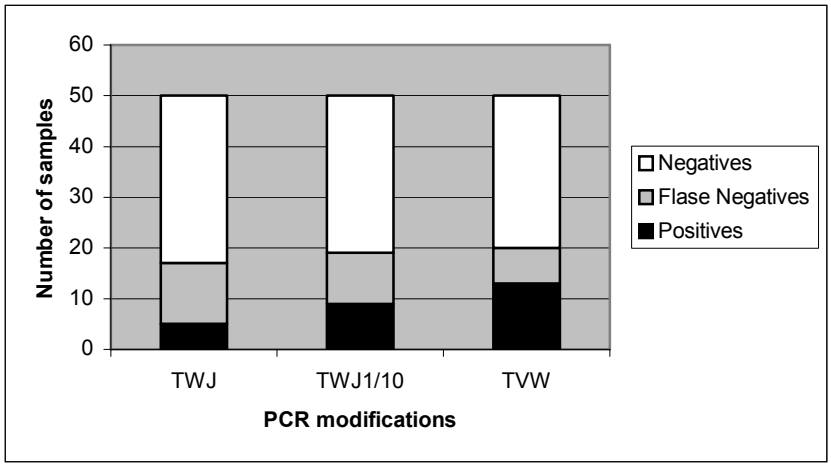


FIG. 5. False negatives: parasitologically positive samples but PCR negatives.

3.9. CATT

Results from this test were obtained from two provinces only, Guarayos and Sara (Table V). The number of positives by the CATT test was greater than the number of positives obtained by TePCR and TvPCR in both provinces, particularly in Guarayos. However, a Kappa agreement analysis between CATT and either TePCR or TvPCR showed a slight agreement in both provinces Guarayos and Sara. The proportion of CATT positives in Guarayos was greater than in Sara. This differed from the TePCR results, which showed a relative higher proportion of positives in Sara than in Guarayos.

3.10. TvELISA.

TvELISA positive samples were found in the four provinces tested, in a greater proportion than the number of TvPCR or TePCR positives. There was no significant agreement between TvELISA and TvPCR or TePCR in Guarayos and Sara, but a slight agreement was seen in Yacuma between TvELISA and both PCR assays. Guarayos had

the highest proportion of TvELISA positives, this was followed by Yacuma, Sara and Velazco having the lowest proportion of seropositives. TvPCR assay showed a similar pattern with the highest proportion of diagnosed positives in Guarayos and the lowest in Sara (Tables V, VI).

TABLE V. TOTAL SAMPLES AND NUMBER OF POSITIVES IDENTIFIED BY TEST

Area	Test									
	TvPCR		TePCR		CATT		TvELISA		TcELISA	
	Samples Number	Pos (%)	Samples Number	Pos (%)	Samples Number	Pos (5)	Samples* Number	Pos (%)	Samples* Number	Pos (%)
Guarayos	251	15 (2.6)	251	6 (2.4)	252	153 (60.7)	167	127 (76.0)	196	143 (56.1)
Sara	241	1 (0.4)	241	6 (2.5)	243	44 (18.1)	126	69 (54.8)	245	93 (38.0)
Yacuma	124	6 (4.8)	124	2 (1.60)	-**	-	163	116 (71.1)	209	198 (94.7)
Velazco	48	0	48	0	-	-	98	47 (47.9)	137	129 (94.2)

* Remaining sample results from accepted Elisa plates ** Blank results are because the tests were not carried out.

TABLE VI. DIAGNOSTIC AGREEMENT BETWEEN PCR AND SEROLOGICAL METHODS FOR THE DETECTION OF BOTH T.VIVAX AND T.EVANSI EXPRESSED BY THE KAPPA STATISTIC+

T.vivax Tests	AREA							
	Guarayos		Sara		Yacuma		Velazco	
	No pos*	Kappa values	No pos	Kappa values	No pos	Kappa values	No pos	Kappa values&
*Tv*PCR vs TvELISA£	7	- 0.074	0	-0.008	6	0.030	0	-
*Tv*PCR vs TcELISA	5	- 0.042	0	-0.008	6	0.019	0	-
*Tv*PCR vs CATT	12	0.039	1	0.036	-#	-	-	-
*Te*PCR vs CATT	5	0.018	3	0.101	-	-	-	-
*Te*PCR vs TcELISA£	5	- 0.014	1	-0.027	2	0.009	0	-
*Te*PCR vs TvELISA	4	- 0.009	2	-0.039	2	0.010	0	-

* Concordant positive samples.
# The test was not carried out. £ *Tc*ELISA: *T.congolense* ELISA. *Tv*ELISA: *T.vivax* ELISA and Kappa agreement was not carried out for this province because all the PCR results were negative. There was no agreement between those tests.

3.11. TcELISA

TABLE VII. PROVINCES' PREVALENCE OF BOTH T.VIVAX AND T.EVANSI BY TEST

	Test prevalence 95% CI						
	Thin smears	MHCT	*Tv*PCR	*Te*PCR	CATT	*Tv*ELISA	*Tc*ELISA
Guarayos	5.2%a	3.6%a	6.0%a	2.39%	60.7%a	76.05%a	56.1%[a]
95% CI	2.8-8.7	1.7-6.7	3.1-9.2	0.88-5.2	54.4-67.0	68.8–82.3	48.9-63.1
Sara	2.4%ab	1.6%ab	0.4b	2.5%	18.1%b	54.8%b	37.96%[b]
95% CI	0.9-5.2	0.4 -4.1	0.00-2.3	0.92-5.3	13.5-23.5	45.7–63.6	31.9-44.4
Yacuma	0.00%b	0.00%b	4.8%ab	1.6%	71.2%a	94.7%[c]	
95% CI	0.0-1.5	0-1.5	1.8-10.2	0.2-5.7	-	63.6–78.0	90.7-97.3
Velazco	0.0%b	0.0%ab	0.0%	0.0%	-	48.0%b	94.16%[c]
95% CI	0.00-2.4	0.00-2.4	0.00-7.4	0.00-7.4		37.8-58.3	88.8-97.4

a,b,c Values in the same column with unlike superscrip differ significantly ($p < 0.05$). The statistical analysis was carried out with X2 or Fisher exact tests

As the other serological tests, high proportions of positives were found in each of the four provinces studied. When this test was compared with TvPCR, no agreement was found in Guarayos and Sara. However, a slight agreement was obtained in Yacuma. When compared with TePCR no agreement was found in Sara and Guarayos with slight agreement in Yacuma. In contrast with the other serological tests, the second highest proportion of TcELISA positives was found in Velazco, where neither PCR nor parasitologically positive samples were obtained. The lowest proportion of TcELISA positives was found in Sara (Table V and VI).

3.12. Prevalence of cattle Trypanosome infection in the Bolivian lowlands

Parasitological evidence of Trypanosome infection was found in three of the four provinces studied: Guarayos, Sara and Yacuma, however antibody to Trypanosomes was found in all the four provinces. Antibody prevalence for *T.vivax* and *T.evansi* was higher than the prevalence reported by the PCR assays or parasitological techniques. All the estimated prevalence by test for each province and the prevalence comparison by test between provinces is summarized in Table VII and Fig. 6.

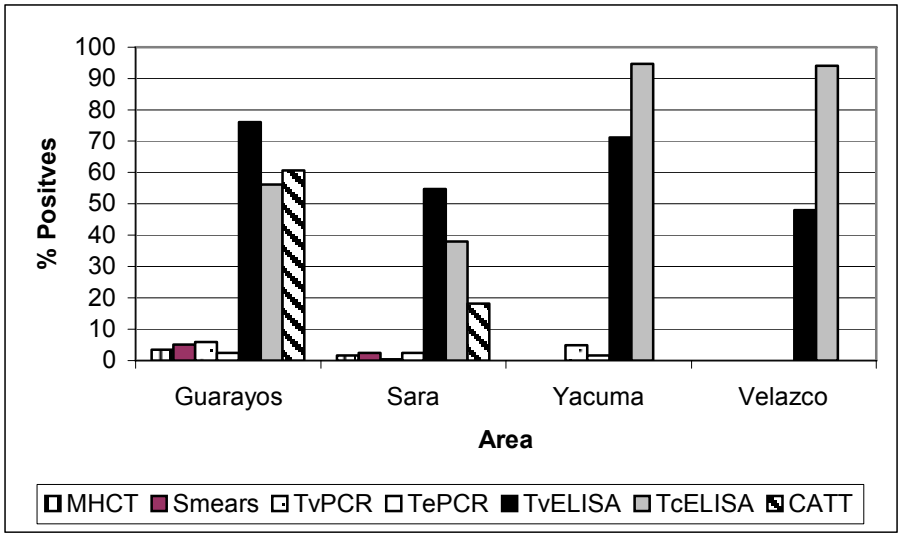


FIG. 6. Prevalence rates obtained by each test in each province.

DISCUSSION

Each primer set only amplified DNA sequences from one of the control DNA preparations under the conditions of the PCR assay. The TBR1/2 set only amplified DNA from the *T.evansi* control preparation, producing a characteristic ladder pattern after electrophoresis with the principal band at 177 bp as described in [8]. The TWJ1/2 primer set only amplified DNA from the *T.vivax* control preparation producing a characteristic 400 bp band as described by [11]. Therefore, the results from the present study provide evidence that the TBR1/2 set detects a DNA sequence related to *T.evansi* while the TWJ1/2 set detects a *T.vivax* — specific sequence. Further evidence of the discriminatory power of these primer sets was obtained from results with extracts from blood spots from an animal parasitologically positive for *T.vivax,* which produced a 400 bp band with the TWJ1/2 primer set, but also a 177 bp ladder with TBR1/2 primer set indicating the presence of a mixed *T.vivax/T.evansi* infection.

The use of PCR assays for the detection of Trypanosome DNA in cattle blood dried as blood spots was also confirmed in this study. In that it was possible to detect evidence of *T.vivax* or *T.evansi* infections by carrying out PCR on eluates from blood spots. PCR products for Trypanosome DNA were obtained with samples from cattle in three of the four provinces studied: Guarayos, Sara and Yacuma, while parasitologically evidence of infection was obtained only from Guarayos and Sara. Therefore, PCR assays were able to detect evidence of infection in areas were parasitological techniques failed to do so. Furthermore, PCR assays were able to detect evidence of *T.evansi* infection, while examination of stained smear observation only identified the presence of *T.vivax*. The fact that PCR assays provided evidence of infection in animals that were classified as uninfected by parasitological techniques suggests that PCR techniques could have an important role to play in the detection of infected animals harbouring a low parasitaemia that would be undetectable by smears or MHCT. The improved detection rates found in the present study agree with those obtained by [11] who reported that PCR assays could detect evidence of Trypanosomes infection during early or late infections, when the parasite levels are undetectable by parasitology and that PCR had a higher threshold sensitivity than parasitological techniques. PCR

identification of parasitologically negative animals was also reported by [12]; [13]; who was able to detect more positive samples for *T.vivax* and *T.evansi* with PCR assays than by microscopical observations of samples from suspected diseased animals during outbreaks of Trypanosomiasis in the Brazilian Pantanal.

The use of nuclear based *Trypanozoon* specific primer sets (TBR1/2) was suitable for the detection of *T.evansi* DNA. The primer set does not distinguish between members of the subgenus *Trypanozoon* but as *T.evansi* is the only member of this subgenus that infects cattle reported in South America, the presence of PCR product has been taken as indicative of *T.evansi* infection. Furthermore, the target sequence for this primers are not found in the kinetoplast making results independent of the parasite kinetoplastic state, which is currently unknown in Bolivia and avoids the problem of failure of primers targeting kDNA as has been reported in South America [17]. The use of the TBR1/2 primer set not only permitted the differentiation between *T.vivax* or *T.evansi* DNA but also the detection of cattle samples harbouring mixed infections, which in this study was not revealed with smears observations as none of the parasitologically positive samples in this study were reported positive for *T.evansi*. This lack of parasitological evidence for *T.evansi* infection is a characteristic of this parasite, which usually causes a chronic disease with low parasitaemia in cattle. Previously in Bolivia *T.evansi* has only been reported in a mixed infection with *T.vivax* in one diseased animal during a severe outbreak in the Bolivian lowlands [1].

Any new diagnostic method needs to be evaluated against existing methods. Ideally this should be against a technique that is able to detect 100% of infected animals — a "gold standard". However, in the case of Trypanosome infection no standard exists. Parasitological evidence continue to be the only absolute indicator of infection, although parasitologically negative animals can be harbouring infection at levels undetectable parasitologically. Validation of the PCR assays in the present study has therefore been carried out against samples from parasitologically positive animals. Kappa agreement analysis between PCR assays and parasitological assays showed differences in agreement in Guarayos and Sara. In Guarayos the agreement between TvPCR with smears and MHCT and TePCR with MHCT was significant while in Sara there was no agreement between the results from these tests. Tests disagreement was associated with the fact that most of the parasitologically positive samples, identified as *T.vivax,* were not detected by the TvPCR assay. All the *T.vivax* positive smear samples from Sara were negative by TvPCR, while only 46% (6/13) of such samples were TvPCR negatives in the samples from Guarayos. To try to improve agreement between parasitological and PCR assays, the effects of modifications to the PCR assay were examined. Diluting the blood spot eluate with distilled water resulted in an improved detection rate by PCR assay; in an addition two *T.vivax* smear positive samples from Guarayos were now classified as positives by TvPCR. The failure to detect under the conditions of the original PCR assay was probably related to the template DNA concentration in the eluate was so high, that resulted in inhibition of the PCR amplification process. A similar observation has been reported where dilution of serum samples improved PCR detection rates. However, this improvement was attributed to the dilution of inhibitory components for the PCR reaction present in sera, such us antigens and antibodies, rather than the parasite DNA dilution. In the present study the presence of haemoglobin in the blood spot eluate might have been inhibitory to the PCR reaction, which was reduced by dilution of the eluate.

As part of the attempts to improve agreement between PCR and parasitological assays, an alternative *T.vivax* primer set was used (TVWA/B). These primers successfully amplified DNA from four of the samples that were classed as PCR negative under the original assay conditions from Guarayos and one from Sara. The latter, it was the only sample from all the smears *T.vivax* positive samples from Sara that was amplified by PCR. The changes made to the template DNA concentrations by dilution of the eluate also improved PCR detection rate of parasitologically negative samples that had produced ambivalent results with the TWJ1/2 primers. As in the case of the parasitologically positive and PCR negative samples, the second set of primers (TVWA/B) resulted in an improved detection rate when compared with the TWJ1/2 primer set regardless the eluate dilution. However, statistical analysis of the significance of these results could not be carried out due to the small sample size. The results obtained during the present study with the different primer sets differ from [18]; who reported the presence of the cDNA sequence targeted by the TWJ1/2 primer set, in several *T.vivax* stocks from Africa and South America. The results from the present study suggest that some stocks of *T.vivax* from Bolivia do not have this sequence in their DNA, in that these were a number of smear positive but PCR negative samples, however, the use of TVWA/B primers resulted in a PCR product. The present results also differ from results obtained by [14]; who used *T.vivax* primer set, TVMR/F, which target a similar cDNA sequence to the TWJ1/2 primers. They obtained an improved detection rate with the TVMR/F primers in relation to TVWA/B. They found that a higher number of *T.vivax* positive samples from Cameroon were misclassified by TVW than TVM and concluded that the DNA sequence targeted by the TVW primer set is not conserved in all *T.vivax* isolates from this region. However, results from the present study suggest that the DNA sequence targeted by the TVWA/B primers is present within all the *T.vivax* population examined in Bolivia. However, the potential confounding problem of genetic diversity on the efficiency of PCR assays needs to be addressed.

Despite the attempts to modify the PCR assay to improve the detection rate, seven parasitologically positive samples failed to produce PCR product with none of the primers at any of the template dilutions. This finding might be due to the presence of different strains of the parasite in this area or technical or procedural failure in processing the samples. Two samples from Guarayos and one from Sara were only detected with the second primer set (TVWA/B) suggesting differences in the genome that could merit further studies. Differences between Trypanosome DNA sequences related to geographical location have been reported for *T.vivax* by PCR assays [19]; [1]; [9]; but these only have been carried out with stocks from Africa where tsetse transmission might promote genetic diversity. Other reasons for the PCR amplification failure could be the following: (1) the Trypanosome DNA was degraded [20], [14]; during or after extraction from the blood spots or during storage and transportation. (2) Low DNA concentrations, therefore insufficient DNA to produce a visible PCR product [14]. This would be unlikely as there was no amplification when the template DNA concentration was increased as a consequence of increased the eluate volume added to the reaction mixture. Furthermore, all these blood spots were from parasitologically positive samples; therefore, a high DNA concentration could be expected from the relatively high parasitaemia required for detection by smears. (3) Failure in the DNA extraction process to produce a DNA of sufficient quality or quantity [11]; or presence of inhibitory components in the sample for the PCR reaction [6]. This would seem to be unlikely, as similarly processed blood spot controls were included in every set of samples that was processed.

Serological tests based on antibody or antigen detection are widely used as diagnostic procedures and antibody based assays were included in the current study of Trypanosome distribution. Although the results from these assays cannot be taken as absolute evidence of infection, the results were compared with those from PCR assays as part of the validation study. Antibodies to Trypanosomes were found in samples from all the provinces tested and in each case the proportion of seropositives was higher than that detected by PCR. No agreement was found between results obtained by PCR assays and antibody ELISA tests, which might be the result of antibody persistence or cross reaction between both Trypanosomes leading to high proportion of positive results, which are not necessary indicative of active infection. The high proportion of positive results found with *T.congolense* ELISA is a strong evidence of cross-reaction phenomenon commonly found in serological tests as *T.congolense* has not been reported from outside Africa. Similar limitations in interpreting results from antibody ELISAs for *T.congolense* or *T.vivax* or *T.brucei* have been reported [5]; [21]. Prevalence rate results by CATT depend on detecting antibody to a commonly occurring predominant VAT for *T.evansi.* The prevalence rates obtained by CATT were significantly lower in relation to the ELISA tests, which would suggest a higher specificity of CATT in relation to ELISA. However, a slight kappa agreement was found between CATT results and those of TePCR or TvPCR suggesting problems of non specificity with the test caused by common VATs between *T.evansi* and *T.vivax*.

The results from the parasitological, serological and PCR assays were used to obtain information in the distribution of Trypanosome infection in the areas of the Bolivian Lowlands. Evidence of *T.vivax* and *T.evansi* infection in cattle was found in Guarayos Sara and Yacuma by parasitology and by detection of DNA by PCR of these parasites. Additionally, high seroprevalence rates were obtained in all four provinces. Overall evidence from this study confirms that Trypanosome infection in cattle is endemic in the provinces of Guarayos and Sara and likely to be so in other provinces. The presence of Trypanosome infection in Guarayos has been established since 1996 when parasitological methods were used during severe outbreaks [1]. Later in Sara, animals with high parasitaemia were detected by smears or MHCT. However, since the initial reporting of disease in cattle in these areas, sporadic outbreaks have been reported (LIDIVET), but it was not until the present study that more detailed information on these parasites has been available through the use of a range of diagnostic methods. Analysis of prevalence rates showed that there were no significant differences in the parasitological and PCR prevalence rates in samples from Guarayos and Sara provinces, except for TvPCR prevalence rates, which was significantly higher in Guarayos than in Sara, this latter finding is possibly linked to the fact that most of the TvPCR false negatives were from Sara. However, the difference in the seroprevalence rates was significantly higher in Guarayos than in Sara. These differences might be related to a higher risk of cattle exposure to Trypanosome infection in Guarayos related to a higher proportion of animals with higher parasitaemia than in Sara, but more likely related to vector abundance. Results from vector abundance studies in these two provinces identified a greater abundance of tabanids in Guarayos than Sara [2]; and hence a potentially greater risk of transmission in Guarayos.

The results from assays used in the present study provided for the first time evidence that *T.vivax* or *T.evansi* infection is present in Yacuma, Beni. Cases of suspected clinical Trypanosomiasis have been reported over a number of years to local veterinarians but these cases could not be confirmed by available diagnostic methods. However, the detection of *T.vivax* and *T.evansi* DNA in samples from Yacuma is a

strong evidence of the presence of these infections in this province. Neither parasitological methods nor PCR assays produced evidence of Trypanosome infection in Velazco, however high seroprevalence rates were obtained. Despite the fact that seropositive results do not necessarily imply active infection in the region, there has been a background of severe Trypanosomiasis outbreaks in this province, which was confirmed by parasitological identification of Trypanosomes during 1996 [1]; [22]. Furthermore, this province is located in the area classified of high tabanids challenge by [2]. And therefore, it is likely that this province is endemic for Trypanosomiasis. However, the factors contributing to the infection patterns, absence of parasite, PCR positives but high seroprevalence — need further study and the implications for control understood.

Although evidence was obtained of an improved detection rate with TVW primers when compared with TWJ primers, problems of availability of reagents in Bolivia did not permit the retesting of all the samples and it is possible that further DNA positives might have been detected if the TVW primers had been applied to all the samples. PCR assays with the different primer sets produced evidence that strain difference might occur between *T.vivax* stocks in Bolivia. However, more work needs to be done to characterize any differences and their significance, especially in relation to the use of particular primers for the diagnosis of *T.vivax* in the country. Genetic differences between stocks in different areas might require the development of local or regional (South America) primer sets which might help to overcome some of the problems associated with PCR miss - identification of infected animals. The availability of such local primer sets would be an essential tool for the successful diagnosis and accurate epidemiological studies. However, the PCR assays as applied in the present study provided specific identification of *T.vivax* or *T.evansi* or mixed infections, and importantly, PCR was able to detect samples that were negative by parasitological means. The present study, therefore, has provided further evidence of the usefulness of PCR assays as a diagnostic tool for Trypanosome epidemiological surveillance in countries such as Bolivia. However, there is a need for careful considering of assay conditions and primer sets for future use in such studies. However at the moments it is likely that a PCR can only be considered to be an additional tool for diagnosis and there will be a continuing need to use parasitological and serological assays in epidemiological studies.

ACKNOWLEDGEMENT

We wish to thank the Joint FAO/IAEA subprogramme in for technical support and financial assistance and to DFID and the LIDIVET-CTVM for additional financial support.

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BOVINE TRYPANOSOMOSIS IN THE BOLIVIAN PANTANAL

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Abstract

Trypanosomosis caused by T.vivax has been a constraint for cattle production in the Bolivian lowlands, since it was introduced in 1996. Flooded areas like the Bolivian Pantanal have a suitable environment for the presence and transmission of Salivarian Trypanosomes and farmers from that region often report Trypanosomosis-like problems on their farms. The objective of the present study therefore was to characterize the epidemiology of bovine Trypanosomosis in the Bolivian Pantanal. In order to achieve this objective, 202 cattle from the province of Angel Sandoval and 209 cattle from the province of German Busch were randomly sampled (the Pantanal is located in both provinces). Fifteen farms in each province were visited, the farmers interviewed and biologic samples collected from their cattle. Samples were submitted for parasitological and PCR evaluation and the prevalence of bovine Trypanosomosis was estimated for each province. Laboratory results were correlated with the sampled animals pack cell volume (PCV) and body condition (BC) scores and the observed T.vivax parasites measured for morphometry analysis. Results from this study show differences in morphometric measures between T.vivax parasites from each province. Differences between provinces were also observed in *T.vivax*-related disease. While in Angel Sandoval the PCV and BC of T.vivax affected animals was significantly lower than those of the *T.vivax* negative animals, in German Busch no differences were observed in the PCV and BC of T.vivax positive or negative animals. The estimated prevalence of T.vivax in Angel Sandoval was 27.23% (IC95%: 20.97-33.49) and in German Busch was 20.10% (IC95%: 14.56-25.64). The estimated prevalence in each province of T.evansi was 1.11% (IC95%: 0.00-2.58) and 5.7% (IC95%: 0.17-11-25) respectively. Based on questionnaire and laboratory results, it was concluded that Trypanosomosis is a primary constraint for cattle production in the Bolivian Pantanal.

1. INTRODUCTION

Bovine Trypanosomosis caused by *T.vivax* was first introduced in Bolivia in 1996. Infected animals imported from Brazil were the source of transmission [1]; and animal movements within the country contributed to disease dissemination within the Bolivian lowlands. The most affected provinces are the departments of Santa Cruz and Beni in the Amazonian region of the Bolivian Lowlands, where weather conditions, mainly rainfall and humidity are suitable to maintain abundant tabanid populations [2]; reviewed in 2004 by [3].

The Bolivian Pantanal is located in the eastern part of the country, in the Bolivian department of Santa Cruz next to the Brazilian states of Mato Grosso and Mato Grosso do Sul. *T.vivax* Trypanosomosis was first introduced into the Bolivian Pantanal in 1997, when the province of German Busch was severely affected [4]. In 1998 the

disease reached the province of Angel Sandoval (the northern part of the Bolivian Pantanal) [3]. Since then, outbreaks of Trypanosomosis have been commonly reported by farmers and veterinarians to the national veterinary service "SENASAG". For the Bolivian Reference Laboratory for Diagnosis of Animal Diseases "LIDIVET," it is very common to assist outbreaks of bovine Trypanosomosis in the provinces and counties close to the department's capital city of Santa Cruz de la Sierra (more than 600 Km western from the Pantanal), where cattle from the Pantanal, especially from Angel Sandoval, are brought for fattening (Fig. 1). To clarify and characterize this situation, the objective of this study was to evaluate the epidemiology of bovine Trypanosomosis in the Bolivian Pantanal.

2. MATERIAL AND METHODS

2.1. Origin of samples

2.1.1. Study area

The Bolivian Pantanal is characterized by large flooded areas during the rainy season (October - March) and is situated in the eastern part of the Bolivian department of Santa Cruz, specifically in the provinces of Angel Sandoval and German Busch, bordering the Brazilian states of Mato Grosso and Mato Grosso do Sul, respectively (Fig. 1).

The Angel Sandoval province is the northern part of the Bolivian Pantanal, covering approximately 32,030 Km^2, and with mean annual rainfall of 1,326 mm, mean relative humidity of 75%, and daily mean temperatures ranging from 22 to 33°C. It has approximately 105,875 bovines distributed among 135 ranches. The province production system is characterized as a cattle breeding region and the estimated calving rate based on the 2003 FMD mass vaccination report was 37.30%. Yearlings and old cattle are continually moved on foot to other provinces of Santa Cruz to be raised and/or fattened (SENASAG, unpublished data).

The German Busch province is the southern part of the Bolivian Pantanal, covering approximately 24,765 Km^2 and with mean annual rainfall of 1200 mm and mean relative humidity of 73%. The annual temperature ranges from 22°C to over 33°C. The cattle population is approximately 35,245 cattle distributed on 133 ranches. The province production system has a complete cycle, indicating cattle are bred, raised and fattened in the province. The estimated calving rate based on the 2003 FMD mass vaccination report was 35.60% and cattle movements are seldom practiced (SENASAG, unpublished data).

2.1.2. Sample size and sample collection

Sample size was estimated for each province considering each province's cattle population, an expected minimum prevalence of 5.8% by PCR (as reported by [2] in 2003) for the Guarayos province), an accepted error of 5%, and a confidence level of 95%. The minimum sample size required for each province was 127.

Sampling was performed in the months of April (Angel Sandoval) and October, 2003 (German Busch). Each province was divided into four geographical strata to ensure even geographical covering of the sampling area. How were farms selected? However, because of the rough terrain to reach the farms, it was difficult to adhere to a strict sampling frame. Fifteen farms were sampled for each province and the sampled

animals were randomly selected. A total of 202 samples were obtained in Angel Sandoval and 209 samples in German Busch.

Whole blood samples were collected from the tail vein of each sampled cattle into vacutainer tubes with EDTA. Samples from Angel Sandoval were kept on ice and 4°C until they reached the Laboratory for DNA extraction, while samples from German Busch were prepared as dried blood spots on Whatman® FTA® cards.

2.1.3. Questionnaire survey and clinical examination

An interview with the farmers or cowboys was conducted to collect preliminary information about the farmer's perception of the main animal health problems in the area and a clinical examination was performed on each sampled animal, including the recording of body condition (BC; scale from 1 to 5; 1 = cachectic and 5 = obese) and pack cell volume (PCV).

2.2. Diagnostic techniques

2.2.1. Parasitological analysis

The microhaematocrit centrifugation technique (MHCT) was performed *in situ*, and thin blood and buffy coat films were prepared from positive samples. Once in the laboratory, thin smears were Giemsa stained, the *Trypanosoma* species identified and measured for morphometry analysis as described by [5].

2.2.2. PCR analysis

2.2.2.1 DNA extraction

DNA was extracted from either whole blood samples (samples from Angel Sandoval) or Whatman® FTA® cards (samples from German Busch) as described by [6]. Due to PCR reagent limitations, only a total of 70 samples randomly selected from every sampled farm from German Busch were tested against *T.evansi.*

2.2.2.2. DNA amplification

The following oligonucleotide primers were used:

T.vivax specific primers-amplification product of 175 bp [7].

TVWA 5' GTG CTC CAT GTG CCA CGT TG
TVWB 5' CAT ATG GTC TGG GAG CGG GT

Trypanosoma Trypanozoon primers used for *T.evansi*-amplification product of 177 bp [8].

TBR1 5' CGA ATG AAT AAT AAA CAA TGC GCA GT
TBR2 5' AGA ACC ATT TAT TAG CTT TGT TGC

The PCR amplifications were conducted in 25 µL reaction mixtures containing as final concentrations: 10 m*M* Tris-HCL pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 µ*M* each of the four deoxynucleoside triphosphates (dNTPs) and 1 unit of Taq DNA polymerase (REDTaq SuperPak DNA Polymerase, Sigma). These concentrations were the same for both primer sets. Primers were supplied to a final concentration of 1 µ*M* (TVWA/B) and 0.4 µM (TBR1/2). Two µL of DNA eluate extracted from whole blood or 1 disk (2 mm) from the samples prepared on FTA® cards were added as template DNA to the final PCR reaction mixture.

A DNA positive control as well as a negative control were used every time a set of field samples were tested. Amplifications were carried out using a Peltier Thermal Cycler (DNA engine DYAD). For TVW A/B, cycles consisted of an initial denaturing step at 94°C for 3 min, followed by 30 cycles of denaturing step at 94°C for 30 s, an annealing step at 60°C for 1 min, and an extension step at 72°C for 30 s. Each amplification was completed with a 72°C incubation for 5 min to ensure the completion of the extension reactions. For TBR1/2, cycles consisted of an initial denaturing step at 94°C for 3 min followed by 30 cycles of denaturing step at 94°C for 45 s, an annealing step at 60°C for 1 min, and an extension step at 72°C for 30 s. A terminal extension at 72°C for 5 min.

The total 25 μL of amplification mixture was loaded into a 1.5% agarose gel. Electrophoresis was processed for 40 min at 100 V. The gel was stained with ethidium bromide and the samples were examined under UV light.

2.3. Data analysis

The prevalence of cattle Trypanosomosis was estimated for each province with its corresponding 95% confidence intervals. The mean and standard deviations were estimated for *T.vivax* morphometry measures as well as for the cattle population PCV values. Median and quartiles of cattle BC scores were also estimated. Means or medians were compared using parametric and non-parametric tests, respectively.

3. RESULTS

3.1. Questionnaire survey

Farmers were asked to identify and categorize the main disease/clinical signs and ectoparasites they considered affected their animals. Farmers from Angel Sandoval reported intoxication problems, Trypanosomosis and reproductive problems (low pregnancy rate and abortion) as the main health problems affecting their cattle, while farmers from German Busch did not specifically identify diseases but they did report the main clinical signs they recognized in their affected animals, including anaemia/emaciation, diarrhoea and intoxication as the main health problems affecting their farms. Farmers from both provinces agreed in identifying horn flies (*Haematobia irritans*), Tabanids and ticks, in that order, as the main ectoparasites. Farmers reported the presence of a high abundance of tabanids most of the year, but mentioned the rainy season (October-April) as the period of higher abundance. Farmers from both provinces reported frequent use of dinimazene aceturate to control “tristeza” (*Babesia bovis*) or Trypanosomosis. One farm from Angel Sandoval reported treatment with diminazene aceturate of a group of bulls (n = 21) suspected of having Trypanosomosis 2 weeks and/or 3 days before our visit. These bulls were sampled and most of the treated animals were positive by parasitology (7/21) and/or PCR (13/21).

3.2. Laboratory results

3.2.1. Parasitological results

Parasitological diagnosis was performed *in situ*, and thin blood/buffy coat films of positive samples were stained and observed for morphometry measures and species identification back in our laboratory. Differences were observed not only in the number of parasitologic positive animals identified in either studied regions but also in the morphometry measures of the observed Trypanosomes.

The proportion of parasitologic positive animals identified in Angel Sandoval (12.38%; n = 202) was higher than that of German Busch (2.35%; n =213). All observed Trypanosomes were classified as *T.vivax* and differences were observed in the parasites measures from both Pantanal regions. *T.vivax* from Angel Sandoval was significantly smaller ($p < 0.05$) than those from German Bush. The mean total length of the parasites from Angel Sandoval was 13.72 µm ± 2.04, while the mean total length of T.vivax from German Busch was 17.07 µm ± 1.04 (Table I).

TABLE I. MORPHOMETRIC MEASURES (µM) OF *T.VIVAX* FROM THE BOLIVIAN PANTANAL

Origin	N	L*	PK	KN	PN	NA	F	Wide	Nucleus
A. Sandoval	106	13.72	0.41	4.85	5.28	5.12	3.29	1.29	1.03
Sd		2.04	0.49	0.85	0.06	1.18	1.29	0.44	0.13
G. Busch	13	17.08	0.62	4.15	4.76	7.38	4.85	2.00	1.69
Sd		2.26	1.21	1.11	1.10	1.34	1.44	0.66	0.47

N = number of parasites measured; L = total length including free flagellum; PK = distance from posterior end to kinetoplast; KN = from kinetoplast to middle of nucleus; PN = from posterior end to middle of nucleus; NA = from nucleus to anterior end; F = free flagellum length. * Significant difference in the mean total length of *T.vivax* from both regions ($p < 0.05$).

3.2.2. PCR results

All samples were tested via PCR against *T.vivax* and *T.evansi*. More samples were found positive to *T.vivax* and only a few animals were positive to *T.evansi*. In Angel Sandoval, 25.74% (n = 202) were positive to *T.vivax* and 1.11% (n = 202) were positive to *T.evansi.* Three parasitological positive samples were negative by PCR. In German Busch, 20.10% (n= 209) were positive to *T.vivax* and 5.71% (n = 70) positive to *T.evansi.* All parasitologically positive samples were also positive by PCR for *T.vivax* identification.

3.2.3. Prevalence and clinical analysis

A parallel approach was used to estimate the prevalence of bovine Trypanosomosis in both sampled regions of the Bolivian Pantanal. A sampled animal was considered positive if it was diagnosed as positive by any of the diagnostic tests performed (parasitology or PCR). In general, this approach was only necessary to estimate the prevalence of *T.vivax* Trypanosomosis in Angel Sandoval, where 3 *T.vivax* positive samples were negative by PCR. The *T.vivax* estimated prevalence in Angel Sandoval was 27.23% (IC95%: 20.97-33.49) and in German Busch was 20.10% (IC95%: 14.56-25.64). The *T.evansi* estimated prevalence was 1.11% (IC95%: 0.00-2.5) and 5.71% (IC95%: 0.17-11.25), respectively.

Every sampled animal was clinically observed and its BC and PCV were recorded. The mean PCV of cattle from Angel Sandoval was 29.99% ± 6.66 and the median for the BC was 2.5 with the 25% and 75% quartile values of 2.0 and 3.0 respectively. The mean PCV of cattle in German Busch was 32.14% ± 5.16 and the median BC was 3 with 2 and 3 as the first and third quartiles. The PCV and BC of *T.vivax* positive animals (by PCR or parasitology) and negative animals were compared by province. A significant difference ($p<0.001$) was observed in the mean PCV of

positive animals compared with the negative animals in Angel Sandoval but not in German Busch (p = 0.27) (Fig. 2a). In the same way, significant differences in the median BC of positive versus negative populations were only found in Angel Sandoval (p<0.001) but not in German Busch (p = 0.16) (Fig. 2b).

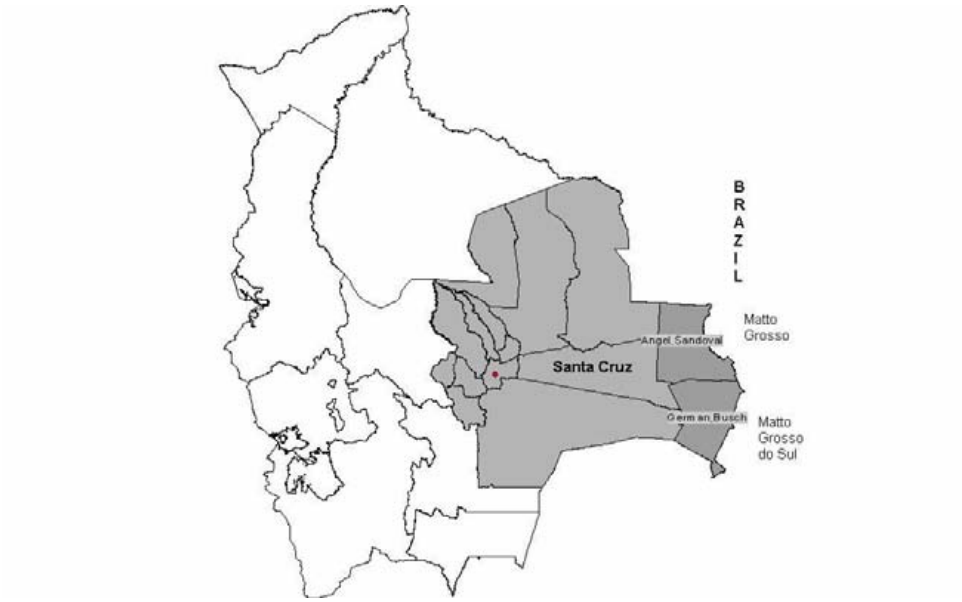

FIG. 1. Map of Bolivia showing the location of the department of Santa Cruz and provinces of Angel Sandoval and German Busch, where the Bolivian Pantanal is located.

The dot is the location of the department capital city, Santa Cruz de la Sierra.

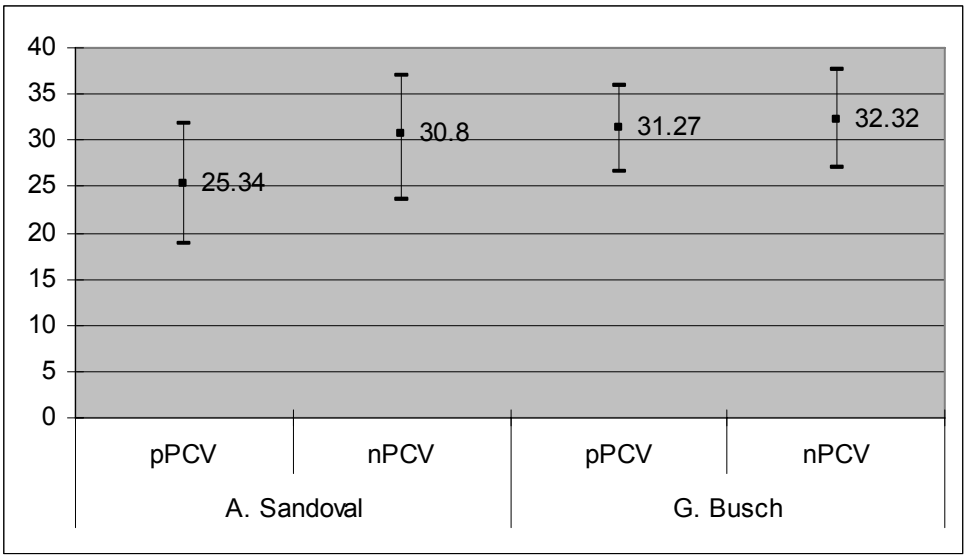

FIG. 2a. Mean PCV values with corresponding standard deviations of T.vivax positive and negative animals from Angel Sandoval and German Bush.

Statistical differences were observed between positive and negative animals only in Angel Andoval (P<0.001). pPCV: positive pack cell volume; nPCV: negative pack cell volume.

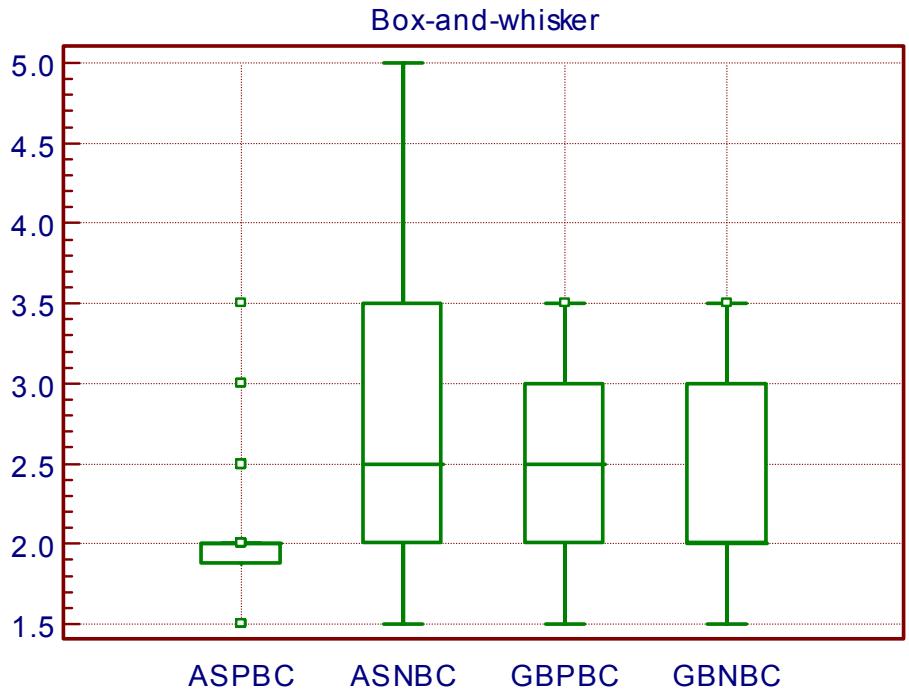

FIG. 2b. Median and quartiles of BC scores of T.vivax positive and negative animals from Angel Sandoval and German Busch.

Statistical differences were observed between positive and negative animals only in Angel Sandoval ($p<0.001$). ASPBC: Angel Sandoval positive body condition, ASNBC: Angel Sandoval negative body condition, GBPBC: German Busch positive body condition, GBNBC: German Busch negative body condition.

4. DISCUSSION AND CONCLUSION

The objective of the present study was to asses the epidemiological situation of bovine Trypanosomosis in the Bolivian Pantanal, which is situated in two provinces of the Bolivian department of Santa Cruz. Samples were collected from both provinces and a questionnaire survey was performed in every sampled farm. Preliminary results obtained with questionnaires indicated the presence of Trypanosomosis in both sampled regions. Farmers from Angel Sandoval were more familiar with the disease than those from German Busch, since they specifically identified Trypanosomosis and correlated the disease with clinical signs of anaemia and emaciation as one of the main problems affecting their cattle. Farmers from German Busch did not identify the disease but did report anaemia and emaciation as the main clinical signs they observed as a common problem in their animals. Farms in both provinces were severely affected with Trypanosomosis when *T.vivax* was first introduced to both regions. *T.vivax* Trypanosomosis was first introduced into German Busch in 1997 [4]; and in 1998 the infection reached Angel Sandoval [3]. In both places, high mortality rates of affected animals, abortion, anaemia and progressive emaciation were observed during the first outbreaks. During this survey, farmers did not reported high mortality rates as recorded during the introduction of the disease, and these results show that the disease is becoming chronic in the region, especially in German Busch province as will be discussed later.

Parasitological and PCR techniques were used to determine the estimated prevalence of bovine Trypanosomosis in both provinces. As expected, more positive animals were identified by PCR than parasitology, which is a result of the higher diagnostic sensitivity of PCR compared to that of parasitological techniques [9]; [10]; [5]. The higher PCR sensitivity allowed the identification of chronically infected animals, which harbour low parasitemias. The selection of TVW A/B primer set for the identification of *T.vivax* was a result of previous evaluations of the use of PCR for diagnosis of *T.vivax* Trypanosomosis [2]. Furthermore, the use of whole blood or FTA card preparations as samples for DNA extraction together with the use of the TVW A/B primer set was evaluated in a small experiment performed in our laboratory with field parasitological positive samples. We found a higher sensitivity of PCR using these kinds of samples with the mentioned primer set compared with other primers and sample preparations reported in the literature for *T.vivax* identification (Gonzales, unpublished data). Later we confirmed these findings with a planned experiment, where we estimated the highest sensitivity of PCR for diagnosis of *T.vivax* Trypanosomosis using the aforementioned sample preparations and primer set [5]. A similar evaluation for the selection of primers for the identification of *T.evansi* was not performed. The TBR ½ primer set [8]; were selected based on our results of the evaluation reported in 2003 [2].

The estimated prevalence of *T.vivax* was higher in Angel Sandoval (27.23%) than that of German Busch (20.10%) but the main difference was observed in the disease situation between both provinces. In Angel Sandoval, the identified affected animals with *T.vivax* (by parasitology and/or PCR) showed statistically significant lower PCV and BC than the animals which reacted negative to the performed test. In contrast, in German Busch, even though lower PCV and BC were recorded for *T.vivax* positive animals, the differences with the PCV and BC of negative animals from positive animals were not significant; furthermore the PCV and BC of *T.vivax* positive and negative animals were similar to the PCV and BC of the *T.vivax* negative animals from Angel Sandoval (Figs 2a and 2b). These findings show that the disease situation in Angel Sandoval remains in a clinical state, while in German Busch the disease is becoming chronic and clinical cases are sporadic. The clinical state of disease in Angel Sandoval can be also explained by the higher proportion of parasitological positive animals (12.38%) found in this province than that found in German Busch (2.35%). Influence of weather conditions between sampling times in each province in the prevalence and disease situation could be discarded, since both regions were sampled at the beginning (German Busch) or at the end (Angel Sandoval) of the rainy season and weather conditions and pastures were similar; furthermore, farmers reported the presence of high abundance of tabanids the whole year, which can be confirmed by a seasonal study of tabanid abundance performed in Guarayos province, which has similar weather conditions to the Pantanal, and found no difference in tabanid abundance between rainy and dry season [11]. An observation which must be carefully considered is the finding in Angel Sandoval of a high proportion of *T.vivax* positive animals (13/21) (section 3.1), which were treated with diminazene aceturate some weeks or days before were sampled. This observation could be related to drug resistance since some animals were treated for a second time 3 days before sampled and remained positive. New infection under field conditions with high parasitemia detectable by parasitological methods would not be expected after such a short time. Resistant *T.vivax* stocks to diminazene aceturate have been already reported in South America [12].

Cattle production system in Angel Sandoval is limited to calf and yearling production and herds of young animals together with old discarded breeders are moved by foot to other provinces within the department of Santa Cruz to be fattened. This movement increases chances of spreading and transmission of the parasite to neighbour farms where herds stop for resting during their journey and to other provinces within the department. This constant activity contributes to keep the parasite and the disease circulating in this region. It is common to observe outbreaks of *T.vivax* Trypanosomosis in fattening farms situated in the provinces close to the capital city (Santa Cruz de la Sierra) right after animals from Angel Sandoval are introduced into these farms. In German Busch, the situation is different, as most of the farmers in the region breed, raise, and fatten their animals on farm. Animal movements by foot are less practiced, therefore cattle are less stressed and even though they harbour *T.vivax*, disease remains in a chronic state.

The morphometric analysis showed differences in length between the *T.vivax* parasites from Angel Sandoval and those from German Busch. The mean length reported for *T.vivax* from Angel Sandoval is the smallest reported to date on the continent, but parasites as small as 11.34 μm have been measured in the Pantanal of the Brazilian state of Mato Grosso, which borders Angel Sandoval [13]. The mean length reported for *T.vivax* from German Busch is similar to that reported by [4]; who measured this protozoa with samples from the first outbreak of bovine Trypanosomosis in this province (1997), and slightly smaller than the parasites measured in the Brazilian neighbour state of Mato Grosso du Sul (18.1 ± 2.04) [14]. Some authors have related shorter forms of *T.vivax* with an acute presentation of disease, reporting them as more pathogenic than longer forms of the parasite. The possibility of different *T.vivax* strains between Angel Sandoval and German Busch cannot be excluded, and should be addressed with further investigation using molecular techniques.

The estimated prevalence of *T.vivax* in each province is the highest reported to date in the Bolivian lowlands. The estimated prevalence by PCR and/or parasitology in other affected provinces in the Bolivian lowlands was 5.98% for Guarayos, 4.84% for Yacuma and 2.42% for Sara [2]. Though the DNA extraction method and the PCR protocol applied to estimate the prevalence in those provinces were not satisfactory and the PCR performed with poor sensitivity as shown by [5], correction of this sensitivity problem and re-estimation of true prevalence would not increase the prevalence as high as the prevalence estimated for the Bolivian Pantanal. The situation in the border Brazilian states of Matto Grosso and Mato Grosso do Sul would likely be similar. Davila [15]; sampled 9 farms from Mato Grosso do Sul, which were selected based on previous findings of *T.vivax* on those farms, and reported the identification by PCR of 44.7% (n = 363) infected bovines. These authors did not find differences in the PCV values of PCR positive animals and PCR negatives, which is similar to the situation we found in the Pantanal of German Busch (border with Matto Grosso do Sul). The results of the present study together with the results reported by [15]; confirm a high prevalence of *T.vivax* Trypanosomosis in the Pantanal region (Brazil and Bolivia).

The prevalence of *T.evansi* in bovines in each province was much lower than that estimated for *T.vivax*. Similar observations were reported at the Brazilian Pantanal. Davila [15]; using PCR, estimated a lower prevalence of *T.evansi* in bovines (8.10%) than *T.vivax* (44.7%). This lower prevalence of *T.evansi* can be related to the low pathogenicity of this parasite for bovines, leaving to a low and undetected parasitemia, which reduces the risk of mechanical transmission of this parasite between bovines. An

observation in this study was that animals identified as positive for *T.evansi* in Angel Sandoval came from farms which raised horses and reported problems of equine Trypanosomosis. These reports were confirmed by identifying *T.evansi* by parasitological or PCR methods in 7 out of 58 horses (Gonzales, unpublished data) sampled while sampling cattle for this study. In German Busch, few to no horses were found at the selected farms for sampling and all the sampled horses (n = 8) were test-negative. Based on the findings in Angel Sandoval, it can be speculated that horses are the main reservoirs of *T.evansi* in the Pantanal and they are the main source of *T.evansi* for mechanical transmission. The above mentioned results of the evaluation performed in horses were not included in the results of this study because of the small sample size. Horses were sampled when they were available in the farms visited for sampling cattle.

In conclusion, a high prevalence of bovine Trypanosomosis was estimated in the Bolivian Pantanal and differences were observed in the disease situations between the provinces of Angel Sandoval and German Busch. The results of this study show that Trypanosomosis is a major constraint for cattle production especially in the region of Angel Sandoval. In this region, low PCV and BC as a result of Trypanosomosis infection have an effect on animal productivity (loss of weight, low pregnancy and calving rates) with corresponding economic impact. Morphological differences between *T.vivax* from both studied provinces, together with the different disease situations could be an indication of intra-species differences between the *T.vivax* circulating in Bolivia and potentially in South America. Further studies using molecular tools are required to answer this question. Studies designed to set up Trypanosomosis control programmes are required for this region, which will also involve assessment of Trypanosomes resistance to treatment with dinimazene aceturate.

ACKNOWLEDGEMENTS

We thank the Joint FAO/IAEA Division of nuclear Techniques in Food and Agriculture for financial support. We also thank L. Lauerman and S. Wells for revising this manuscript and his helpful comments.

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EXPERIMENTAL INFECTION OF BUFFALOES

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Abstract

This study examines experimental infection of buffaloes with a local Trypanosome and analysis of samples for parasites and consequences of infection by conventional as well as molecular and serological methods.

1. MATERIALS AND METHODS

A total of eight buffaloes were purchased from the farmers of ThaiBinh province upon negative microscopic examination and mice innoculation for Trypanosomiasis. Seven buffaloes were innoculated a dose of local strain of *Trypanosoma spp*., and one was kept as control. Bleedings were of one week interval for 6 w, thereafter the buffaloes were treated with Berenil, bled for post treatment at 1, 2, 3 and 7 d. Three types of blood samples were collected: (1) drops on Whatman paper; (2) coagulated blood for serum; and (3) heparin-anticoagulated blood for hematocrit, injection of mice and DNA preparation.

1.1. The appearance of parasite in the blood stream

To see whether or not experimental inoculation gave rise to Trypanosoma, 0.2 mL of uncoagulated blood was inoculated into the peritoneum of each mouse. Most of the inoculated mice died (Table II). The presence of Trypanosomes was confirmed by microsopic examination after 3 or 7 d in all inoculated mice, showing that all buffaloes were infected and the parasites appear in their blood stream as soon as a week after infection.

1.2. Detection of parasite using PCR-based methods

DNA preparations were carried out with 2 different methods: (a) using Qiagen Kit (200 µL final volume), and (b) following the methods of Peter-Henning Clausen (250 µL final volume). These DNA samples were stored at -30°C for further PCR analysis. Beside, the a third type of blood sample was colleted by dropping the blood on to Whatman Paper for test field capacity of the designed methods in future.

1.3. Detection and evolution of serum Abs of infected buffaloes using ELISA methods

A 96-well microtiter plate (Nunc) was coated with 2µg/mL Rotat 1.2-Ag (provided by Filip Buscher, Belgium) at 37°C for 1 h, wash three times using PBS-0,05% Tween20. Plates were blocked with 2% milk for 1 h at 37°C. Washing was made three times prior to application of the test sera. Sera were collected weekly (Table I) and stored at -30°C for multiple usage. Sera were diluted to 1/1000 in PBS, of which 100 µL were used in ELISA, incubated at 37°C for 30 min. Again, washing 3 times, HRP-conjugated anti-cow Ab diluted 1/8000 (DAKO-P0159) was added and incubated at 37°C for 30 min. Triple washes were done again. Development of color made using

peroxidase solutions A+B (1/1), in dark, at 37°C for 30 min. Reaction was stopped by adding H_2SO_4, 2 N, and read OD values with an ELISA reader 450 nm.

Results (Fig. 1) show that there is an increase in antibody titer in all infected buffaloes after 2 weeks of infection. The peak was reached at 4-5 w post infection. In contrast, the antibody titre of the control remained at the background level in the whole experimental period. Variation among buffaloes also occurred. The drop of antibody titer of buffalo N° 4 needs to be verified.

1.4. Evaluation of an Indirect ELISA kit for Trypanosoma antibody detection

Based on the shared antigens amongst *Trypanosoma spp.,* i.e. between the *T. congolense* and *T.vivax* and the *T.evansi*, the *T. congolense* and *T.vivax* precoated plates (provided as a kit by the IAEA) were tested for prospective application for diagnosis of Trypanosomiasis in Vietnam.

Before testing on the field serum samples, the pre-coated plates were used in an ELISA to test the immune response of the infected animals. The serum samples from the infected buffaloes were diluted 1/100 in PBS, 0.05% Tween 20, of which 100 µL were placed on wells of the precoated plate. The conjugate and color development was done as before. The *T.vivax* precoated plates gave only weak signals in all instances (results not shown).

The *T. congolense*-precoated plates gave a higher signal (Fig. 2); however, unlike the immune response to the local isolate strain (Fig. 1), there seems to be unresponsive to the *T. congolense* antigen used. Further tests on the sera of buffalos that are Trypanosoma-positive in a mice-inoculation test also gave inconsistent results. Therefore, the antigenicity of the *T.evansi* isolated in Vietnam may be not compatible to the *T. congolense*, *T.evansi* being used in a precoated ELISA plates.

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TABLE I. SCHEDULES SAMPLING BLOOD OF INFECTED BUFFALOES

Code	Description
D0	Before experimental infection
D1	1 w post infection
D2	2 w post infection
D3	3 w post infection
D4	4 w post infection
D5	5 w post infection
D6	6 w post infection
DT1	1 d after treatment
DT2	2 d after treatment
DT3	3 d after treatment
DT7	7 d after treatment

TABLE II. POST INFECTION OF MICE USING BLOOD SAMPLES OF *TRYPANOSOMA SPP.* INNOCULATED BUFFALOES

	Post infection (weeks)						
N°	0	1	2	3	4	5	6
3	-	3	3	7	7	3	7
4	-	3	3	7	7	3	7
5	-	3	3	3	7	3	7
7	-	3	3	7	7	3	7
8	-	3	3	7	7	3	7
9	-	3	3	3	7	3	7
10	-	3	7	7	7	3	7
Control-	-	-	-	-	-	-	-

0.2mL corresponding blood samples were inoculated each mouse. Numbers indicate the post infection date when Trypanosoma spp., was found.

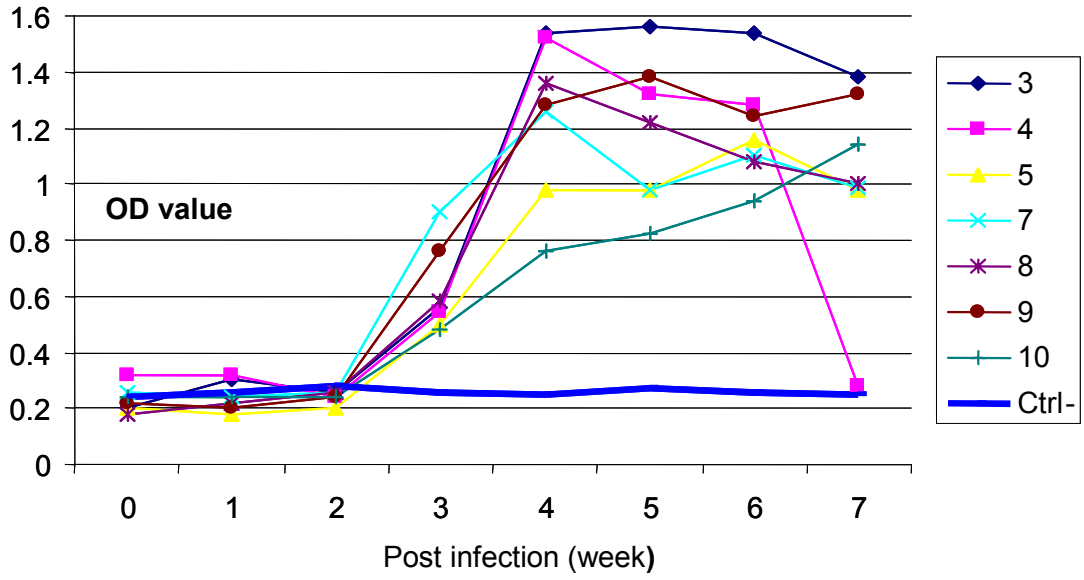

FIG. 1. Development of serum Antibodies of infected buffaloes.

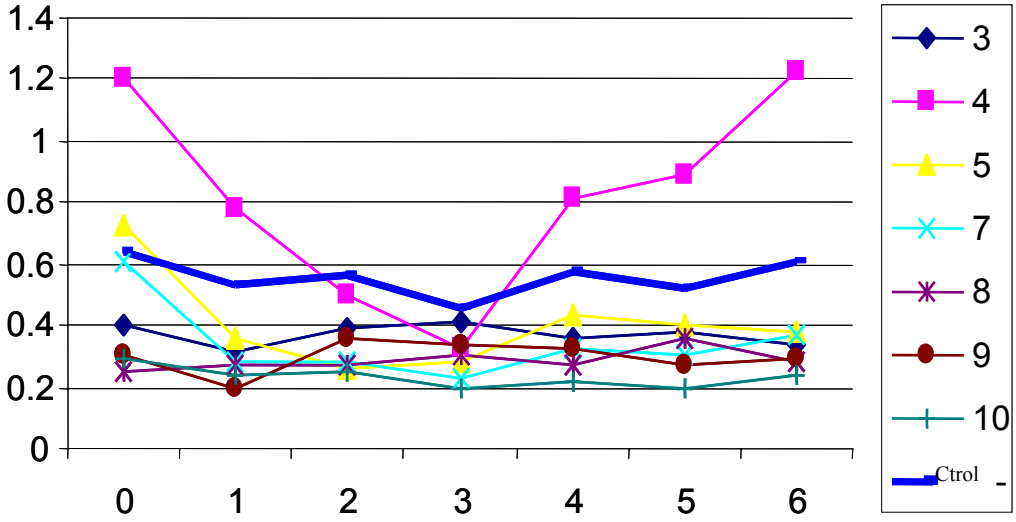

FIG. 2. Development of antibody in T.evansi-infected buffaloes to the T. congolense antigen.

DETECTION AND CLASSIFICATION OF TRYPANOSOMA CRUZI GENOTYPES IN ANIMALS OF AN ENDEMIC AREA OF CHILE

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Abstract

Blood samples from 200 sylvatic and peridomestic animals from an endemic area of Chile were subjected to PCR amplification of *Trypanosoma cruzi* minicircle sequences. This method enabled to detect parasite DNA in animals of the species. (*Thylamis elegans, Octodon degus, Phyllotis darwini,* and *Abrothrix olivaceuss*) as representatives of sylvatic animals, and Capra hircus as representative of the peridomestic one. Altogether, 51% of the sylvatic and 36% of the peridomestic animals were infected with *T.cruzi* Amplified DNA products obtained in this study were then studied by Southern analysis with a panel of four radioactive probes prepared from genotyped *T.cruzi* clones in the endemic areas of Chile and pertaining to *T.cruzi* lineages I and II. Most of the animal are infected at a rate of 35% with *T.cruzi I*, however other 85% are infected with *T.cruzi II*. This method is able to detect mixed infections with two or more different genotypes this figure raise to approximately 40% in this sample.

1. INTRODUCTION

Chagas disease, whose etiological agent is the protozoon parasite *Trypanosoma cruzi*, is endemic in South America. Parasitemia in chronic cases is low; hence its diagnosis is mainly performed by serologic assays. However, the validation of serological tests with parasitological diagnosis as PCR is necessary since sensitivity of PCR targeted to amplify kinetoplast DNA molecules as minicircles is sensitivity as conventional serology. Moreover, the application of PCR detection of *T.cruzi* kDNA and amplification of DNA segments are valuable as molecular markers to define parasite genotypes circulating in sylvatic, peridomestic or domestic habitats. Our protocol for kDNA amplification yields products derived from the minicircle variable region. These sequences have proven useful in strain typing of *T cruzi* by hybridization tests. The present work investigative the presence of *T. cruzi*, and the characterization of *T.cruzi* strains circulating in animals of an endemic chagasic area of Chile.

2. MATERIAL AND METHODS.

2.1. Blood samples

These where collected (0.5 to 1.0 mL.) from animals anesthetized with isofurane in the presence of heparin as anticoagulant, and boiled for 15 min before use.

2.2. DNA extraction and PCR amplification.

Half mL of blood was processed for DNA extraction with the E.Z.N.A. kit. The amplification reactions were performed essentially as previously described with primers 121 and 122. PCR products of 330 bp were analysed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Amplified DNAs were

then transferred to nylon membranes, denatured, cross-linked with UV irradiation, and hybridized using a total kDNA from *T.cruzi* as a universal probe, and labelled by the random priming method with [α^{32}P] dCTP [1]

2.3. Generation of *T.cruzi* specific probes

The four genotype–specific probes corresponding to the *T.cruzi* clones were sp104cl$_1$ (*T.cruzi* I), and CBB cl$_3$, v195cl$_1$, and NRcl$_3$ corresponding to *T.cruzi* IIb, IIc, and IId, respectively. They were prepared with parasite DNA by PCR and the oligonucleotides CV$_1$ and CV$_2$, which are directed from the constant regions of the minicircles as described [2]. Finally DNA products were digested with the restriction endonucleases Sau 96I and Sca I to remove part of the oligonucleotide primer. This procedure generated 250bp probes which are purified by electrophoresis on low melting point agarose.

2.4. Southern blot analysis

Agarose gel containing PCR products are transferred to nylon membranes as described in [1]; and hybridized overnight with the 250 bp genotype-specific radioactive DNA in a hybridisation solution of 10mL (buffer 2X SSE, 5X, Denhardt´s solution) containing 0.5 x 10^6 cpm of ^{32}P-labelled DNA probe/membrane (specific activity 50-80 x 10^6 cpm /ug DNA). Washing was carried out in 2 x SSE, 0.1% SDS at 55°C. The membranes where probed with radioactive DNA autoradiographed and reused with another labelled probe previous to removal and decay of the old probe.

3. RESULTS

3.1. Detection of *T.cruzi* infected animals

Results for sylvatic animals indicated that 43.3% were positives. Results from *Capra hircus* indicated that 26.2% were positives. Additional positives were detected by Southern analysis in samples that resulted PCR negative. In sylvatic animals 51% were positives with the radioactive probe, and 35.7% of *C. hircus* were positives. It is possible to observe based on the reported results that the percentage of positive cases increased when PCR and hybridization results are combined.

3.2. Classification of *Trypanosoma cruzi* genotypes

DNA amplified from infected animals was subjected to Southern analysis with the four specific probes. Results of positive cases with the *T.cruzi* I*, T.cruzi* IIb, IIc, and IId for *T. elegans* are 33,3% 16.7%, 50% and 16.7%, respectively. Equivalent results for *O. degus* with *T.cruzi* I, *T.cruzi* IIb, IIc, and IId are 47.8%, 43.5%, 21.7%, and 39%, respectively. Meantime results for *A. olivaceus* with *T.cruzi* I, *T.cruzi* IIb, IIc, and IId are 26.9%, 42.3%, 8%, and 15%, respectively. Finally results for the sylvatic animal *P. darwini* infected with *T.cruzi* I, *T.cruzi* IIb, IIc, and IId are 44%, 28%, 44%, and 40%, respectively. The only one peridomestic animal infected with *T.cruzi* is *C. hircus*, and results of reservoirs infected with *T.cruzi* I, *T.cruzi* IIb, IIc, and IId are 33.3%, 20%, 46.7%, and 13.3% respectively. It is important to point out that not all infected animals are characterized by this method with the panel of four probes, suggesting that other different *T.cruzi* genotypes are also circulating in these reservoirs. There are animals as *C.hircus* where non identified *T.cruzi* genotypes is close to 0%, meantime in others as *A. olivaceus* and *P. darwini* the non identified *T.cruzi* genotypes range 30-40%. Finally

is worth mentioning that percentages of detected genotypes are over 100%, a clear indication that several animals are infected with more than one *T.cruzi* genotype, that is, a mixture of parasites.

4. DISCUSSION

This work describes the PCR assay to detect *T.cruzi* in blood samples of sylvatic and peridomestic animals in a way to improve and confirm serological diagnosis. This method proved useful and more sensitive when a combined hybridization assay is performed with a universal probe. The difference between PCR and Southern analysis results might reflect the very low parasitemia in approximately 8-10% of the animals, which are detected positive by the hybridisation signal, but not by ethidium bromide staining. The other importance to target kinetoplast minicircle DNA by PCR is to determine the infective *T.cruzi* genotype on each mammalian reservoir with amplified DNA by a panel of well characterized *T.cruzi* genotype specific probes and hybridization tests. Results of *T.cruzi* genotypes circulating in sylvatic animals and *C. hircus* varied. Both *T.cruzi* I and *T.cruzi* II lineages are heterogeneous (IIb, IIc, IId), and frequently animals are infected with mixtures of genotypes which circulate and are transmitted in nature Interestingly sylvatic animals carry *T.cruzi* I, but also *T.cruzi* II genotypes which are characteristic of the domestic transmission cycle, a situation completely different as previously thought. This study is relevant to understand the molecular epidemiology of Chagas disease in the wild transmission cycle and to determine reservoirs of parasites with potential risk for humans.

ACKNOWLEDGMENTS

This work was supported by FONDECYT, Chile 104-0762 and IAEA-CHI-11767.

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EVALUATION ET VALIDATION DES AMORCES ITS POUR L'AMELIORATION DU DIAGNOSTIC PCR DES TRYPANOSOMOSES ANIMALES AFRICAINE

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Abstract

Nous avons évalué 2 couples d'amorces présélectionnés (TRYP-A et TRYP-B) amplifiant l'ITS1 de l'ADN ribosomal (ADNr) des Trypanosomes en vue de l'amélioration du diagnostic des Trypanosomoses animales africaines (TAA). Les essais ont été conduits à la fois sur ADN purifiés et sur les échantillons de terrain (sang frais de bovin et de souris, sang sur papier filtre, organes de glossines) traités au Chelex100. Après investigations, le couple TRYP-B a été retenu au regard de sa sensibilité vis à vis des échantillons de terrain. Nous rapportons ici, à notre connaissance le premier test PCR unique panTrypanosomique identifiant de façon univoque les principaux Trypanosomes responsables des TA par l'utilisation d'échantillons de terrain: *Trypanosoma congolense* (type savane, forêt et kilifi), *T. simiae, T. godfreyi, T.vivax, T.brucei (brucei brucei, brucei gambiense et brucei rhodesiense), T.evansi* et *T.equiperdum*. En plus de la réduction du coût de la PCR, la technique a été simplifiée, ne nécessitant pas une manipulation complémentaire comme la RFLP ou une nested-PCR. Ceci réduit par la même occasion le temps de traitement des échantillons analysés. D'autres investigations sont toutefois nécessaires pour une validation définitive du test à large échelle dans la perspective d'une utilisation en masse par les laboratoires de diagnostic des services de développement.

We evaluated 2 preselected primers (TRYP-A and TRYP-B) amplifying the first internal transcribed spacer of ribosomal DNA (rDNA) for improving the PCR diagnosis of African animal Trypanosomosis (AAT). Assays have been conducted both on purified DNA and fields samples treated with chelex-100. After investigations, TRYP-B primers have been selected for his sensitivity to fields samples. Our results report, to our knowledge, the first panTrypanosomic diagnosis test which is specific, sensitive and cheaper than those reported previously. The main Trypanosomes of African Trypanosomosis: *Trypanosoma congolense (savannah type, forest* and *kilifi), T. simiae, T. godfreyi, T.vivax, T.brucei (brucei brucei, brucei gambiense et brucei rhodesiense), T.evansi et T.equiperdum*, have been clearly detected. Moreover, our test doesn't need any supplementary analysis such as RFLP or nested-PCR. Thus, the time necessary to treat samples is highly reduced. However further investigations should be conducted to a definitive validation before transferring this diagnostic tool in veterinary laboratories and extension services.

Objectifs spécifiques

(1) Mettre au point et valider la PCR ITS pour le diagnostic et les études épidémiologiques avec des amorces présélectionnées : TRYP A et TRYP B.

(2) Réduire le coût de la technique PCR.

(3) Simplifier la technique par rapport à la nested-PCR, la PCR-RFLP et réduire le délai d'obtention des résultats.

Les résultats attendus sont:

(1) Un protocole sensible pour les espèces de Trypanosomes pathogènes et utilisable avec les échantillons de terrain.

(2) Une discrimination des couples d'amorces TRYP A et TRYP B.

(3) Un protocole à coût réduit et accessible aux laboratoires des services de développement.

1. MATERIELS

1.1. ADN de référence

En plus des ADN fournis par la cryothèque du CIRDES, de l'ADN a été extrait à partir des prélèvements périodiques effectués sur des bovins par les équipes de terrain du CIRDES.

1.2. Échantillons de terrain

Les Buffy coats (interface globules rouges/globules blancs) sont préparés à partir du sang prélevé sur des bovins naturellement infectés d'une part et sur des souris expérimentalement infectées d'autre part. Entrent également dans cette catégorie, les prélèvements effectués sur les vecteurs.

1.3. Les oligonucléotides amorces-TRYP-A et TRYP-B

Les couples d'amorces TRYP-A (ITS1A-F et ITS1B-R) TRYP-B (ITS1C-F et ITS1B-R) sont censés amplifier l'ITS1 de l'ADNr des Trypanosomes. L'amorce directe (F) se fixe sur le gène 18S alors que la reverse (R) se fixe sur le gène 5.8S. Les amorces ITS1C-F et ITS1B-R ont 20 oligonucléotides. Les T_m sont de 60°C pour ITS1C-F et 58°C pour ITS1B-R ; soit une T_m théorique de 59°C pour le couple (TRYP-B). L'amorce ITS1A-F est un 18-mer de T_m 60°C, soit une T_m de 59°C pour TRYP-A.

Les tailles des produits d'amplification des espèces détectées sont indiquées dans le Tableau I.

TABLEAU I. TAILLE DES PRODUITS D'AMPLIFICATION DES TRYPANOSOMES PAR TRYP-A ET TRYP-B

Espèces	Tailles des produits avec TRYP-A (pb)	Tailles des produits avec TRYP-B (pb)
T.vivax	600	230
T.evansi	810	550
T.equiperdum	810	550
T.brucei	810	550
T.congolense forêt	1070	710
T.congolense kilifi		650
T.congolense savane		700
T.simiae		400
T.theleiri	710 (faible)	Pas d'amplification

2. METHODES

2.1. Infection expérimentale des souris

Un lot de souris (NMRI) est infecté par voie intra péritonéale soit par une seule souche (infection simple) soit par des combinaisons doubles ou triples (infection mixte) de souches de Trypanosomes pathogènes (*T.vivax Zaria/81/Y486/699*, *T.brucei FAR/80/CRTA/1*, *T.congolense* savane SER71/CRTA219). L'infection mixte se justifie par le fait que l'on trouve dans la nature des animaux infectés par deux ou plusieurs Trypanosomes. 500µL de chacune des associations suivantes ont été inoculés aux souris: *T.congolense* savane*/T.vivax, T.congolense* savane*/T. brucei, T. brucei/T.vivax, T.congolense savane/T. brucei/T.vivax*. La parasitémie est quotidiennement surveillée et, lorsqu'elle atteint 10^9 Trypanosomes/mL le sang est recueilli pour la préparation des Buffy coat.

2.2. Collecte et traitement des Buffy coat [1]

Après le prélèvement, le sang est centrifugé à 3.000 t/minute pendant 10 min dans un microtube à hématocrite. Les Trypanosomes se trouvant à l'interface érythrocytes-leucocytes, sont récupérés dans un tube contenant de l'eau distillée.

2.3. Traitement au Chelex 100®

A 30 µL d'un mélange d'eau distillée et de Buffy coat sont ajoutés 30µL de Chelex-100® 5%. La solution est vigoureusement vortexée avant d'être portée successivement à 56°C et 95°C, respectivement pendant 60 et 30 min. La dernière étape du traitement consiste en une légère centrifugation.

2.4. Extraction et Evaluation de la pureté de l'ADN

Les ADN Trypanosomiens sont extraits suivant le protocole du kit d'extraction PROMEGA® décrit dans les lignes qui suivent. La pureté de l'ADN est évaluée par la mesure de l'absorbance à 260nm qui est la longueur d'onde maximale d'absorption des acides nucléiques et à 280nm, longueur d'onde d'absorption maximale des protéines. Ces dernières absorbant aussi à 260nm, le ratio $R=A_{260nm/}\ A_{280nm}$ permet d'évaluer la contamination par les protéines. R doit être compris entre 1.8 et 2 pour les ADN purs [2].

2.5. Filtration des Trypanosomes

Le sang total est passé à travers une colonne de DEAE-cellulose selon la méthode de [3]. Les globules rouges chargés négativement, seront retenus plus rapidement que les Trypanosomes. L'éluât (contenant les Trypanosomes) est recueilli dans des erlenmeyers.

2.6. Extraction de l'ADN (kit PROMEGA)

Les Trypanosomes obtenus sont lysés par addition de 300 µl à 900 µl de Cell lysis. Le mélange est incubé pendant 10 min à la température ambiante puis centrifugé pendant 20 s à 13000 g. Le culot de centrifugation est récupéré et vortexé avant de procéder à la lyse des noyaux par addition de 300 Ml de Nuclei lysis.

Après avoir mélangé à l'aide d'une micropipette, 100 µL de Protein precipitation y sont ajoutés. Le mélange est ensuite centrifugé à 13,000 g pendant 3 minutes. Le surnageant est transféré dans un tube de 1.5 mL contenant 300 µL d'isopropanol.

L'ensemble est délicatement mélangé jusqu'à l'obtention d'une masse homogène avant centrifugation pendant 1 min jusqu'à ce que l'ADN soit visible. L'isopropanol est 'lavé' avec 300 µL d'éthanol 70%. L'éthanol est aspiré et séché après une centrifugation à 13.000 g en 1 minute.

La dernière étape du traitement consiste à réhydrater l'ADN par la solution DNA Rehydratation et à l'incuber à 65°C pendant 1 h.

2.7. Contrôle de la qualité de l'ADN.

La qualité de l'ADN récolte est contrôle par migration de 8 a 10µL de l'ADN extrait sur un gel d'agarose 1.5%, afin d'attester de son intégrité. L'absence de traînée sur le gel indique que l'extraction n'a pas altéré l'ADN.

2.8. Quantification de l'ADN.

La concentration des ADN est évaluée par la relation : $[ADN]=DO_{260nm}\times d\times 50\mu g/mL$ [2] ; représente le facteur de dilution. Les mesures ont été effectuées à l'aide d'un spectrophomètre

2.9. Programmes d'amplification

Les caractéristiques des programmes testés sont données ci-dessous :

Programme 1
(1) Mélange réactionnel: 1.5 m*M* de $MgCl_2$, 200 µ*M* de dNTP, 1 µ*M* Primers, 0,5 unité Taq polymerase.
(2) Programme d'amplification : 94°C/2 min, 94°C/30 s, 50°C/45 s, 72°/30 s, 38 cycles, 4°C à l'infini.

Programme 2
(1) Mélange réactionnel: 35 m*M* de $MgCl_2$, 490 µ*M* dNTP, 0,2 µ*M* Primers, 0,75 unité Taq polymerase, 1.5 mg/mL BSA.
(2) Programme d'amplification : 95°C/5min, 94°C/1 min, 57°C/1 min, 72°C/1.2 min, 35 cycles, 72°C/10 min, 4°C à l'infini.

Programme 3
(1) Mélange réactionnel: 1.5 m*M* de $MgCl_2$, 200 µ*M* dNTP, 0,2 µ*M* Primers, 1 unité Taq polymerase, 1.5 mg/mL BSA.
(2) Programme d'amplification: 95°C/5 min, 94°C/1 min, 57°C/1 min, 72°C/1.2 min, 35 cycles, 72°C/10 min, 4°C à l'infini.

L'essentiel des essais a été conduit dans un thermocycleur PTC 100 M.J. Research™ (annexe 3d).

2.10. Conditions d'électrophorèse sur gel d'agarose.

2.10.1. Gel de migration

La préparation du gel de migration se fait suivant le protocole de [2]. Un gel d'agarose de 2% a été utilise: 2.5g d'agarose (SIGMA) sont suspendus dans 125 mL de Tris-borate EDTA (TBE) 1X (0.09 *M* Tris-base, 0.09 *M* acide borique, 2 m*M* EDTA, qsp eau distillée).

2.10.2. Electrophorèse

Après avoir été mélangés avec 2 µL de tampon de charge, 10 µL de chaque amplifia et le contrôle négatif sont délicatement déposés dans les puits puis la migration se fait à 120V pendant 90 min, le gel est photographié sous UV.

2.11. Evaluation des amorces TRYP-A et TRYP-B

La capacité des couples d'amorces TRYP A et TRYP-B à discriminer les différents Trypanosomes en une seule PCR est évaluée par l'amplification d'une collection d'ADN

2.11.1. Optimisation de la technique

L'optimisation de la méthode consiste en la variation (dans les deux sens) de certains paramètres comme (i) les concentrations des réactifs (dNTPs, Taq polymérase, $MgCl_2$ essentiellement) et (ii) la température de fusion des amorces, le nombre de cycles. Elle vise aussi à augmenter le seuil de détection des parasites.

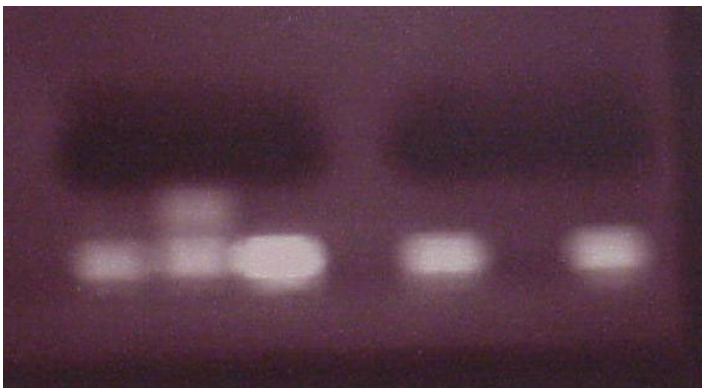
3. RESULTATS

3.1. Infection expérimentale des souris.

Le sang est récolté dans des tubes stériles contenant de l'héparine pour ensuite servir à la collecte des buffy-coat. Dans chaque cas (infection simple ou mixte) deux lots de souris ont été nécessaires pour avoir une bonne parasitémie (~10^9 Trypanosomes/mL). Ainsi, en microscopie photonique le nombre par champs de *T.congolense* savane prédomine pour l'association *T.congolense savane/T.vivax*; *T.brucei* et *T.vivax* ont poussé à des proportions quelques peu équivalentes. Quant à l'association *T.brucei*/*T.congolense* savane*,* le nombre de *T .brucei* par champs est de loin plus élevé que celui de *T.congolense* savane. L'association des trois espèces n'a pas donné de parasitémie suffisante (moins de 20 Trypanosomes/champs) au bout de 72 h de cultures. Les infections monovalentes se sont révélées plus longues, près de 12 jours après inoculation.

3.2. Extraction, purification et contrôle de la qualité de l'ADN

Après extraction, le rapport $R=A_{260nm}/A_{280nm}$ et la migration (120V pendant 30 minutes) sur gel d'agarose 1,5% ont permis respectivement d'évaluer la pureté et l'intégrité de l'ADN (Fig. 1). Dans certains cas, l'électrophorèse n'a montré aucun produit de migration (lignes 4 et 6).



1 2 3 4 5 6 7

FIG. 1. Contrôle de la qualité de l'ADN après extraction. Migration de quelques ADN extraits par le protocole du kit PROMEGA.

3.3. Quantification des ADN

Le Tableau II donne les DO_{260nm} des ADN étudiés ainsi que les concentrations qui en sont déduites. Le facteur de dilution est le même pour toutes les espèces: d =51 (20µl d'ADN dans 1 mL d'eau distillée).

TABLEAU II. DO260NM, CONCENTRATIONS DES ADN (µG/µL).

Trypanosomes	DO260nm	Concentrations (µg/µL)
TCS kigoni	0,340	0,867
TCF	0,212	0,540
TCK	0,450	1.147
TB	0,199	0,507
TE	0,233	0,594
TV	0,191	0,487
DAL972	0,120	0,306
058	0,231	0,589
TV*	0,016	0,043
TB*	0,142	0,364
TCS*	0,148	0,379

TCS: *T.congolense savane*; TCF : *T.congolense forêt*; TCK: *T.congolense kilifi*; TB : *T. brucei*; 058 : *T.b.rhodesiense*; DAL: *T. b. gambiense*; TV : *T.vivax* ; TE *: T.evansi*.
* : échantillons utilisés pour déterminer la limite de sensibilité sur ADN purs.

3.4. Test de spécificité

Les résultats obtenus suite aux tests avec le couple TRYP-A se sont révélés très peu satisfaisants aussi avions nous poursuivi les investigations avec le couple TRYP-B. De même les essais conduits avec les programmes 1 et 2 ont manqué de sensibilité pour les échantillons de terrain, c'est le Programme 3 qui a donc servi à l'analyse des échantillons de terrain.

L'électrophorèse en gel d'agarose (2%) des produits d'amplification de l'ITS1 de l'ADNr (Fig. 2) montre des bandes dont la taille est évaluée par un marqueur de

poids moléculaire (SL100): 550 pb pour l'ensemble du sous-groupe *Trypanozoon* (Lignes 4-8), 724 pb pour *T.congolense* forêt, 700 pb pour *T.congolense* savane, 650 pb pour *T.congolense* kilifi, 300 pb pour *T.godfreyi*, *T.simiae* 400 pb, *T.vivax* 233 pbLes tailles des amplifias mesurées s'accordent avec celles prévues (voir tableau 1) avec cependant quelques différences mineures : 233 pb contre 230 attendus (*T.vivax*) et 724 pb contre 710 pb attendus (*T.congolense* forêt). Ces légers écarts peuvent être attribués à la méthode utilisée pour évaluer la taille des fragments. Elle donne en effet une valeur approchée de la taille des amplifias. Un séquençage des bandes sera beaucoup plus informatif.

L'amplification spécifique des différents Trypanosomes par le couple d'amorces TRYP-B est une donnée intéressante en vue de son optimisation.

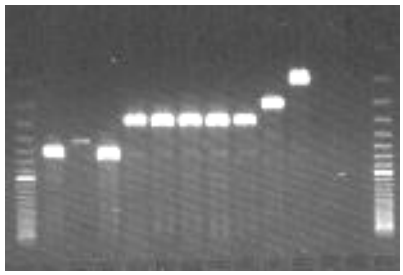


FIG. 2. Amplification de l'ITS1 des Trypanosomes avec TRYP-B et le Programme 3.

Ligne 2:*T.congolense* savane; ligne 3: *T.congolense* kilifi; ligne 4: *T.congolense* foret; ligne 5: *T.evansi*; ligne 6: *T.b.brucei*; ligne7: *T. .gambiense*; ligne 8: *T.b.rhodesiense* ; ligne 9: *T.equiperdum*; ligne 10: *T. simiae*; ligne 11: *T.vivax*; ligne 12: animal sain; ligne 13: contrôle négatif ; ligne 14: SL100.

3.5. Optimisation des paramètres efficaces : $MgCl_2$, Taq polymerase, dNTPs, amorces, Tm

Les essais conduits avec le Programme 1 n'ont pu détecter que très peu d'échantillons (Photo 3) (2 *T.vivax* et 1 *T.congolense* savane) et aucun produit n'a été détecté pour *T.b.brucei*.

100pb
200pb

1000pb

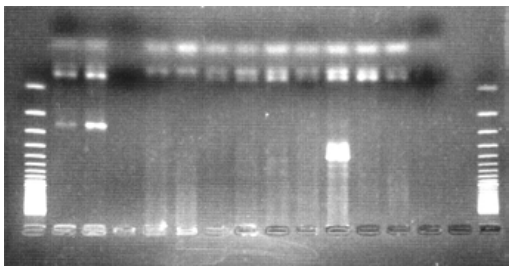


FIG. 3. Amplification des échantillons de terrain avec TRYP-B et le Programme 1.

2-6 (T.vivax); 7-11 (T.congolense savane); 12-14 (T. brucei); 15 : contrôle négatif.1 et 16: SL100.

Ce programme a donc été abandonné au profit des Programmes 2 et 3. Plusieurs essais ont été conduits en faisant varier les concentrations des réactifs ($MgCl_2$, amorces,

Taq polymerase et dNTPs) et la T_m. La concentration des ions Mg^{2+} est passée de 35 m*M* à 1.5 m*M* la réduction de moitié de la concentration des amorces (0.2 à 0.1 µ*M*) n'affecte pas l'amplification des ADN purifiés mais s'avère totalement inefficace en présence d'échantillon de terrain. La variation de la T_m (Programme 2) n'altère pas en elle-même l'amplification de l'ITS1 (Fig. 4). La présence de bandes d'amplification non spécifiques de même taille que les produits PCR du sous-groupe *Trypanozoon* (lignes 4-8) introduit un biais qui peut interférer avec l'analyse des résultats et les conclusions y découlant. La réduction de 0.25U de la concentration de la Taq polymerase a donné des résultats passables lors du passage sur les échantillons de terrain. Au terme des différents ajustements, le tampon Qiagen 10 X (10m*M* Tris; 1.5 m*M* $MgCl_2$; 50 m*M* KCl) a été préféré au tampon d'origine, la concentration des dNTPs (Eurogentec) a été ramenée à 200 m*M*, celle des amorces (Proligo) et du BSA (Sigma) ont été conservées 0.2 µ*M* et 1.5 mg/mL respectivement, et la Taq polymerase (Qiagen) a été portée à 1U.

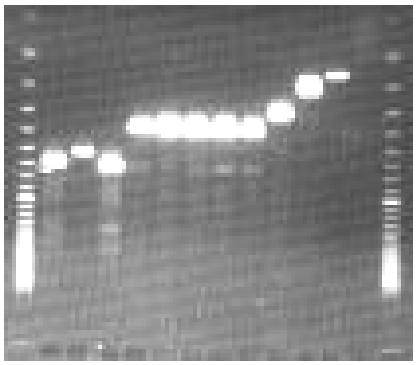


FIG. 4. Produits d'amplification avec TRYP-B et le Programme 2.
La disposition des échantillons est la même que sur la Fig. 2.

3.6. Evaluation sur échantillons de terrains: Programme 3-TRYP-B

La révélation sous U.V après séparation sur gel d'agarose 2% des produits PCR des BUFFY-COAT de terrain montrent des bandes d'amplification caractéristiques des échantillons testés (Fig. 5). L'intensité des signaux est comparable à celle obtenue avec les ADN purifiés. Deux échantillons de *T.brucei* présentent toutefois des bandes de faible intensité (lignes 15 et 16).

FIG. 5. Produits d'amplification des échantillons de terrain avec TRYP-B et le Programme 3.

1 et 19 (SL100); 2-7 (T.vivax); 8-12 (T.congolense savane); 14-17 (T.b.brucei); 18 contrôle négatif.

3.7. Evaluation sur organes de glossines

Fig. 6 montre les produits d'amplification des organes de glossines. Certains échantillons ont des bandes de très faible intensité (lignes 6, 8).

FIG. 6. Amplifias des organes de glossines avec TRYP-B et le Programme 3.

2-7:T.vivax, 8-13 T.congolense savane, 14 contrôle négatif, 1et 15 marqueur de poids moléculaire.

3.8. Seuil limite de détection-Dilutions d'ADN purifiés

Les ADN utilisés par ce test ont été préalablement dosés au spectrophotomètre tel que décrit dans le chapitre précédent. Les concentrations d'ADN de départ sont :

T.congolense savane Kigoni/ILRAD/776: 0.379 µg/µL
T.brucei FOL/02/CIRDES/01: 0.364 µg/µL
T.vivax FOL/03/CIRDES/02: 0.043 µg/µL

Six dilutions successives (1/10, 1/100, 1/1000/,1/10000, 1/100000, 1/1000000) sont réalisées. Après la troisième dilution (10^{-3}) il n'y a plus de signal pour *T.congolense* savane et *T.vivax* alors que la disparition du signal survient après la quatrième dilution (10^{-4}) pour *T.b.brucei* (Fig. 7).

Ramenée à la concentration, la limite inférieure de détection des espèces susmentionnées est :

T.congolense savane Kigoni/ILRAD/776 : 0.379 ng/µL
T.brucei FOL/02/CIRDES/01: 0.0364 ng/µL
T.vivax FOL/03/CIRDES/02: 0.043 ng/µL

FIG. 7. Produits PCR des dilutions d'ADN.

T.B (2-7), TCS (8-15), T.V (16-23), Contrôle négatif (24), 1et 25 marqueur de poids, SL100.

3.9. Dilutions au sang de Buffy coat

Un pool de 26 dilutions au sang des buffy coat a été réalisé (annexe 2). La limite inférieure de sensibilité du test est de 5 Trypanosomes/mL soit 0. 005 Trypanosome/µL de sang. Il est à signaler la décroissance simultanée du signal avec celle de la concentration (Figs 8 et 9).

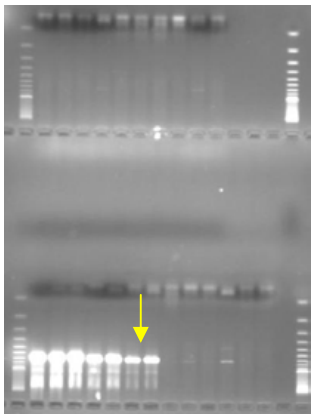


FIG. 8. Dilutions au sang de T.congolense.

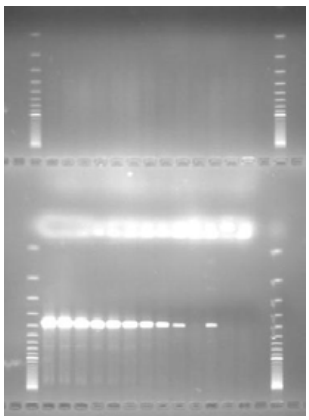


FIG. 9. Dilutions au sang de T.brucei.

Haut du gel : 16 à 26 : échantillons de sang selon les dilutions; 27 : contrôle négatif.
Bas du gel : 2 à 15 : échantillons de sang selon les dilutions ; 1 et 16 (marqueurs de poids SL100)
Voir tableau des dilutions en annexe 2.

3.10. Détection d'une infection mixte

La séparation sur gel d'agarose 2% des produits d'amplifications des infections mixtes (Fig. 10) montrent que notre technique est susceptible de détecter une infection mixte dans les conditions de terrain. En effet chacun des ADNs présents dans le mélange a été amplifié de façon spécifique. Dans le cas des échantillons de terrain les associations de *T.brucei* avec *T.vivax*, *T.congolense* savane d'une part et l'association des trois donnent une bande d'amplification épaisse mais dont la taille se situe autour de 550 pb. Cela peut être attribué à la densité de l'ADN. A signaler également que [4] ont fait la même observation.

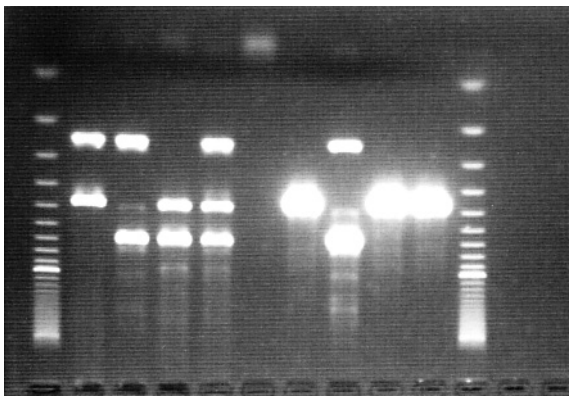


FIG. 10. Détection d'une infection mixte

Lignes 1 et 11 marqueur de poids; 2-5 mélanges échantillons de terrain : 2. (T.brucei/T.vivax), 3. (T.congolense savane/ T.vivax), 4. (T.brucei/T.congolense savane), 5. (T.congolense savane/ T.brucei/ T.vivax); 7-10, mélanges ADN purs: 7. (T.brucei/T.vivax), 8. (T.congolense savane/T.vivax), 9. (T.brucei/T.congolense savane), 10. (T.congolense savane/ T.brucei/ T.vivax); ligne 6. contrôle négatif.

4. DISCUSSION

L'objectif assigné à ce travail était d'évaluer la faisabilité d'un diagnostic PCR unique des TAA qui soit à la fois spécifique et utilisable en condition de terrain. A-t-il été atteint ?

A ce stade des investigations le choix s'est porté sur le couple TRYP-B, l'autre couple n'ayant pas donné satisfaction avec les échantillons de terrain. L'optimisation des paramètres critiques nous a conduit à retenir le Programme 3. Tous les ADN de référence ont été détectés de façon spécifique et univoque (Fig. 2). Les tailles des bandes caractéristiques des différents Trypanosomes sont en accord avec celles attendues. Ici et comme pour les études précédentes [5]; [6] ; les amplifias des membres du sous-genre *Trypanozoon* ont la même taille (550 pb). Cette observation avait justifié le recours à une étape supplémentaire de restriction enzymatique RFLP [7]; [8]; pour élucider le polymorphisme de l'ITS1 dans ce sous-groupe. Les profils de migration ont en effet permis de séparer les différentes espèces.

Contrairement aux *Trypanozoon*, les *Nanomonnas* se révèlent beaucoup plus hétérogènes : 724 pb (*T.congolense* forêt), 650 pb (*T.congolense* kilifi), 700 pb (*T.congolense* savane) et 400 pb (*T.simiae*). Nous estimons par ailleurs ici et ce, pour la première fois, la taille des produits PCR de *T.godgreyi* par le couple d'amorces TRYP-B: environ 300 pb.

T.vivax, seul représentant du groupe *Dutonnella* a été détecté de manière spécifique autour de 233 pb et avec un signal clair. Cela constitue une avancée par rapport aux résultats de [9] où le signal de ce Trypanosome majeur était extrêmement faible.

A l'évidence, notre technique est spécifique aux niveaux des espèces et des sous-espèces : dans l'espèce *T.congolense,* les bandes d'amplification des différentes sous-espèces se distinguent clairement les unes des autres.

Nous inférons de ces premiers résultats que c'est le polymorphisme de longueur de l'ITS et non de séquences qui permet de discriminer les organismes entre eux. Renforce cela, le groupe *T.congolense* où une dizaine de paires de bases suffit à distinguer *T.congolense 'forêt* (724 pb) de *T.congolense* 'savane' (700 pb).

La question de la spécificité étant réglée, il reste à explorer le volet de la sensibilité de notre technique. La sensibilité est entendue ici comme la détection des échantillons de terrain.

Pour les études épidémiologiques l'ADN des échantillons de terrain ne subit pas toutes les étapes d'extraction et de purification par les méthodes couramment utilisées. Dans notre laboratoire les échantillons sont principalement traités au Chelex-100.

Les résultats des tests aussi bien chez l'hôte (Fig. 5) que chez le vecteur (Fig.6) montrent que notre technique est sensible dans les conditions de terrain. A notre connaissance, c'est la première fois qu'est rapportée une telle résolution pour ce genre de tests. A signaler également que la récolte des échantillons ne semble pas affecter la technique d'autant plus qu'aussi bien les échantillons récoltés sur papier filtre que ceux récoltés dans de l'eau distillée ont été amplifiés avec succès.

Dans le même registre, la limite inférieure de détection (Figs 8 et 9) est de 5 Trypanosomes/mL soit 0,005 organismes/µL pour *T.congolense* savane et *T.brucei*; ce seuil est 4 fois plus bas que celui rapporté par la revue de [4]. Deux observations découlent de cela (i) le test pourrait être utilisé pour débusquer les infections occultes (phase chronique) au cours desquelles la parasitémie est très faible; (ii) au delà de ce seuil, aucune infection n'est détectée malgré la présence des Trypanosomes dans l'analyte. En outre, malgré la présence d'inhibiteurs apportés par le sang dans lequel sont dilués les buffy coat, la technique parvient à révéler la présence des Trypanosomes.

En l'absence d'une infection mixte naturelle, nous avons réalisé des mélanges d'ADN purifiés d'une part et d'échantillons de terrain d'autre part. L'électrophorèse en gel d'Agarose (2%) sépare de façon spécifique les ADN contenus dans ces différents mélanges aussi bien pour les ADN purs que pour les échantillons de terrain (Fig. 10). Même si nous pouvons nous satisfaire de ces résultats, nous n'en pouvons tirer une conclusion définitive car, seule une infection mixte en conditions naturelles peut trancher la question. Ce résultat est cependant évocateur de ce que la méthode peut potentiellement détecter les infections mixtes d'une part , et d'autre part servir au suivi d'un traitement Trypanocide ainsi que cela a déjà été fait au CIRDES par [10] avec la PCR monospécifique.

L'une des entraves à l'utilisation à large échelle de la PCR est son coût onéreux en comparaison avec les autres méthodes diagnostic. D'après nos évaluations (Tableau III) l'analyse d'un échantillon d'hôte revient à 770 f CFA en PCR-ITS contre 1900,36 f CFA en PCR monospécifique. Autrement dit, le prix d'une réaction en PCR monospécifique permet d'analyser environ 2.5 fois plus d'échantillons en PCR-ITS. De plus, chez le vecteur le nombre de réactions d'amplification passe de 15 à 3, réalisant ainsi une économie de 3390,441 f CFA.

Ces résultats donnent un aperçu de la puissance de la technique. En effet en dehors des espèces prises comme références (*T.congolense* savane, *T.vivax*, *T.brucei*), la PCR ITS peut également détecter *T.equiperdum*, *T.evansi*, *T.godfreyi*, *T.simiae* et les sous-espèces de *T.brucei* en une seule réaction

Au delà de la réduction du nombre de PCR par échantillons et donc du coût de la technique, notre test se présente comme un outil fiable orientant vers une utilisation rationnelle des Trypanocides, réduisant du coup le phénomène de chimiorésistance engendré par l'usage incontrôlé et disproportionné de ces médicaments [11]. De plus, le temps consacré au traitement d'un échantillon est considérablement réduit. Le traitement d'un échantillon en PCR monospécifique nécessite en effet dans le meilleur des cas 48 h sinon 72 heures, alors que dans le même temps 6 heures suffisent pour être fixé sur le statut d'un échantillon.

Les résultats rassemblés dans ce travail aussi encourageants qu'ils soient, ne sauraient occulter les problèmes que pose la sensibilité de la technique. La recherche des seuils de détection des ADNs et des échantillons de terrain instruit de ce que la méthode utilisée est limitée par la quantité d'ADN dans l'analyte. En effet de façon classique l'ADN devant être analysé en PCR subit toutes les étapes d'extraction, de purification et de quantification, mais dans le souci de simplification et pour se rapprocher le plus possible des conditions de terrain, les étapes de purification et de quantification sont volontairement éliminées. Grand soin doit, par conséquent, être porté à la préparation des échantillons et du mélange réactionnel. Pour ce qui est précisément du mélange réactionnel, nous avons noté que la concentration de la Taq polymerase est un paramètre central pour le succès du test. Il est à signaler également que l'amplification de façon spécifique des ADN n'est possible que si le programme d'amplification tient compte des propriétés thermodynamiques des amorces [12]. Des arbitrages sensibles de la concentration des réactifs, des températures et des temps d'hybridation et de polymérisation doivent donc être opérés en amont de la mise au point d'un test PCR [13].

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ANNEXE 1A: ECHANTILLONS UTILISES PENDANT LES ESSAIS

Annexe 1a : ADN de référence utilisés

Espèces de Trypanosomes	Codification de la souche
T.vivax	FOL/03/CIRDES/02
	LAHI/02/CIRDES/01
	GUYANE*
	FOL/02/CIRDES/02
	FOL/02/CIRDES/01
T.congolense 'savane'	SAT/87/CRTA/238.1 B.752
	KIGONI/ILRAD/776
T.congolense kilifi	K60 1A/KENYA
T.congolense forêt	KOMOE/87/CRTA/153
T.b.brucei	FARAKOBA/81/CRTA/4
	FOL/02/CIRDES/01
T.brucei gambiense	DAL 972
T.brucei rhodesiense	058
T.simiae	KEN 2
T.godfreyi	KEN 7
T.equiperdum	BoTAT

ANNEXE 1B: ECHANTILLONS DE TERRAIN PRELEVES CHEZ L'HOTE MAMMIFERE

Espèces de Trypanosomes	N° de l'échantillon	Date
T.vivax	4932	15/07/03
	4935	15/07/03
	4937	15/07/03
	3604	24/01/01
	3438	5/12/00
T.congolense 'savane'	DI20	28/05/02
	PI 13	31/0502
	3448	31/10/00
	W14	17/02/02
	W5	17/07/02
T.brucei brucei	3440	24//07/01
	5260	25/10/01
	5252	25/10/01
	5260	14/02/02

ANNEXE 1C : ECHANTILLONS DE TERRAIN PRELEVES SUR LES GLOSSINES.

Espèces de Trypanosomes	N°	Date de collecte
T.vivax	FB1 5P	16/10/02
	FB2 1P	16/10/02
	FB2 3P	16/10/02
	FB5 1P	16/10/02
	FT 1P	16/10/02
	FT 4P	16/10/02
T.congolense savane	DI 18 1204P	11/04/02
	A25 084P	08/05/02
	2IM	12/06/02
	IM6	12/02/02
	IM GPG	12/02/02
	IM GPG	13/02/02

ANNEXE 2: DILUTIONS AU SANG DES BUFFY COAT

Concentration finale (Trypanosomes/mL)	Numéro de la dilution 5 FOLD RANGE
25,000,000	1 UNDILUTED
5,000.0000	2 1/5
1,000.000	3
200,000	4
40,000	5
8,000	6
1,600	7
400	8
100	9
25	10
5	11
2.5	12
0,5	13
0.1	14
0.02	15
0.004	16
0.0008	17
0.00016	18
0.00032	19
0.0000064	20
0.00000128	21
0.000000256	22
$5.12.10^{-8}$	23
$1.024.10^{-8}$	24
$2.04.10^{-9}$	25
$4.09.10^{-10}$	26

DETECTION DE *TRYPANOSOMA CONGOLENSE TYPE SAVANE* PAR LA PCR-ELISA DANS DES ECHANTILLONS DE SANG DE BOVIN

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Abstract

La PCR-ELISA a été mise au point pour détecter des souches de Trypanosoma congolense type savane (TCS) dans des échantillons de buffy coats bovins de terrain. Les résultats obtenus par la PCR-ELISA et par PCR classique sont comparés et la sensibilité de ces deux techniques est comparée à celle de la méthode de Murray [1]; pour la détection TCS dans 257 échantillons. Les produits PCR sont marqués à la DIG-DUTP pendant l'amplification de la séquence de l'ADN satellite. Une sonde d'ADN spécifique marquée à la biotine a permis la détection des amplifias par ELISA dans des microplaques sensibilisées à la streptavidine. Il ressort que la PCR-ELISA et la PCR classique sont plus sensibles que la méthode de Murray. En effet, pour les 257 échantillons analysés par les trois techniques, la PCR-ELISA et la PCR ont détecté TCS respectivement dans 98 et 97 échantillons, tandis que la méthode de Murray n'a révélé que 39 cas positifs à TCS. De même, la sensibilité et la spécificité de la PCR-ELISA et de la PCR classique sont comparables. Ainsi, pour l'ensemble des 334 échantillons analysés au cours de cette étude, la PCR-ELISA et PCR classique ont détecté TCS respectivement dans 38.62% et 39.22% des échantillons. Aux termes de l'étude, le coût d'analyse d'un échantillon de buffy coat bovin par PCR-ELISA a été évalué à 1993 FCFA, soit €3.04.

Abstract

PCR-ELISA was set up to detect strain of Trypanosoma congolense type savannah in field samples of buffy coats. Results of PCR-ELISA and PCR were compared and the sensibility and specificity of both techniques were also compared with those of the method of Murray [1] for the detection of TCS in 257 samples. The PCR products were labelling with DIG-dUTP during amplification cycles of the repetitive satellite DNA. A DNA biotinyled capture probe was used to detect the amplicon by ELISA in streptavidine coated microplates. Both of PCR-ELISA and PCR were more sensible and more specific than the method of Murray. Indeed, for the 257 samples analysed by the three techniques, PCR-ELISA and PCR have detected TCS in 98 and 97 samples respectively, whereas the method of Murray has detected TCS in only 39 samples. In addition, PCR-ELISA and PCR had almost the same sensibility and specificity. So, PCR-ELISA and PCR have respectively detected TCS in 38.62% and 39.22% of all the 334 samples analysed by both techniques during this study. At the end of this study, the cost of analyse by PCR-ELISA of a sample of buffy coat, was evaluated at 1993 FCFA or €3,04.

Objectif spécifique

La PCR-ELISA, basée à la fois sur les méthodes de la PCR et de l'ELISA, a été récemment utilisée pour la détection des Trypanosomes chez l'hôte mammifère et chez les insectes vecteurs [2]; [3]. Pour notre part, la PCR-ELISA est utilisée pour détecter *T.congolense* type savane dans des buffy coats bovins de terrain. Les résultats obtenus par la PCR, la PCR-ELISA et la méthode de Murray, sont comparés et discutés afin d'évaluer l'avantage comparatif de la PCR-ELISA.

1. MATERIEL ET METHODES

1.1. Sites géographiques et échantillons

Les 334 échantillons de buffy coats bovins sont repartissent en deux lots distincts. Le premier lot composé de 257 échantillons, est collecté dans la région de Ouangolodougou, sud-ouest du Burkina. Le second lot totalise 77 échantillons issus d'un stock de prélèvements de buffy coats du nord Ghana, récolté sur du papier filtre. Les échantillons ont été traités au Chelex 100® pour l'extraction d'ADN. Le sud ouest Burkina et le nord Ghana sont deux zones où la prévalence de la Trypanosomose est assez élevée à cause de la pression glossinienne élevée.

1.2. Les souches de Trypanosomes témoins

L'ADN pur utilisé dans le cadre de cette étude, est constitué par 6 souches de *T.congolense* type savane stockés au CIRDES (cf. tableau I).

TABLEAU I. LES DIFFERENTES SOUCHES DE *T.CONGOLENSE* TYPE SAVANE

Stock d'origine	**Code**	**Hôte d'origine**	**Pays / année d'isolement**
Samandéni/82/CRTA/32.1	Sam.32.1	Bovin	Burkina(1982)
Karankasso/83/CRTA/66	Kar.83	Bovin	Burkina (1983)
Satiri/86/CRTA/91	Sat.91.2	Bovin	Burkina(1986)
IL 3000	IL 3000	Bovin	Kenya (1983)
Kigoni/ ILRAD/776	ILRAD/776	Bovin	Kenya (1976)
Serengeti/71/STIB/212	IL 3575	Lion	Tanzanie (1971)

1.3. Les oligonucléotides

Les amorces TCS1 (5'-CGA-GCG-AGA-ACG-GGC-AC-3') et TCS2 (5'-GGG-ACA-AAC-AAA-TCC-CGC-3') permettent d'amplifier la séquence l'ADN satellite de *T.congolense* type Savane [2]. Une sonde oligonucléotidique, *''olicongo''*, de 25 nucléotides, marquée à biotine (*Proligo Primers and Probes*) a été dessinée entre les 228ème et 257ème bases dans la séquence de l'ADN satellite: *olicongo*: (Biotin-5'-CGG-GCC-TAT-TTG-ACC-GGC-ATA-GTG-A-3').

Séquence de l'ADN satellite de TCS [2]

1 GTTCCAAAAA TGGTTGTGCG GGATTTGTTT GTCCCTAATT TTCATCGAAA ACGCCGAAAT GCGTTTTAAA

71 AATGATCAAA TTTCGAGCGA GAACGGGCAC TTTGCGATTT TCCCAAAATT CACCTTTTTG GGCCCAAATG

141 GGCAAAAACC GGTTTTTTTG AAAATGGTCA AAAATGTCAA AAACGCAAAA ATTCGAAAAA CGCGTATTTG

211 GCACGTATTT GTCGTTTTCG GGCCTATTTG ACCGGCATAG TGATTTTTCA AAATTTTGCA AAAAATTGTG

281 TCAAAAACTT TTTCTAATTT TTGCAAATTT TCAAAAAAAA ATTTGTAAAA AAATATTTTT TTTTGACTTT

351 TTGGGCGAAA ATTTTTTCT

1.4. Amplification PCR et marquage à la digoxigénine

Les réactifs utilisés pour l'amplification sont contenus dans le kit PCR-ELISA DIG-Labeling (*Roche Diagnostics*). Le mélange réactionnel pour chaque échantillon contient : 2 µL de tampon à 10 X; 2 µL de DIG-Labeling-Mix (2 m*M* (dATP, dCTP et dGTP) ; 1,9 m*M* (dTTP) et 1 m*M* (*DIG-dUTP*)] ; 0,25 µL de TCS1 et TCS2 à 20 µ*M* chacune; 0,1µL de Taq à 5U/µL, 13,4 µL d'eau stérile et 2 µL de l'échantillon à tester. L'amplification se fait dans un thermocycleur (*MJ Research Inc*) selon le programme suivant : d'abord, une dénaturation initiale à 95°C pendant 5mn ; ensuite 30 cycles dont chaque cycle comprend une dénaturation à 95°C pour 45 s, une hybridation à 60°C pour 1 min et une élongation à 72°C pour 2 min; enfin une élongation finale à 72°C pendant 11 min.

1.5. Détection des produits PCR sur gel d'agarose

Un mélange de 9 µL de produits PCR et 1µL du loading buffer sont déposés sur un gel d'agarose 1.5% (14cm X 14cm) contenant du bromure d'éthidium (BET). La migration se fait à 120V pendant 1h dans une cuve à électrophorèse (*Horizontal MGU*) contenant du Tris Borate EDTA (TBE). Un marqueur de poids d'ADN est utilisé pour déterminer la taille des produits obtenus. La lecture du gel se fait dans une chambre noire munie d'une caméra aux rayons ultra violets (*Vilber Lourmat TCP*).

1.6. Détection des produits PCR par ELISA

Les produits PCR sont détecter avec le kit PCR-ELISA DIG-Detection, 5 pack (*Roche Diagnostics*) et des microplaques sensibilisées à la streptavidine StreptaWell® (*Roche Diagnostics*). Les analyses sont effectuées en double exemplaire. Pour chaque échantillon, 5 µL des produits PCR et 20 µL de solution de NaOH à 1 *N* sont mélangés dans un tube stérile de 250 µL. Le mélange est gardé à température ambiante pendant 10 min puis sont ajoutés 225 µL de la solution d'hybridation contenant la sonde *olicongo* à la concentration de 7.5 pmol/mL. Dans chaque puits de microplaque, sont pipetés 100 µL du mélange, puis les microplaques sont incubées sous agitation à 37°C pendant 90 min. Après, les puits sont vidés par aspiration, puis lavés 4 fois avec la solution de lavage, PBS-Tween (10 m*M* Na_2HPO_4, 10 m*M* NaH_2PO_4, 0.1 m*M* Na_2EDTA, pH 6.8 ; 0.05%, Tween-20) (*Roche Diagnostics*). Ensuite, 100 µL de la solution d'anticorps anti digoxigénine conjugué à la peroxydase, dilués au centième, sont introduits par puits, et les microplaques sont incubées sous agitation à 37°C pendant 30 min. Par la suite, les microplaques sont lavée à nouveau 4 fois. Enfin, 100 µL du substrat (ABTS®) sont pipetés dans chaque puits, et les microplaques sont incubées une dernière, à 37°C pendant 10 min à l'abri de la lumière. Les cas positifs se colorent en vert. Mais l'absorbance des échantillons testés est mesurée à 405nm au spectrophotomètre (*Labsystem Multiskan MCC/340*).

2. RESULTATS

2.1. Mise au point de la PCR-ELISA

Pour la mise au point de la PCR-ELISA, 6 souches de *T.congolense* type savane et des échantillons de terrain dont le statut est connu, ont été testés. La PCR-ELISA a détecté avec succès, toutes les 6 souches de *TCS* (Fig. 1).

FIG. 1. Détection des ADN purs des 6 souches de Trypanosoma congolense type savane.
A=Blanc, B=négatif, C=ILRAD/776, D=Sat.91.2, E=IL3575, F=IL3000, G=SAM.32.1, H= Kar.83.

De même, pour les échantillons de terrain, les résultats de la PCR-ELISA et ceux de la PCR classique concordent (Figs 2 et 3).

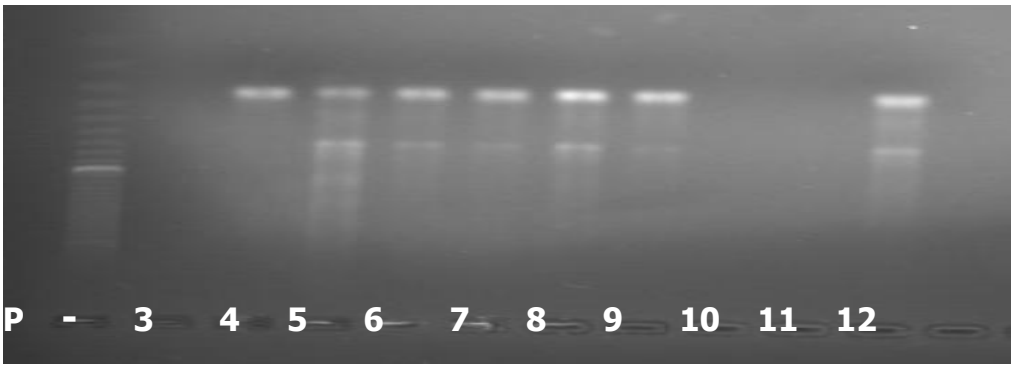


FIG. 2. Détection d'amplicon marqué à la digoxigénine par migration sur gel.
MP= marqueur de poids, -=négatif, 3=positif; 4; 5; 6; 7; 8 et 11 sont positifs, tandis que 9; 10 et 12 sont négatifs.

FIG. 3. Détection par ELISA d'amplicon marqué à la digoxigénine.
A1=Blanc, A2=négatif, A3=positif; A4; A5; A6; A7; A8 et A11 sont positifs, tandis que A9; A10 et A12 sont négatifs : les résultats de la PCR et de la PCR-ELISA concordent parfaitement.

2.2. Résultat de la PCR-ELISA

La PCR-ELISA a détecté *T.congolense* type savane dans 33 échantillons sur les 77 du nord Ghana et dans 98 échantillons sur les 257 de Ouangolodougou. Pour les résultats montrés à la Fig. 4, les cas positifs peuvent être mis en évidence par observation directe de la plaque à l'œil nu.

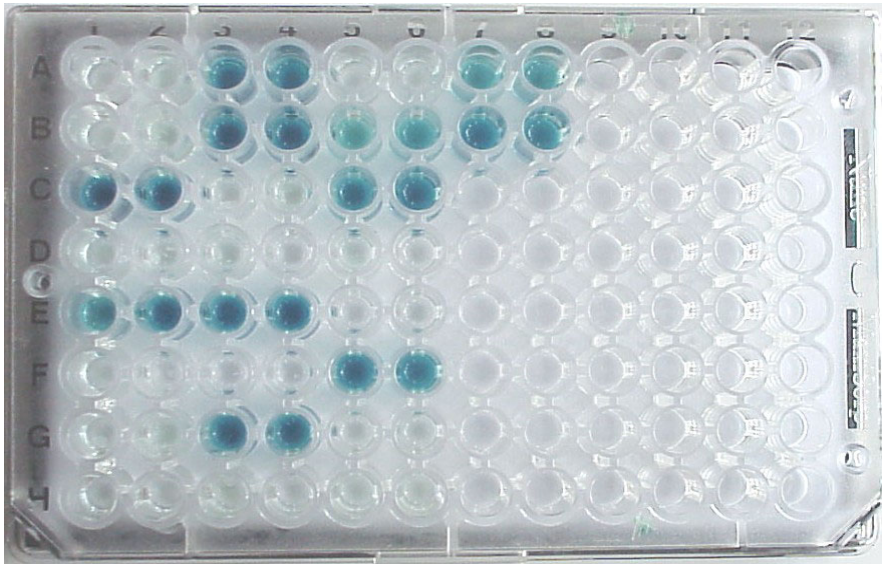


FIG. 4. Photo d'une microplaque de PCR-ELISA : les cas positifs sont représentés par les puits colorés en vert.

Mais la lecture des microplaques au spectrophotomètre, permet de détecter les cas positifs en fonction des densités optiques (Tableau II).

TABLEAU II. DENSITES OPTIQUES DES ECHANTILLONS ANALYSES PAR PCR-ELISA

	1	2	3	4	5	6	7	8
	0,000	0,007	2,099	2,123	0,019	0,020	0,669	0,658
A	Blanc		6		14		22	
	0,003	0,005	2,129	2,053	0,323	0,330	1,834	1,864
B	Négatif		7		15		23	
	1,972	2,071	0,004	0,010	0,912	0,898		
C	Positif		8		16			
	0,019	0,011	0,002	0,005	0,000	0,019		
D	1		9		17			
	0,536	0,663	2,063	2,110	0,008	0,007		
E	2		10		18			
	0,027	0,002	0,006	0,012	2,270	2,135		
F	3		11		19			
	0,026	0,009	2,180	1,994	0,026	0,005		
G	4		12		20			
	0,026	0,009	2,180	1,994	0,026	0,005		
H	5		13		21			

Les numéros en gras sont les échantillons positifs

2.3. Résultats de la PCR classique

Pour les échantillons du nord Ghana, la PCR classique a révélé 32 cas positifs sur les 77 échantillons et 97 cas positifs sur les 257 échantillons de Ouangolodougou. Les cas positifs apparaissent sur la photographie du gel à 320 pb (Fig. 5).

FIG. 5. Révélation sur gel d'agarose de produits PCR marqué à la DIG-dUTP
Les échantillons 12, 16, 19 et le 21 sont positifs, tandis que les autres sont négatifs

4.2.4. Résultats de parasitologie

Sur les 257 buffy coats bovins de Ouangolodougou, La méthode de Murray [1]; a détecté *T.congolense* dans 39, soit une prévalence de 15.17%. Les échantillons du nord Ghana n'ont subi aucune analyse de parasitologie. Tous les résultats de l'étude sont regroupés dans tableau III.

TABLEAU III. RECAPITULATIF DE LA PREVALENCE OBTENUE PAR LES DIFFERENTES TECHNIQUES

Lieu	Effectif	PCR positif		PCR-ELISA positif		Parasitologie positive	
Ouangolodougou	257	97	37.74%	98	38.13%	39	15.17%
Nord Ghana	77	32	41.56%	33	42.86%	-	-
Total	334	129	38.62%	131	39.22%	-	-

3. DISCUSSION

Grâce à l'utilisation d'amorces spécifiques pour l'amplification et d'une sonde d'ADN pour la détection, la PCR-ELISA est une technique de diagnostic d'une grande sensibilité et d'une haute spécificité. En effet, tous les échantillons positifs en parasitologie (méthode de Murray) ont été révélés positifs en PCR classique et en PCR-ELISA. De même, tous les échantillons positifs en PCR classique l'ont été en PCR-ELISA. Les résultats de cette étude montrent que les techniques de PCR classique et de PCR-ELISA sont tous les deux plus sensibles que la méthode de [1] que la PCR-ELISA et la PCR classique ont une sensibilité et une spécificité comparables. Des études antérieures utilisant la PCR classique et la PCR-ELISA pour la détection des Trypanosomes, ont donné des résultats similaires [2]; [3].

La PCR-ELISA permet de lever le doute quant à l'interprétation des résultats faiblement positifs par la technique de migration sur gel d'agarose ; comme en témoignent les Figs 6 et 7.

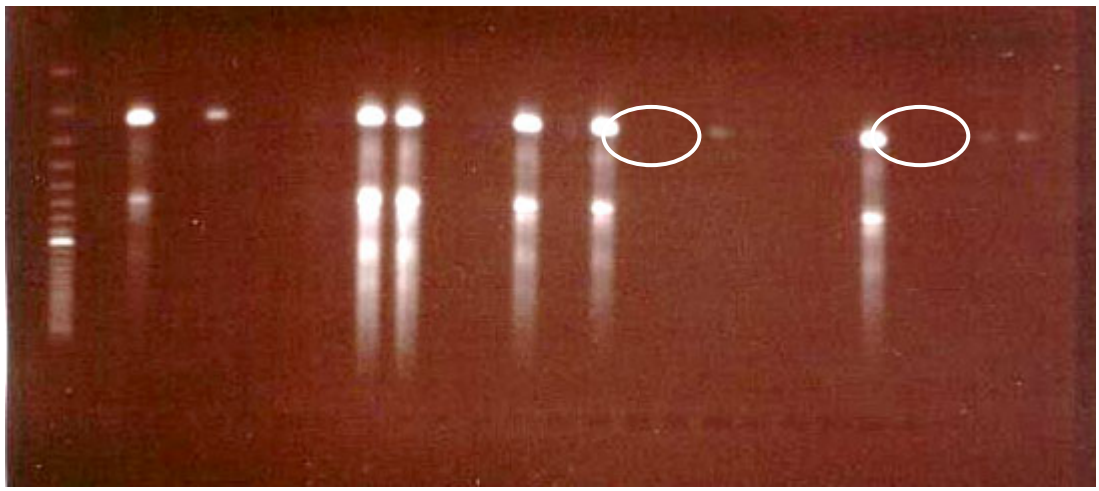
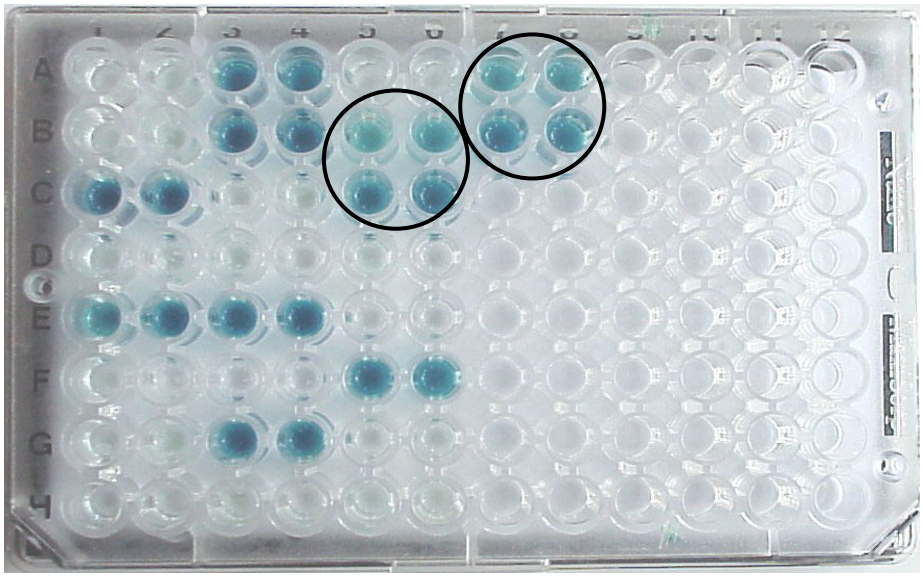


FIG. 6. Photo d'un gel d'agarose : dans les entourés blancs:cas positifs peu évidents.



Légende

	1	2	3	4	5	6	7	8
A	Blanc		**6**		14		**22**	
B	-		**7**		**15**		**23**	
C	**+**		8		**16**			
D	1		9		17			
E	**2**		**10**		18			
F	3		11		**19**			
G	4		**12**		20			
H	5		**13**		21			

FIG. 7. Photo d'une microplaque : dans les entourés noirs : cas positifs bien visibles.

La PCR-ELISA peut être utilisée comme un outil de diagnostic de routine. En effet, grâce à l'utilisation de microplaques, de micropipettes multicanaux et du spectrophotomètre, elle permet de gagner du temps par analyse de plusieurs échantillons à la fois. Aussi, la PCR-ELISA est-elle appliquée au dépistage du Syndrome Immuno Déficience Acquise (SIDA) [4]; et au diagnostic des maladies ré émergentes comme la tuberculose [5]; [6]. La PCR-ELISA pourrait aussi être utilisée comme diagnostic de référence dans l'épidémiosurveillance des maladies vétérinaires comme la peste bovine ou la péri pneumonie contagieuse bovine (PPCB) dans les pays en phase d'éradication.

De plus, contrairement à la PCR classique, la PCR-ELISA évite l'emploi du bromure d'éthidium et de sondes radioactifs qui sont des substances cancérigènes. Enfin, la PCR-ELISA ne nécessite pas des investissements supplémentaires dans des laboratoires qui pratiquent déjà la PCR et l'ELISA.

Toutefois, le coût de la PCR-ELISA demeure l'une des contraintes actuelles à une large application de cette technique comme outil de diagnostic dans des laboratoires disposant de peu de moyens. En effet, au cours de la présente étude, le coût d'analyse d'un échantillon a

été évalué à €3.04, presque 4 fois le coût de la PCR classique estimé à €0.8 [7]. Cependant, un coût raisonnable, pourrait être atteint, si à la place des kits PCR-ELISA, les réactifs nécessaires sont séparément commandés.

4. CONCLUSIONS

La PCR-ELISA a été détecté avec succès toutes les 6 souches de *T.congolense* type savane lors de la mise au point. Elle a également détecté des souches de *T.congolense* type savane dans des échantillons de terrain. La PCR-ELISA peut être appliquée au diagnostic de routine des Trypanosomoses animales, à condition de trouver les paramètres optimum à sa réalisation, en l'occurrence le coût.

5. CONCLUSION GENERALE

L'évaluation des amorces ITS fournies par l'équipe de Davila , Rio de Janeiro, Brésil a donné des résultats intéressants. Un couple d'amorces (TRYPB) s'est avéré plus performant mais il a fallu procéder à des modifications dans le protocole initial proposé.

La PCR-ELISA a été mis au point pour *T.congolense type savane*.

Les travaux ont fait l'objet de formation universitaire de deux étudiants qui ont soutenus leurs diplômes de DEA grâce aux résultats qu'ils ont obtenus par leurs travaux :

Monsieur Ludovic Mewono a soutenu son DEA a l'université de Ouagadougou le 28 février 2004 sur le 1er thème : «*Evaluation et validation des amorces ITS pour l'amélioration du diagnostic PCR des Trypanosomoses animales africaines ».*

Monsieur Adama Sow a soutenu son DEA a l'université de Dakar le 30 octobre 2004 sur le thème: «*Mise au point et la validation de la PCR-ELISA pour la détection de l'ADN de Trypanosoma congolense type savane (TCS)».* Monsieur Adama Sow a pu bénéficier d'une bourse de l'AIEA pour suivre un cours sur la PCR, à Pretoria, en mai 2004.

En perspectives nous comptons valider la PCR pan-pathogénique sur un large échantillonnage de prélèvements venant directement du terrain en vue de pouvoir sauter les étapes d'extraction et de purification d'ADN.

La continuation de la mise au point de la PCR-ELISA se poursuit avec les autres espèces de Trypanosomes pathogènes du bétail (*T.vivax*, *T.brucei* et les sous types de *congolense* dont Forest et Kilifi. De même, l'amélioration du protocole est en cours en vue de réduire le coût par la sensibilisation des plaques ELISA directement au labo et la commande séparée de certains réactifs au lieu du kit entier à chaque commande.

REMERCIEMENTS

Nos sincères remerciements s'adressent à l'AIEA, sa section Santé et Production Animale pour le soutien financier et technique au programme, et surtout à John Crowther pour la disponibilité et la bonne coordination du programme. Nous remercions également A. Davila, de l'Institut Oswaldo Cruz, Rio de Janeiro, au Brésil, pour nous avoir fourni les premiers échantillons des amorces ITS et stimuler la coopération Sud-Sud.

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MOLECULAR MARKERS FOR THE DIFFERENT (SUB)-SPECIES OF THE TRYPANOZOON SUBGENUS

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Abstract

Recently developed PCRs based on the internal transcribed spacer 1 (ITS-1) allow differentiation of the Trypanozoon subgenus, *T.vivax* and *T.congolense* based on differences in length of the amplification products [1]; [2]. These assays also allow the detection of mixed infections by one single PCR assay.

PCR assays for differentiating the different members of the Trypanozoon subgenus, however, remained a challenging issue. During the past years, species specific PCR's for identifying the different taxa within the Trypanozoon subgenus have been developed. For the detection of the two human pathogenic Trypanosomes, PCR-SRA for T.b.rhodesiense [3; 4] and PCR-TgsGP gene for *T.b.gambiense* [5] were developed. For animal Trypanosomiasis, a *T.evansi* specific PCR based on the RoTat 1.2 VSG was developed [6]. Only for *T.b.brucei* and *T.equiperdum*, no specific markers could be identified. However, the results do point out that *T.equiperdum* is more closely related to *T.b.brucei* than to *T.evansi* and even might be a particular strain of *T.b.brucei* [7].

1. INTRODUCTION

Diagnosis of Trypanosomosis usually starts with clinical suspicion or with the detection of antibodies in the blood of the examined mammalian host. Conclusive evidence of infection however relies on detection of the parasite in the blood or tissue fluids of infected humans or animals. Unfortunately, parasitological techniques cannot always detect ongoing infections as the level of parasitaemia is often low and fluctuating, particularly during the chronic stage of the diseases [8].

As an alternative to parasitological tests, DNA detection based on PCR is being investigated. *Trypanozoon* specific primers have been designed previously: TBR primers which target a 177 bp repeat [9]; pMUTEC primers targeting a retrotransposon [10], ORPHON primers that target the spliced leader sequence [11], and the PCR-ESAG6/7 that target the transferrin receptor [12]; [13]. PCR tests for diagnosis of *T.congolense* and *T.vivax* infections exist as well [14]; [15].

Hence, to differentiate between the three major pathogenic Salivarian Trypanosomes at least three sets of primers were necessary. To obtain the same result with one single PCR, Desquesnes and colleagues developed an assay based on the ribosomal region [2]. In parallel, [1] developed a PCR for amplification of the ITS-1 region, with primers and conditions different from those used by Desquesnes *et al.* [2]. Both these PCRs allow the differentiation of the *Trypanozoon* subgenus from *T.congolense* and *T.vivax* and might lead to a pan-

Trypanosoma assay in those regions where concomitant infections of different pathogenic Salivarian Trypanosomes occur.

PCR assays for differentiating the different members of the *Trypanozoon* subgenus, however, remained a challenging issue. In this mini-review we will give an overview of the work carried out during the last few years at the Institute of Tropical Medicine Antwerp and collaborating institutes, in this field.

2. MATERIALS AND METHODS

2.1. Trypanosome populations

A large collection of Trypanosomes, including clones and stocks from *T. b. gambiense, T.b.rhodesiense*, *T.b.brucei*, *T.evansi* and *T.equiperdum, T. congolense, T.theileri* and *T.vivax* was used in the different experiments. They are isolated from different host and regions, all over world (Table I). All populations were kept as cryostabilates in liquid nitrogen.

2.2. DNA preparation

Bloodstream form Trypanosome populations were grown in mice and rats and subsequently purified according to Lanham and Godfrey [16]. Pure Trypanosome pellets were stored at -80°C. Twenty µL of Trypanosome pellet (approximately 2.10^7 cells) were resuspended in 200 µL of Phosphate Buffered Saline (PBS) (8.1m*M* $Na_2HPO_4.2H_20$, 1.4m*M* $NaHPO_4$, 140 m*M* NaCl, pH 7.4) and the Trypanosome DNA was extracted using the commercially available QIAamp DNA mini kit (Westburg, Leusden, The Netherlands), resulting in pure DNA in 200 µL of TE buffer. The typical yield of DNA extracted from a 20 µL pellet was 150 ng/µL or 30 µg total DNA. Obtained extracts were diluted 200 times in water and divided into aliquots of 2 mL in microcentrifuge tubes for storage at -20°C.

2.3. Polymerase chain reactions (PCRs)

2.3.1. PCR EGAG 6/7 [13]

Twenty µL of sample was mixed with 30 µL of a PCR-mix consisting of: 1,5 U Taq DNA recombinant polymerase (Gibco BRL, UK), 10X PCR buffer (Gibco BRL, UK), 3,0 m*M* $MgCl_2$ (Gibco BRL, UK), 200 µ*M* of each of the four dNTPs (Roche, Mannheim, Germany) and 0.5 µM of each ESAG 6/7 primer. The ESAG 6/7 primers were used for amplification of a 237 bp fragment from *T.evansi* genomic DNA, ESAG 6/7 Forward ACA TTC CAG CAG GAG TTG GAG and ESAG 6/7 Reverse CAC GTG AAT CCT CAA TTT TGT [17]. Cycling conditions were as follows: a first denaturation step of 4 min at 94ºC was followed by 35 cycles consisting of 1 min denaturation at 94ºC, 1 min primer-template annealing at 55ºC and 1 min polymerization at 72ºC. The last extension step of 5 min at 72°C was performed to polymerize all remaining single strand DNA fragments (ssDNA).

Twenty µL of the PCR product were electrophoresed on a 2% agarose gel (25 min at 100V) with a 100 bp marker (Gibco BRL, UK) as size marker. The gels were stained with ethidium bromide (2 µL/50mL gel) and analysed on a U.V. transilluminator.

2.3.2. PCR ORPHON [11]

The sequence of the ORPHON primers is as follows:

ORPHON5J-F 5' GAT CCC TCT CCA CCA ATC GAC CG 3'

ORPHON5J-R 5' AAC TGC CCC GAC CTC CGC AGT 3'

To each 20 µL of sample, 30 µL of a PCR cocktail was added, consisting of 25 µL of 2x Goldstar PCR-mix (Eurogentec) enriched with 400 µ*M* of each of the four deoxynucleotides and 3.0 m*M* $MgCl_2$, 0.5 µL 1U/µL Goldstar DNA polymerase (Eurogentec), and 20 p*M* of each primer.

The amplification programme was as follows: 50 cycles of 30 s at 94°C, 90 s at 68°C; 1 cycle of 120 s at 72°C. PCR products were electrophoresed (30 min at 180-200 V) in 2% agarose gels (Biozym) previously stained by submersion in an ethidium bromide solution (1.5 mg/l Tris acetate EDTA buffer, pH 8.0). Signals were made visible by UV illumination and photographed with a Polaroid camera.

2.3.3. PCR ITS-1 [1]

Primers were derived from the *T.evansi* 18S DNA sequence (D89527). Primer sequences were identified within the end region of the 18S ribosomal subunit and the beginning of the 5.8 small subunit. Primer sequences were as follows;

ITS-1 Forward 5' TGT AGG TGA ACC TGC AGC TGG ATC 3'

ITS-1 Reverse 5' CCA AGT CAT CCA TCG CGA CAC GTT 3'

For amplification, 20 µL of extracted DNA were mixed with 30 µL of a PCR-mix containing: 1 U Taq DNA recombinant polymerase (Promega, UK), PCR buffer (Promega, UK), 2.5 m*M* $MgCl_2$ (Promega, UK), 200 µ*M* of each of the four dNTPs (Roche, Mannheim, Germany) and 0.8 µ*M* of each primer (Gibco BRL, UK). All amplifications were carried out in a Biometra® T3 thermocycler. Cycling conditions were as follows: denaturation for 4 min at 94ºC, followed by 35 amplification cycles of 1 min denaturation at 94ºC, 1 min primer-template annealing at 54ºC and 1 min polymerization at 72ºC. A final elongation step was carried out for 5 min. at 72°C. Finally, 20 µL of the PCR product and 10 µL of a 100 bp size marker (MBI Fermentas, Germany) were subjected to electrophoresis in a 2% agarose gel (25 min. at 100V). Gels were stained with ethidium bromide (0.5 µg/mL) (Sigma, USA) and analyzed on an Imagemaster Video Detection System (Pharmacia, UK).

2.3.4. PCR-SRA [3]

Primers were derived from the sequence of the serum resistance associated gene (SRA) (accession number Z37159). Primer sequences were as follows:

PCR-SRA-*f* 5'ATA GTG ACA AGA TGC GTA CTC AAC GC

and

PCR-SRA-*r* 5'AAT GTG TTC GAG TAC TTC GGT CAC GCT.

They amplify a 284 bp fragment between nucleotides 383-667 of the *SRA* gene. All PCR amplifications were performed using 10 ng of the DNA extracted from purified parasites. The DNA templates were amplified in 50 µL of PCR reaction mixture containing 1

x PCR buffer (20 m*M* Tris-HCl pH 8.7, 100 m*M* KCl, 50 m*M* $(NH_4)_2SO_4$), 1.5 m*M* $MgCl_2$, 200 µ*M* of each of the four dNTPs, 1 µ*M* of each of the primers and 2.5 units of HotStar Taq DNA polymerase (Qiagen). All PCR amplifications were performed on a T3 Thermocycler (Biometra). PCR conditions were as follows: sample incubation for 15 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at 68°C and 1 min at 72°C, and a final extension at 72°C for 10 min. A 20 µL sample of each PCR product was analysed by electrophoresis in a 2% agarose gel. The gels were stained with ethidium bromide (1 $\mu g.mL^{-1}$) (Sigma) and analysed on an Imagemaster Video Detection System (Pharmacia).

2.3.5. PCR-TgsGP [5]

The primers were derived from the sequence of the *T.b.gambiense* specific glycoprotein (TgsGP) [18]. Using the Genbank homology search program, the primer sequence was derived from the region lacking any significant similarity with already known DNA sequences. Primer sequences were as follows:

PCR-TgsGP-*f* 5' GCT GCT GTG TTC GGA GAG C

and

PCR-TgsGP-*r* GCC ATC GTG CTT GCC GCT C.

All PCR amplifications were performed using 10 ng of extracted DNA. Amplification was performed using 50 µL of a reaction mixture containing 1x PCR buffer (20 m*M* Tris-HCl pH 8.7, 100 m*M* KCl, 50 m*M* $(NH_4)_2SO_4$, Q solution), 1.5 m*M* $MgCl_2$, 200 µ*M* each of the four dNTPs, 1 µ*M* each of the primers and 2.5 units of HotStar Taq DNA polymerase (Qiagen, Westburg, Leusden, The Netherlands). All PCR amplifications were performed using T3 Thermocycler supplied by Biometra (Westbur, Leusden, Netherlands). The PCR amplification using TgsGP derived primers was performed by incubating the samples for 15 min at 95°C followed by 45 cycles of 1 min at 94°C, 1 min at 63°C and 1 min at 72°C, and a final extension at 72°C for 10 min. A 20 µL sample of each PCR product was analysed by electrophoresis in a 2% agarose gel. Additionally, the detection limit was increased by a second PCR amplification that was performed using 1 µL of the first one.

2.3.6. PCR-RoTat 1.2 [6]

Primers were derived from the RoTat 1.2 VSG sequence (AF317914), recently cloned and sequenced by [19]. Primer sequences were identified within the region (608-812 bp) lacking homology with any other known VSG sequences. Primer sequences (in 5'-3' direction) and annealing temperatures were as follows:

RoTat 1.2 Forward GCG GGG TGT TTA AAG CAA TA, $T_{ann.}$ 59°C and

RoTat 1.2 Reverse ATT AGT GCT GCG TGT GTT CG, $T_{ann.}$ 59°C.

Twenty µL of extracted DNA were mixed with 30 µL of a PCR-mix containing: 1 U Taq DNA recombinant polymerase (Promega, UK), PCR buffer (Promega, UK), 2.5 m*M* $MgCl_2$ (Promega, UK), 200 µ*M* of each of the four dNTPs (Roche, Mannheim, Germany) and 0.8 µ*M* of each primer (Gibco BRL, UK). All amplifications were carried out in a Biometra® Trio-block thermocycler. Cycling conditions were as follows: denaturation for 4 min. at 94ºC, followed by 40 amplification cycles of 1 min denaturation at 94°C, 1 min primer-template annealing at 59ºC and 1 min. polymerization at 72°C. A final elongation step was carried out for 5 min. at 72°C. Twenty µL of the PCR product and 10 µL of a 100 bp size marker (MBI Fermentas, Germany) were subjected to electrophoresis in a 2% agarose gel (25 min.

at 100V). Gels were stained with ethidium bromide (0.5 µg/mL) (Sigma, USA) and analyzed on an Imagemaster Video Detection System (Pharmacia, UK).

2.3.7. Multiple-endonuclease genotyping approach (MEGA) [7]

A fine-scale genotyping approach involving multiplex endonucleases in combination with a pair of cognate adapters was used according to Agbo and colleagues [20]. Briefly, 100-250 ng genomic DNA were digested for 4 h using 10 U each of *Bgl*II, *Bcl*I, *Acs*I and *Mun*I endonucleases in two successive double digestion reactions. The final digestion products were precipitated and reconstituted in 10 µL distilled water. Ten µL of a buffer containing 660 m*M* Tris HCl, 50 m*M* $MgCl_2$, 10 m*M* Dithiothreitol, 10 m*M* ATP, pH7.5, and 20 p*M* of each *Bgl*II and *Mun*I adapters were added. The *Bgl*II adapter also ligated to the overhang sites created by *Bcl*I, while *Mun*I adapter also ligated to the *Acs*I site. One µL (400U) of T4 DNA ligase (New England Biolabs) was added and the mixture incubated for 2 h at 25ºC. Pre-selective amplification was performed in a total volume of 20 µL containing 4 µL of 1:1-diluted ligation product, 1 U of Taq polymerase (Roche Molecular Biochemicals, Almere, The Netherlands), 10X PCR buffer (100 m*M* Tris HCl pH 9.0, 50 m*M* KCl, 1% triton X-100, 0.1% w/v gelatin), 2.5 m*M* $MgCl_2$, 200 µ*M* of each dNTP and 5 p*M* of each *Bgl*II ($^{5'-}$GAGTACACTGTCGATCT) and *Mun*I ($^{5'-}$GAGAGCTCTTGGAATTG) primers. The reaction mix was incubated for 2 min at 95ºC, and subjected to 20 cycles of PCR (30 s at 95ºC, 30 s at 56ºC and 2 min at 72ºC). Four µL of 1:20-diluted pre-selective products were used as template for selective reaction with *Mun*-0/*Bgl*-A selective primer combination (in which the *Mun* primer was fluorescently labelled). The PCR program was essentially the same as for pre-selective amplification, except that the last cycling step was followed by 30 min incubation at 60ºC. The final products were diluted 1:1 with TE, and Genescan-500 internal lane standard (PE Applied Biosystems) was added. One µL of the mix was resolved in a 7.3% denaturing sequencing gel using a model ABI 373A automated DNA sequencer. Gels were routinely prepared by using ABI protocols and electrophoresed for 5 h. Gel patterns were collected with GenScan software (PE Applied Biosystems) and sample files were transferred to GelCompar II software (Applied Maths, Kortrijk, Belgium).

3. RESULTS

3.1. PCR ESAG 6/7 and PCR ORPHON

Both PCRs are specific for the *Trypanozoon* group: *T. brucei*, *T.evansi* and *T.equiperdum* DNA can be detected while *T.congolense*, *T.vivax* and *T.theileri* samples remained negative. Detection limit of the PCR ESAG6/7 was determined at 100 Trypanosomes/mL using spiked mouse blood, while 200 Trypanosomes/mL was the detection limits when spiked water buffalo blood was used, i.e. five Trypanosomes per PCR reaction (data not shown). The detection limit of the ORPHON PCR was determined at fifty Trypanosomes per reaction, which corresponds to an analytical sensitivity of 2,000 parasites/mL.

3.2. ITS-1

As shown in Fig. 1, all Trypanosomes from the subgenus *Trypanozoon* yielded a band of approximately 450 bp (lane 1-15); the *T.vivax* yielded a 150 bp band (lane 16), while the *Trypanosoma congolense* Savannah yielded a larger band of approximately 650 bp (lane 17).

Double and triple mixed infections were simulated by mixing 10 µL (approximately 7.5 ng) of *T.b.brucei* (AnTat 1.8), *T.congolense* (TRT 17, Savannah) and *T.vivax*

(ILRAD 700) DNA. For each preparation, the obtained PCR pattern reflected the species composition of the mixture Fig. 2).

3.3. PCR-SRA

The expected 284 bp *SRA*-PCR product was obtained with the DNA of the 24 different populations considered as *T.b.rhodesiense*. All other 72 non- *T.b.rhodesiense* populations were negative, thus confirming the specificity of the *SRA*-PCR for the *T.b.rhodesiense* subspecies. An overview of the PCR-SRA results with different human African Trypanosomiasis strains (*T.b.rhodesiense* and *T b.gambiense*) is shown in Fig. 3.

3.4. PCR-TgsGP

In total, 73 different Trypanosome populations have been analysed by the TgsGP-PCR. A specific PCR product was obtained with 13 out of 15 *T b.gambiense* populations. All other 58 non-*T.b.gambiense* populations remained negative, thus confirming the specificity of the TgsGP-PCR for *T.b.gambiense* within the collection of tested *Trypanosoma sp*. After a single PCR reaction, the detection limit reached 1000 Trypanosomes/mL blood. This detection limit was lowered to 10 Trypanosomes/mL blood when the TgsGP-PCR was repeated using an aliquot of the first PCR reaction product. An overview of the PCR-TgsGP results with different human African Trypanosomiasis strains is shown in Fig. 4.

3.5. PCR RoTat 1.2

As shown in Fig. 5, the RoTat 1.2 PCR yielded a 205 bp amplicon in the positive control (lane 1) as well as in all other *T.evansi* populations (lanes 3-8). Moreover, the same fragment was found in seven out of the nine *T.equiperdum* populations tested. Only the *T.equiperdum* BoTat 1.1 (lane 10) and the *T.equiperdum* OVI strain (lane 11) were PCR negative. All other tested Trypanosome populations, including six *T.b.brucei*, eight *T.b.gambiense*, five *T.b.rhodesiense,* two *T. congolense*, one *T.vivax* and one *T.theileri,* were negative. (lanes 18-40). As a negative control, a PCR-mix without template DNA was included (lane 2). The PCR was able to detect as few as 10 Trypanosomes per PCR reaction, which corresponds with a lower detection limit of 50 Trypanosomes per mL. In principal, this limit can still be lowered if a blood sample of 200 µL extracted with the QIAamp DNA mini kit is eluted in less than 200 µL.

3.6. MEGA, a modified AFLP

In the UPGMA clustering data obtained from the modified AFLP analysis, all *T.evansi* are grouped in one cluster with a similarity of 85-95%, together with eight out of ten *T.equiperdum* strains. The *T.b.brucei* group appeared as a heterogeneous cluster, including the *T.equiperdum* BoTat 1.1 and OVI strains. Based on the modified AFLP data, the level of similarity of these two latter strains was calculated at 74%. In this analysis, OVI seems closely related to *T.b.brucei* KETRI 2494, while BoTat 1.1 shares more homology with *T.b.brucei* AnTat 2.2 (Fig. 6).

4. DISCUSSION AND CONCLUSION

PCR ITS-1, a pan-*Trypanosoma* assay. A single PCR was developed that detected all pathogenic *Trypanosom*a spp. Unfortunately, this pan-*Trypanosoma* test cannot discriminate within the subgenus *Trypanozoon.* As well as other pan-*Trypanosoma* tests, such

as the PCR-ITS [2] and PCR-RFLP [21], this PCR targets conserved DNA sequences within the genome. So far, these techniques seem to work for discriminating species within the *Trypanosoma* genus except for the closely related taxa within the *Trypanozoon* subgenus. Apparently, *T. brucei*, *T.evansi* and *T.equiperdum* are too similar to observe any differences within the 18S and ITS regions. Pan-*Trypanosoma* assays may have a potential for distinguishing *T. congolense*, *T.vivax* and *T.brucei* and to detect mixed infections in bovine or small ruminants.

A *T.b.rhodesiense* specific marker. The *SRA*-based PCR was shown to be specific for *T.b.rhodesiense*, as a 284 bp specific PCR product was generated with 24 of the 25 *T.b.rhodesiense* used in this study, whereas this fragment was never detected in other subspecies and species. Other recently used analytical techniques such as isoenzyme analysis and RFLP failed to identify an unequivocal criterion to differentiate *T.b.rhodesiense* from *T.b.brucei* [22-24]. As a large variety of game and domestic animals serve as a reservoir for both subspecies, our PCR test could be used for identification of human infective and non-human infective Trypanosomes within the animal reservoir and the vector [22]; [24-26].

A *T.b.gambiense* specific marker. The obtained results showed that the expected 308 bp specific PCR product was generated solely with *T.b.gambiense* parasites, and that no cross-reactivity occurred with any other DNA templates used in this study. Interestingly, two *T.b.gambiense* strains i.e. ABBA and LIGO scored negative in PCR amplification. These strains however had previously already been classified as being distinct from the conventional *T.b.gambiense* parasites, and were grouped together with the Nigerian *T.b.brucei* subspecies [27]. This classification had been made based on the cluster analysis of the restriction enzyme polymorphism pattern using the ribosomal non-transcribed spacer region. As both strains were negative by TgsGP-based PCR but were previously characterized as human infective, we have re-tested their resistance to normal human serum. Both strains appeared to be completely human serum sensitive and as such were presumed to be non-infective to human, possibly representing a *T.b.brucei* subspecies. On the other hand, two other *T. b. gambiense* strains used in this study i.e. OUSOU and KOBIR that were previously classified together with the ABBA and LIGO strains, did contain the TgsGP gene and were confirmed to be completely resistant to normal human serum. Besides the diagnostic value, the developed TgsGP based PCR test can also serve as a useful tool for disease, vector and reservoir control, indeed rendering possible the differentiation of *T.b.gambiense* from *T.b.rhodesiense* and *T.b.brucei.* As such, the TgsGP-based PCR can be used for epidemiological purposes as well.

A new marker for *T.evansi*. Species-specific markers for *T.evansi* have been previously developed, based on kDNA mini-circle sequences [28-30]. These assays however could not detect dyskinetoplastic *T.evansi* strains since their kDNA is severely reduced or even absent. Bayana Songa and Hamers [31]; and Verloo and colleagues [32]; already identified the RoTat 1.2 VSG as an interesting candidate for species-specific diagnosis of *T.evansi*. Several serological antibody detection tests have been developed and tested both on camels [33]; and water buffaloes [34]. Results indicated the usefulness of the RoTat 1.2 VSG for diagnostic purposes of classic *T.evansi* strains. Only for *T.evansi* type B a problem of diagnosis may appear since this type does not contain the RoTat 1.2 VSG gene [35]; [36]. However, so far this *T.evansi* type B has only been reported in one locality in Kenya. All other isolates in our collection, originating from all over the globe, are from the classic type A and contain the RoTat 1.2 VSG gene. Hence, in general, the RoTat 1.2 VSG gene may be considered as a molecular marker for classic *T.evansi* type A.

Regarding the results of this study, another problem with species-specificity appears unless we accept that the *T.equiperdum* reacting with RoTat 1.2 VSG (gene) are misclassified *T.evansi*. In that case, the RoTat 1.2 VSG (gene) is a potential marker for all *T.evansi* strains, including the dyskinetoplastic strains.

Fitting in *Trypanosoma equiperdum.* Data provided in this study indicate that the *T.equiperdum* collection is not as homogenous as previously believed and the generally followed concept that *T.equiperdum* is very closely related to *T.evansi* and more distant from *T.b.brucei* seems to be incorrect. If we accept the presence of RoTat 1.2 gene to be a specific *T.evansi* marker, only two strains of *T.equiperdum, in casu* the BoTat 1 and the OVI strain, are non *T.evansi* and are more closely related to *T.b.brucei* than to *T.evansi*. Thus the problem of differentiating *T.equiperdum* from the rest of the *Trypanozoon* species shifts from *T.evansi* (the general belief) to *T.b.brucei* (the new concept).

TABLE I. DIFFERENT TRYPANOSOME POPULATIONS, PRESENT IN THE INSTITUTE OF TROPICAL MEDICINE, ANTWERP

(sub)-species	Trypanosome populations	Origin	Isolation year	Original host
T.b.brucei	AnTat 1.8	Uganda	1966	bushbuck
T.b.brucei	AnTat 2.2	Nigeria	1970	tsetse
T.b.brucei	AnTat 5.2	Gambia	1975	bovine
T.b.brucei	AnTat 17.1	D.R.Congo	1978	sheep
T.b.brucei	Ketri 2494 ITMAS 270881	Kenya	1980	tsetse
T. b .brucei	J10 ITMAS 250500A	Zambia	1973	Hyena
T.b.brucei	TSW 196 ITMAS 300500A	Côte d'Ivoire	1978	Pig
T. b. gambiense	AnTat 9.1 ITMAP 1788	Cameroon	1976	man
T. b. gambiense	LiTat 1.3	D.R.Congo	1952	man
T. b. gambiense	AnTat 11.6	D.R Congo	1974	man
T. b. gambiense	AnTat 22.1	Congo/Brazza.	1975	man
T. b. gambiense	JUA ITMAS 010799	Cameroon	1979	man
T. b. gambiense	BAGE ITMAP 2569	D.R.Congo	1995	man
T. b. gambiense	NABE ITMAP 2569	D.R.Congo	1995	man
T. b. gambiense	PAKWE ITMAP 2570	D.R.Congo	1995	man
T. b. gambiense	SEKA ITMAP 2568	D.R.Congo	1995	man
T. b. gambiense II	KOBIR ITMAS 260600	Côte d'Ivoire		man
T. b. gambiense II	OUSOU ITMAS 220600	Côte d'Ivoire		man
T. b. gambiense II	ABBA ITMAS 190600A	Côte d'Ivoire		man
T. b. gambiense II	LIGO ITMAS 190600B	Côte d'Ivoire		man
T.b.rhodesiense	AnTat 25.1	Rwanda	1971	man
T.b.rhodesiense	0404	Rwanda	1970	man
T.b.rhodesiense	STIB 847 ITMAS 050399A	Uganda	1990	man
T.b.rhodesiense	STIB 848 ITMAS 190399	Uganda	1990	man
T.b.rhodesiense	STIB 849 ITMAS 050399B	Uganda	1991	man
T.b.rhodesiense	STIB 850 ITMAS 050399C	Uganda	1990	man
T.b.rhodesiense	STIB 851 ITMAS 080399C	Uganda	1990	man
T.b.rhodesiense	STIB 882 ITMAS 080399A	Uganda	1993	man
T.b.rhodesiense	STIB 883 ITMAS 080399B	Uganda	1994	man

T. congolense	TRT 17 ITMAS 020699	Zambia	1997	bovine
T. congolense	IL 1180			
T.vivax	ILRAD 700	Nigeria		bovine
T.evansi	AnTAR 3 ITMAS 180274A	South America	1969	Capybara
T.evansi	AnTat 3.1 ITMAS 070799	South America	1969	Capybara
T.evansi	AnTat 3.2 ITMAS 270280A	South America	1969	Capybara
T.evansi	AnTat 3.3 ITMAS 161189A	South America	1969	Capybara
T.evansi	AnTat 3.4 ITMAS 301189A	South America	1969	Capybara
T.evansi	AnTat 3.5 ITMAS 310180A	South America	1969	Capybara
T.evansi	RoTat 1.2 ITMAS 020298	Indonesia	1982	Buffalo
T.evansi	MHRYD/BR/86/E18 020297	Brazil	1986	capybara
T.evansi	CAN 86 K ITMAS 140799B	Brazil	1986	Dog
T.evansi	Colombia ITMAS 150799	Colombia	1973	Horse
T.evansi	Vietnam WH ITMAS 101298	Vietnam	1998	Buffalo
T.evansi	STIB 816 ITMAS 140799A	China	1978	Camel
T.evansi	KETRI 2479 ITMAS 100883A	Kenya	1980	Camel
T.evansi	KETRI 2480 ITMAS 110297	Kenya	1980	Camel
T.evansi	KETRI 2481 ITMAS 010883C	Kenya	?	?
T.evansi	KETRI 2485 ITMAS 080981B	Kenya	?	Camel
T.evansi	Philippines ITMAS 060297	Philippines	1996	Buffalo
T.evansi	Kazakstan ITMAS 060297	Kazachstan	1995	Camel
T.evansi	Merzouga56 ITMAS 120399D	Morocco	1998	Camel
T.evansi	Merzouga 94 original stab.	Morocco	1998	Camel
T.evansi	Merzouga 93 ITMAS 150399C	Morocco	1998	Camel
T.evansi	Zagora I.3 ITMAS 010399B	Morroco	1997	Camel
T.evansi	Zagora I.5 ITMAS 040399A	Morroco	1997	Camel
T.evansi	Zagora I.10 ITMAS 220299	Morroco	1997	Camel
T.evansi	Zagora I.17 ITMAS 040399B	Morroco	1997	Camel
T.evansi	Zagora I.28 ITMAS 040399	Morroco	1997	Camel
T.evansi	Zagora I.31 ITMAS 120399A	Morroco	1997	Camel
T.evansi	Zagora I.75 ITMAS 010399C	Morroco	1999	Camel
T.evansi	Zagora I.81 ITMAS 010399D	Morroco	1998	Camel
T.evansi	Zagora I.86 original stab.	Morroco	1997	Camel
T.evansi	Zagora II.28 ITMAS 150399B	Morroco	1997	Camel
T.evansi	Zagora II.42 original stab	Morroco	1998	Camel
T.evansi	Zagora II.52 ITMAS 120399B	Morroco	1998	Camel
T.evansi	Zagora II.111 original stab	Morroco	1998	Camel
T.evansi	Zagora II.114 original stab	Morroco	1998	Camel
T.evansi	Zagora II.115 original stab	Morroco	1998	Camel
T.evansi	Zagora III.25 ITMAS 120399C	Morroco	1998	Camel
T.equiperdum	AnTat 4.1 ITMAS 210983A	unknown	unknown	unknown
T.equiperdum	Alfort ITMAS 241199A	unknown	unknown	unknown
T.equiperdum	SVP ITMAS 241199B	unknown	unknown	unknown
T.equiperdum	Hamburg ITMAS 251199A	unknown	unknown	unknown
T.equiperdum	ATCC 30019 ITMAS 020301	France	1903 ?	Horse
T.equiperdum	ATCC 30023 ITMAS 280201	France	1903 ?	Horse
T.equiperdum	STIB 818 ITMAS 010999	P. R. China	1979	Horse
T.equiperdum	American ITMAS 220101	unknown	unknown	unknown
T.equiperdum	Canadian ITMAS 290101	unknown	unknown	unknown
T.equiperdum	OVI ITMAS 241199C	South Africa	1975	Horse
T.equiperdum	BoTat 1.1 ITMAS 240982A	Morocco	1924	Horse

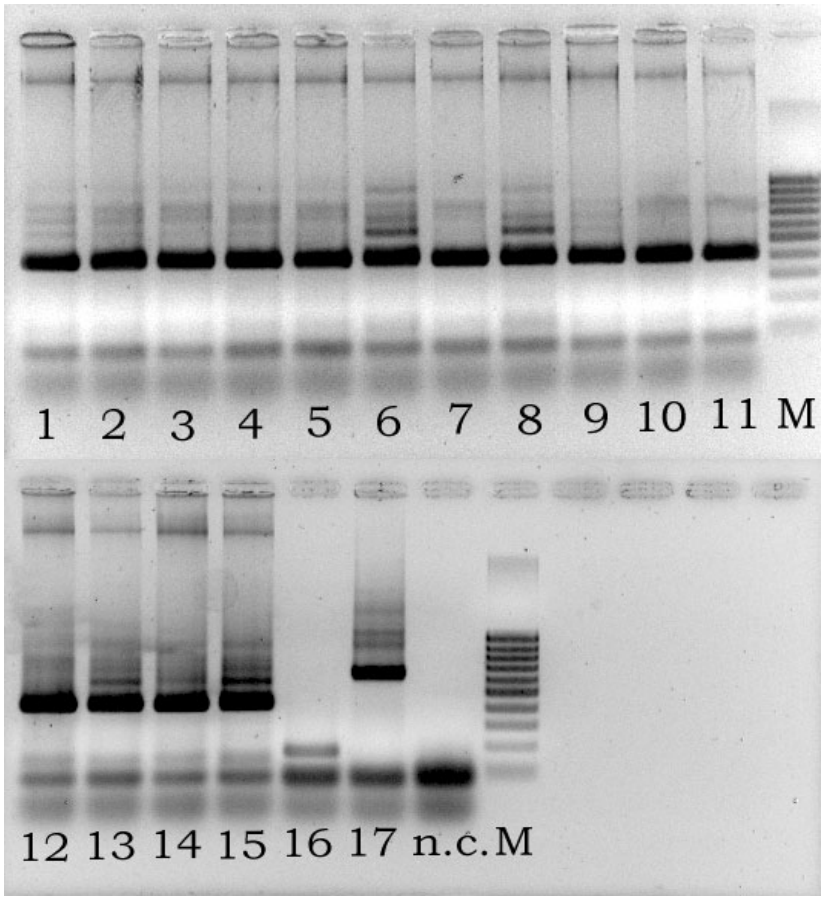

FIG. 1. PCR results obtained with the different Trypanosome populations.

Lanes 1-5, *T.evansi* strains respectively RoTat 1.2, AnTat 3.1, STIB 816, CAN 86K, Merzouga; Lanes 6-12 *T.equiperdum* strains respectively BoTat 1.1, AnTat 4.1, OVI, STIB 818, SVP, Hamburg, Alfort, lane 13 *T.b.brucei* KETRI 2494, lane 14, *T. b. gambiense* AnTat 9.1, lane 15 *T.b.rhodesiense* STIB 850, lane 16 *T.vivax* ILRAD 700, lane 17 *T.congolense* Savannah TRT 17.

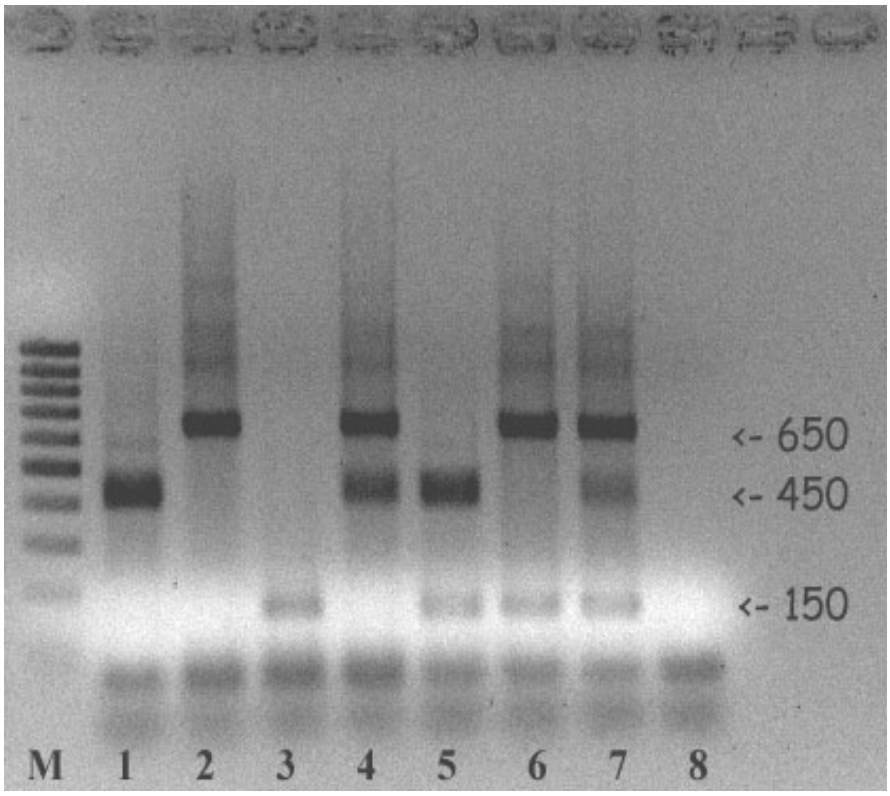

FIG. 2. PCR results for mixed preparations of T.b.brucei, T.vivax and T.congolense Savannah DNA.

Lane 1: *T .b. brucei* AnTat 1.8; lane 2: *T.congolense* TRT 17; lane 3: *T.vivax* ILRAD 700; lane 4: *T.congolense + T .b. brucei*; lane 5: *T.b.brucei* + ***T.vivax***; lane 6*: T.congolense + T.vivax*; lane 7: *T.congolense + T.b.brucei + T.vivax;* lane 8: neg. control; M: 100 bp molecular marker (MBI Fermentas, Germany)

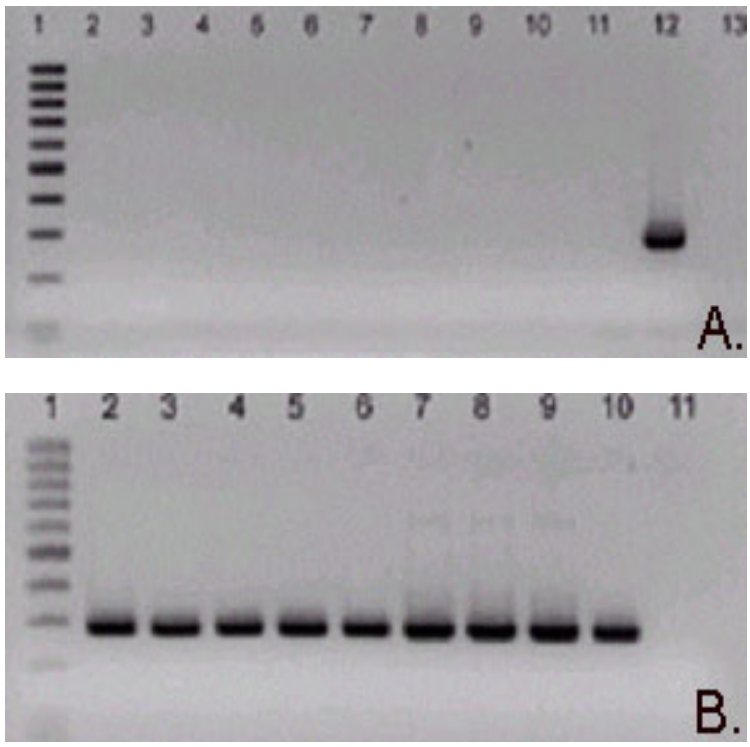

FIG. 3. PCR-SRA, a T.b.rhodesiense specific PCR. Part A.

Lane 1 Marker, lanes 2-11 are *T.b.gambiense*, respectively JUA, AnTat 11.17, AnTat 22.1, SEKA, AnTat 9.1, AnTat 11.6, LiTat 1.6, LiTat 1.3, BAGE, NABE, lane 12 *T.b.rhodesiense* STIB 850, lane 13 white blood cells. ***Part B***. lane 1 Marker, lanes 2-11 are *T.b.rhodesiense*, respectively STIB 883, STIB 882, AnTat 12.1, AnTat 25.1, STIB 847, STIB 884, STIB 851, STIB 850, STIB 849, JUA

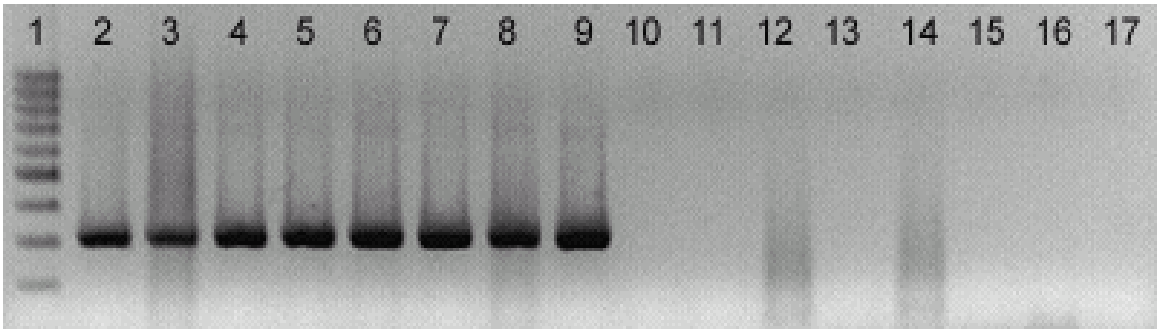

FIG. 4. PCR-TgsGP, a T .b. gambiense specific PCR..

Lane 1 Marker, lanes 2-9 are *T.b.gambiense*, respectively JUA, LiTat 1.3, AnTat 11.17, AnTat 22.1, SEKA, AnTat 9.1, BAGE, LiTat 1.6; lanes 10-17 are *T.b.rhodesiense*, respectively STIB 883, STIB 882, AnTat 12.1, AnTat 25.1, STIB 847, STIB 884, STIB 851, STIB 850, STIB 849, JUA

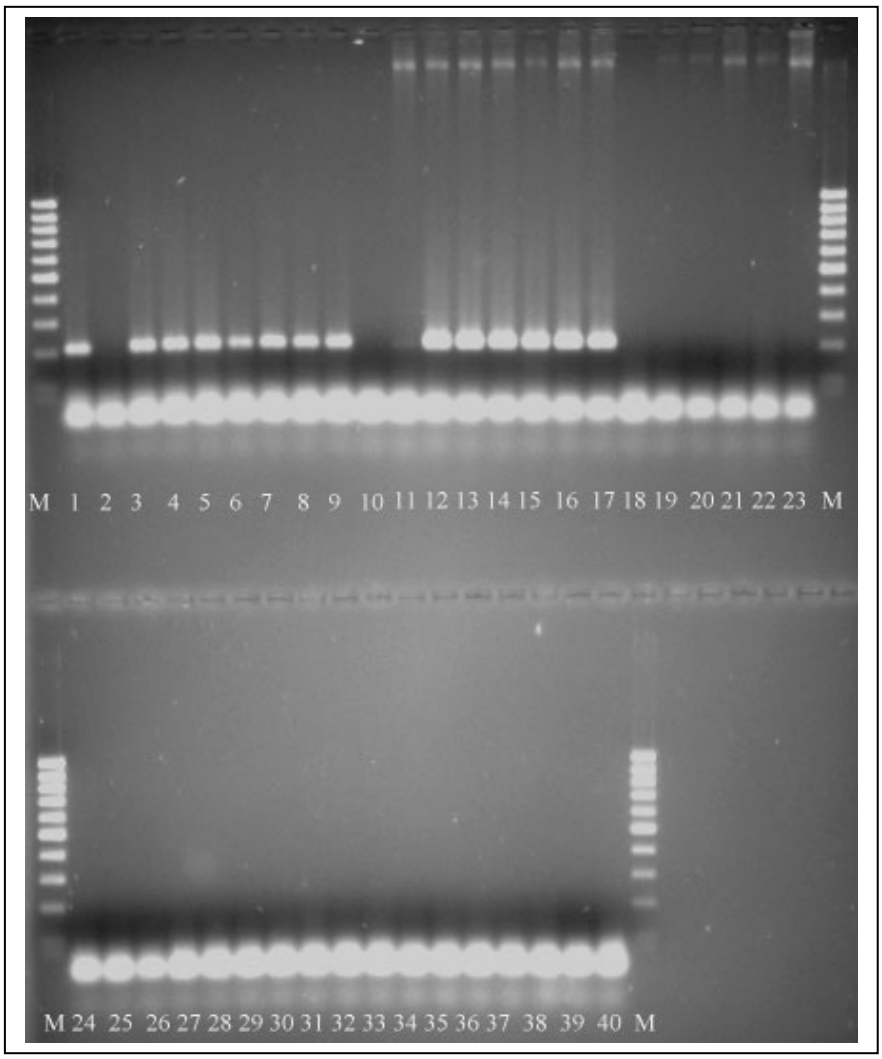

FIG. 5. PCR RoTat 1.2 specificity results for the different Trypanosoma (T.) species and subspecies in this study.

Lane 1 pos. control RoTat 1.2, Lane 2 neg. control, Lanes 3-8 (*T.evansi*) are, respectively, AnTat 3.1, STIB 816, Zagora I.17, Colombia, Merzouga 56, CAN 86K; Lanes 9-17 (*T.equiperdum*) are, respectively, AnTat 4.1, BoTat 1.1, OVI, STIB 818, Alfort, Hamburg, SVP, Am. Strain, Can. Strain ; Lanes 18-23 (*T.b.brucei*) are, AnTat 1.8, AnTat 2.2, AnTat 5.5, KETRI 2494, TSW 196, STIB 348; Lanes 24-31 (*T. b. gambiense*) are, respectively, AnTat 9.1, AnTat 11.6, AnTat 22.1, NABE, SEKA, ABBA, LIGO, LiTat 1.6; Lanes 32-36 (*T.b.rhodesiense*) are STIB 884, STIB 850, AnTat 25.1/S, Etat 1.2/S, AnTat 12.1/S ; Lanes 37-38 (*T. congolense*) are IL1180, TRT 17; Lane 39 (*T.vivax*) is ILRAD 700 and Lane 40 (*T.theileri*) is MELSELE ; Lanes M 100 bp molecular marker (MBI Fermentas, Germany)

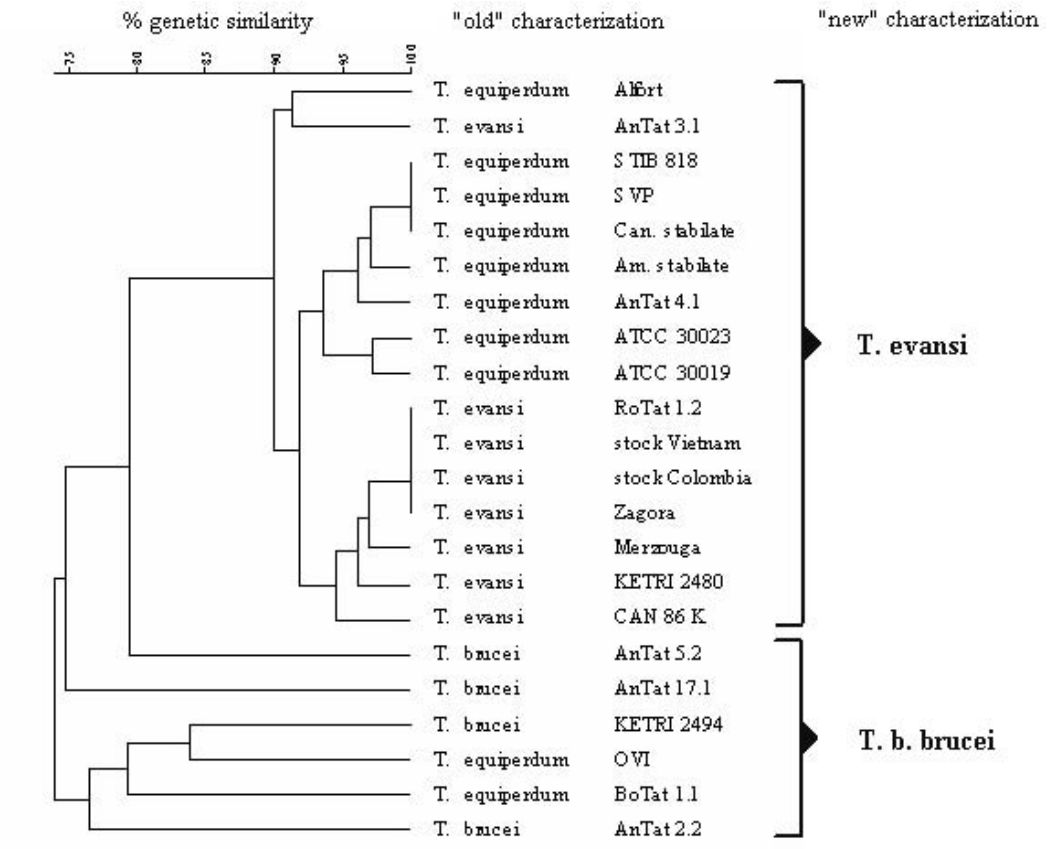

FIG. 6. UPGMA cluster analysis based on the MEGA results.
All *T.evansi* and 8 out of 10 *T.equiperdum* cluster out in one group with a 90-100% genetic similarity. All these strains also contain the RoTat 1.2 VSG gene. Thus, in our newly proposed characterization, this group are *T.evansi* while the other strains can be characterized as *T.b.brucei*.

ACKNOWLEDGEMENTS

This study received financial support from the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT) and the International Livestock Research Institute (ILRI, Nairobi). We wish to thank T. De Waal, T. Baltz, R. Brun, P-H. Clausen, D. Kinker and J. Hagebock for their willingness to provide us with *T.equiperdum* strains.

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PROTOCOLS

SPECIFIC ENZYMATIC AMPLIFICATION OF DNA IN VITRO: THE POLYMERASE CHAIN REACTION (PCR)

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PCR is an *in vitro* method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated. It involves two oligonucleotide primers that flank the DNA fragment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase. These primers hybridize to opposite strands of the target sequence and are oriented so that DNA synthesis by the polymerase proceeds across the region between the primers. Since the extension products themselves are also complementary to and capable of binding primers, successive cycles of amplification essentially double the amount of the target DNA synthesized in the previous cycle. The result is an exponential accumulation of the specific target fragment, approximately 2^n, where *n* is the number of cycles of amplification performed.

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1. AMPLIFICATION OF *TRYPANOSOMA (TRYPANOZOON) BRUCEI*-DNA

1.1. Basic equipment

Thermal Cycler

Microcentrifuges (2)

Pipettes 1-10 μL, 10-100 μL, 100-1000 μL

Freezer

Balance, accurate to 0.1 mg

PH meter

Laminar flow hood with UV option

Agarose gel apparatus, minigel size

Electrophoresis power supply

UV Transilluminator and camera

1.2. Reagents

Taq DNA Polymerase (e.g. AmpliTaq Gold©, Perkin Elmer)

Oligonucleotide primers

Deoxynucleotide triphosphates, dNTPs (dATP, dCTP, dGTP, dTTP)

PCR buffer

Mineral oil, light, sterile

Glass-distilled, sterile water (DNase and RNase free)

TBE electrophoresis buffer

Agarose

Ethidium bromide

Bromphenol blue

DNA marker (e.g. 100 bp ladder)

1.3. Supplies

Microcentrifuge tubes, 0.5 mL, with sealing, sterile

Pipette tips with sealing filter, 0.5-10 μL, l0-100 μL, 100-1000 μL

Pipette tips without filter 0.5-10 μL

1.4. Procedure-Sample preparation

The essential criteria are that the sample contains at least one intact DNA strand encompassing the region to be amplified and that impurities are sufficiently dilute so as not to inhibit polymerization. Lysing cells in a hypotonic solution or with surface-active compounds (e.g. Tritons) is a quick and effective method for preparing DNA for PCR:

(1) Mix 250 μL blood with 250 μL lysis buffer (0.32 *M* Sucrose, 0.01 *M* Tris-Cl (pH 7.5), 5 m*M* $MgCl_2$, 1% Triton X-100).

(2) Centrifuge at 13,000 x g for 20 s.

(3) Remove supernatant and add 500 μL lysis buffer; mix by vortexing.

(4) Repeat steps 2 and 3 twice.

(5) Centrifuge at 13000 x g for 20 s., remove supernatant, and resuspend in 250 μL PCR buffer (1x).

(6) Add 1.5 μL of proteinase-K (10 mg/mL), mix by vortexing.

(7) Incubate at 56^{o}C for 1 h.

(8) Incubate at 95^{o}C for 10 min to inactivate the proteinase-K. Store frozen at -20^{o}C.

1.5. Protocol

1.5.1. Preparation of the reaction mixture

Example. The detection of Trypanosomal DNA [Trypanozoon] with a nuclear repeat primer set TBR-1/TBR-2; Moser *et al*., 1989, Artama *et al*., 1992)

Reagents

10 x PCR buffer	100 m*M* Tris-HCl (pH 8.4 at 20°C), 500 m*M* KCl, (stock solution) 1% Triton X-100
100 x dNTP stock	100 m*M* dNTP´s (25 m*M* dATP, 25 m*M* dCTP, 25 m*M* dGTP, 25 m*M* dTTP)
TBR-1	1 μ*M*, 5´-CGAATGAATATTAAACAATGCGCAGT-3´
TBR-2	1 μ*M*, 5´-AGAACCATTTATTAGCTTTGTTGC-3

$MgCl_2$-stock sol. 50 m*M* $MgCl_2$

Taq polymerase 5000 units/mL, AmpliTaq Gold®, Perkin Elmer

DNA samples test samples, positive and negative controls

1.5.2. PCR Procedure

A 25 µL reaction mixture containing 10 m*M* Tris-HCl, 50 m*M* KCL, 0.1% Triton X-100, 3 m*M* $MgCl_2$, 1 m*M* dNTP´s, 1 µ*M* each of the oligonucleotide primers, and 1 unit of *Taq* DNA polymerase is prepared as follows:

(a) Place 10 µL of 1/6.7 prediluted 50 m*M* $MgCl_2$ into each PCR tube,

(b) Add 5 µL of template (DNA sample),

(c) Prepare the reaction mixture: (volumes given for one sample, prepare for 10 samples)

- 2.5 µL 10 x PCR buffer
- 0.2 µL TBR-1 (= 1µ*M*)
- 0.185 µL TBR-2 (= 1µ*M*)
- 0.25 µL 100 m*M* dNTP´s
- 6.665 µL H_20
- 0.2 µl *Taq* polymerase

10 µL Total volume

(d) Mix and centrifuge (30 sec)

(e) Transfer 10 µL of the reaction mixture to each PCR tube. Mix and overlay with one drop of mineral oil. Centrifuge at 10,000 x g and 2 min. Transfer tubes immediately to the thermal cycler.

1.5.3. Cycling parameters

PCR is performed by incubating the samples at three temperatures corresponding to the three steps (denaturation, annealing, and extension) in a cycle of amplification.

	Temperature^{o}C	Time (min)	Time (seconds)
Step1	95	10	0
Step 2	60	2	0
Step 3	72	0	30
*Step 4	94	1	0
*Step 5	60	0	30
*Step 6	72	0	30
Step 7	72	0	0
Step 8	40	Pause**	

* Steps 4–6 are repeated 35 times; ** transfer samples to a refrigerator (4°C)

1.6. Electrophoresis of DNA products

1.6.1. Reagents

Agarose	Molecular biology quality
Ethidium bromide	C_{21} H_{20} N_3Br, 10mg/mL, (Mutagen!), store at 4^0C.
TBE-buffer, (10x)	1 *M* Tris, 1 *M* Boric acid (H_3BO_3), 20 m*M* EDTA, 50 m*M* $MgCl_2$, pH: 8.1, store at 4^0C.
Sample-buffer	4 x TBE-buffer, 10% Ficoll 400 (Pharmacia), sodium dodecyl sulphate, Pharmacia), 0.4 mg/mL Bromophenol blue, (Sigma), store at 4^0C.
100 bp DNA ladder	15 blunt-ended fragments between 100 and 1500 by in multiples of 100 bp (Gibco)

1.6.2. Procedure

(a) Prepare stock Tris-borate/EDTA electrophoresis buffer (10xTBE buffer), pH 8.1 store at 4^oC.

1 *M* Tris $C_4H_{11}NO_3$	121.1 gm
1 *M* H3BO3	61.8 gm
20 m*M* EDTA	7.44 gm
50 m*M* $MgCl_2$ x 6 H20	10.16 gm
H_2O	to 1000 mL

(b) Prepare a 25 mL 2% agarose gel, containing 2.5 µl of ethidium bromide in 1 x TBE buffer (use the 8 place comb). Fill the chamber with buffer solution (1 x TBE) until the top surface of the gel is submerged approximately 1 mm (about 400 mL).

(c) Add 5 µL of sample buffer to each sample, centrifuge (10,000 x g for 20 s).

(d) Prepare the 100 bp DNA ladder (50 µL 100 bp ladder, 650 µl H20, 300 µl sample buffer, mix).

(e) Load the samples (e.g. 10 µL) and the DNA ladder (e.g. 10 µL) into the agarose gel wells; connect the electrophoresis chamber to a 60V power supply and start the run (app. 45 min.).

(f) The separated DNA products are detected by UV illumination. Trypanosoma brucei ssp. containing a DNA sequence of 173 bp that is amplified by these TBR primers (Moser et al., 1989). Photographs of the products can be taken under UV illumination using a camera (e.g. Polaroid©).

1.7. References

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2. AMPLIFICATION OF *TRYPANOSOMA CONGOLENSE FOREST* TYPE DNA

2.1. Basic equipment

Thermal Cycler

Microcentrifuges (2)

Pipettes 1-10 μL, 10-100 μL, 100-1000 μL

Freezer

Balance, accurate to 0.1 mg

PH meter

Laminar flow hood with UV option

Agarose gel apparatus, minigel size

Electrophoresis power supply

UV Transilluminator and camera

2.2. Reagents

Taq DNA Polymerase (e.g. AmpliTaq Gold©, Perkin Elmer)

Oligonucleotide primers

Deoxynucleotide triphosphates, dNTPs (dATP, dCTP, dGTP, dTTP)

PCR buffer

Mineral oil, light, sterile

Glass-distilled, sterile water (DNase and RNase free)

TBE electrophoresis buffer

Agarose

Ethidium bromide

Bromophenol blue

DNA marker (e.g. 100 bp ladder)

2.3. Supplies

Microcentrifuge tubes, 0.5 mL, with sealing, sterile

Pipette tips with sealing filter, 0.5-10 μL, l0-100 μL, 100-1000 μL

Pipette tips without filter 0.5-10 μL

2.4. Procedure- Sample preparation

The essential criteria are that the sample contains at least one intact DNA strand encompassing the region to be amplified and that impurities are sufficiently dilute so as not to inhibit polymerization. Lysing cells in a hypotonic solution or with surface-active compounds (e.g. Tritons) is a quick and effective method for preparing DNA for PCR:

(1) Mix 250 μL blood with 250 μL lysis buffer (0.32 M Sucrose, 0.01 *M* Tris-HCl (pH 7.5), 5 m*M* $MgCl_2$, 1% Triton X-100).

(2) Centrifuge at 13,000 x g for 20 s

(3) Remove supernatant and add 500 μL lysis buffer; mix by vortexing.

(4) Repeat steps 2 and 3 twice.

(5) Centrifuge at 13,000 x g for 20 sec., remove supernatant, and resuspend in 250 μL PCR buffer (1x).

(6) Add 1.5 μL of proteinase-K (10 mg/mL), mix by vortexing.

(7) Incubate at 56^{o}C for 1 h.

(8) Incubate at 95^{o}C for 10 min to inactivate the proteinase-K.

(9) Store frozen at –20^{o}C.

2.5. PCR protocol

2.5.1. Preparation of the reaction mixture

Example-the detection of Trypanosomal DNA [*Trypanosoma congolense forest*] with the primer set TCF-1 and TCF-2; Masiga et al. 1992)

Reagents

10 x PCR buffer (stock solution)	100 m*M* Tris-HCl (pH 8.4 at 20°C), 500 m*M* KCl, 1% Triton X-100
100 x dNTP stock	100 m*M* dNTP´s (25 m*M* dATP, 25 m*M* dCTP, 25 m*M* dGTP, 25 m*M* dTTP)
TCF-1	1 μ*M*, 5´- GGACACGCCAGAAGGTACTT –3´
TCF-2	1 μ*M*, 5´- GTTCTCGCACCAAATCCAAC –3´
$MgCl_2$-stock sol.	50 m*M* $MgCl_2$
Taq polymerase	AmpliTaq Gold®, 5000 units/mL, Perkin Elmer

DNA samples test samples, positive and negative controls

2.5.2. Procedure

A 25μL reaction mixture containing 10 m*M* Tris-HCl, 50 m*M* KCL, 0.1% Triton X-100, 3 m*M* $MgCl_2$, 1 m*M* dNTP´s, 1 μ*M* each of the oligonucleotide primers, and 1 unit of Taq DNA polymerase is prepared as follows:

(a) Place 10 μL of 1/6.7 prediluted 50 mM $MgCl_2$ into each PCR tube,

(b) Add 5 μL of template (DNA sample),

(c) Prepare the reaction mixture: (volumes given for one sample)

2.5 μL	10 x PCR buffer,
0.15 μL	TCF-1 (= 1μ*M*)
0.15 μL	TCF-2 (= 1μ*M*)
0.25 μL	100 m*M* dNTP´s
6.75 μL	H_20
0.2 μL	Taq polymerase
10 μL	Total volume

(d) Mix and centrifuge (30 s)

(e) Transfer 10 μL of the reaction mixture to each PCR tube. Mix and overlay with one drop of mineral oil. Centrifuge at 10,000 x g and 2 min. Transfer tubes immediately to the thermal cycler.

2.5.3. Cycling parameters

PCR is performed by incubating the samples at three temperatures corresponding to the three steps (denaturation, annealing, and extension) in a cycle of amplification.

	Temperature ^{o}C	Time (min)	Time (s)
Step 1	95	10	0
Step 2	60	2	0
Step 3	72	0	30
*Step 4	94	1	0
*Step 5	60	0	30
*Step 6	72	0	30
Step7	72	0	0
Step8	4	Pause**	

* Steps 4-6 are repeated 35 times

** Transfer to a refrigerator (4°C)

2.6. Electrophoresis of DNA products

2.6.1. Reagents:

Agarose	Molecular biology quality
Ethidium bromide	C_{21} H_{20} N_3Br, 10mg/mL, (Mutagen!), store at 4^{o}C.
TBE-buffer, (10x)	1 *M* Tris, 1 *M* Boric acid (H_3BO_3), 20 m*M* EDTA, 50 m*M* $MgCl_2$, pH: 8.1, store at 4^{o}C.
Sample-buffer	4 x TBE-buffer, 10% Ficoll 400 (Pharmacia), 0.4% sodium dodecyl sulphate, Pharmacia), 0.4 mg/mL Bromophenol blue, (Sigma), store at 4^{o}C.
100 bp DNA ladder	15 blunt-ended fragments between 100 and 1500 by in multiples of 100 bp (Gibco)

(g) Prepare stock Tris-borate/EDTA electrophoresis buffer (10xTBE buffer), pH 8.1 store at 4^{0}C.

1 *M* Tris $C_4H_{11}NO_3$	121.1 gm
1 *M* H_3BO_3	61.8 gm
20 m*M* EDTA	7.44 gm
50 m*M* MgCl2 x 6 H_20	10.16 gm
H_2O	to 1000 mL

(h) Prepare a 25 mL 2% agarose gel, containing 2.5 μl of ethidium bromide in 1 x TBE buffer (use the 8 place comb). Fill the chamber with buffer solution (1 x TBE) until the top surface of the gel is submerged approximately 1 mm (about 400 mL).

(i) Add 5 µL of sample buffer to each sample, centrifuge (10,000 x g, 20 s).

(j) Prepare the 100 bp DNA ladder (50 µL 100 bp ladder, 650 µl H_20, 300 µL sample buffer, mix).

(k) Load the samples (e.g. 10 µL) and the DNA ladder (e.g. 10 µL) into the agarose gel wells; connect the electrophoresis chamber to a 60V power supply and start the run (app. 45 min.).

(l) The separated DNA products are detected by UV illumination. Trypanosoma brucei ssp. containing a DNA sequence of 173 bp that is amplified by these TBR primers (Moser et al., 1989). Photographs of the products can be taken under UV illumination using a camera (e.g., Polaroid©).

2.7. References

KATAKURA, K.C., LUBINGA, H., Y. T. CHITAMBO. Detection of Trypanosoma congolense and T.brucei subspecies in cattle in Zambia by polymerase chain reaction from blood collected on a filter paper. Parasitology Research, 83: 241-245 (1997).

LEFRANCOIS, T., SOLANO, P., BAUER, B., KABORE, I., TOURÉ, S.M., CUNY, G., DUVALLET, G. Polymerase chain reaction characterization of Trypanosomes in Glossina morsitans submorsitans and G. tachinoides collected on the game ranch of Nazinga, Burkina Faso. Acta Tropica, 72**:** 65-77 (1999)

MASIGA, D.K., SMYTH, A.J., HAYES, P., BROMIDGE, T.J., GIBSON, W. Sensitive detection of Trypanosomes in tsetse flies by DNA amplification. International Journal for Parasitology, 22: 909-918 (1992).

MOSER, D.R., COOK, G.A., OCHS, D.E., BAILEY, C.P., MCKANE, M.R., DONELSON, J.E: Detection of Trypanosoma congolense and Trypanosoma brucei subspecies by DNA amplification using the polymerase chain reaction. Parasitology, 99: 57-66 (1989).

J.M. REIFENBERG, D. CUISANCE, J.L. FREZIL, G. CUNY, G. DUVALLET. Comparison of the susceptibility of different Glossina species to simple and mixed infections with Trypanosoma (Nannomonas) congolense savannah and riverine forest types. Medical and Veterinary Entomology, 11: 246-252 (1997).

REIFENBERG, J.M., SOLANO, P., DUVALLET, G., CUISANCE, D., SIMPORE, J., CUNY, G. Molecular characterization of Trypanosome isolates from naturally infected domestic animals in Burkina Faso. Veterinary Parasitology, 71: 251-262 (1997).

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3. AMPLIFICATION OF *TRYPANOSOMA CONGOLENSE SAVANNAH* DNA

3.1. Basic equipment

Thermal Cycler

Microcentrifuges (2)

Pipettes 1-10 μL, 10-100 μL, 100-1000 μL

Freezer

Balance, accurate to 0.1 mg

PH meter

Laminar flow hood with UV option

Agarose gel apparatus, minigel size

Electrophoresis power supply

UV Transilluminator and camera

3.2. Reagents

Taq DNA Polymerase (e.g. AmpliTaq Gold©, Perkin Elmer)

Oligonucleotide primers

Deoxynucleotide triphosphates, dNTPs (dATP, dCTP, dGTP, dTTP)

PCR buffer

Mineral oil, light, sterile

Glass-distilled, sterile water (DNase and RNase free)

TBE electrophoresis buffer

Agarose

Ethidium bromide

Bromophenol blue

DNA marker (e.g. 100 bp ladder)

3.3. Supplies

Microcentrifuge tubes, 0.5 mL, with sealing, sterile

Pipette tips with sealing filter, 0.5-10 μL, l0-100 μL, 100-1000 μL

Pipette tips without filter 0.5-10 μL

3.4. Procedure-Sample preparation

The essential criteria are that the sample contains at least one intact DNA strand encompassing the region to be amplified and that impurities are sufficiently dilute so as not to inhibit polymerization. Lysing cells in a hypotonic solution or with surface-active compounds (e.g. Tritons) is a quick and effective method for preparing DNA for PCR:

(1) Mix 250 μL blood with 250 μL lysis buffer (0.32 *M* Sucrose, 0.01 *M* Tris-HCl (pH 7.5), 5 m*M* $MgCl_2$, 1% Triton X-100).

(2) Centrifuge at 13,000 x g for 20 sec.

(3) Remove supernatant and add 500 μL lysis buffer; mix by vortexing.

(4) Repeat steps 2 and 3 twice.

(5) Centrifuge at 13,000 x g for 20 sec., remove supernatant, and resuspend in 250 μL PCR buffer (1x).

(6) Add 1.5 μL of proteinase-K (10 mg/mL), mix by vortexing.

(7) Incubate at 56°C for 1 h.

(8) Incubate at 95°C for 10 min to inactivate the proteinase-K. Store frozen at -20°C.

3.5. PCR protocol

3.5.1. Preparation of the reaction mixture

Example-The detection of Trypanosomal DNA [*Trypanosoma congolense* savannah] with the primer set TCN-1 and TCN-2; Moser *et al*. 1989)

Reagents

10 x PCR buffer	100 m*M* Tris-HCl (pH 8.4 at 20°C), 500 m*M* KCl, (stock solution) 1% Triton X-100
100 x dNTP stock	100 m*M* dNTP´s (25 m*M* dATP, 25 m*M* dCTP, 25m*M* dGTP, 25 m*M* dTTP)
TCN-1	1 μ*M*, 5´- TCGAGCGAGAACGGGCACTTTGCGA -3´
TCN-2	1 μ*M*, 5´- ATTAGGGACAAACAAATCCCGCACA -3´
$MgCl_2$-stock sol.	50 m*M* $MgCl_2$

Taq polymerase 5000 units/mL, Perkin Elmer, AmpliTaq Gold®

DNA samples test samples, positive and negative controls

3.5.2. Procedure

A 25μL reaction mixture containing 10 m*M* Tris-HCl, 50 m*M* KCL, 0.1% Triton X-100, 3 m*M* $MgCl_2$, 1 m*M* dNTP´s, 1 μ*M* each of the oligonucleotide primers, and 1 unit of *Taq* DNA polymerase is prepared as follows:

(a) Place 10 μL of 1/6.7 prediluted 50 m*M* $MgCl_2$ into each PCR tube.

(b) Add 5 μL of template (DNA sample).

(c) Prepare the reaction mixture: (volumes given for one sample).

2.5 μL	10 x PCR buffer,
0.2 μL	TCN-1 (= 1μ*M*),
0.2 μL	TCN-2 (= 1μ*M*),
0.25 μL	100 m*M* dNTP´s
6.65 μL	H_20
0.2 μL	*Taq* polymerase 10 μL Total volume

(d) Mix and centrifuge (30 s)

(e) Transfer 10 μL of the reaction mixture to each PCR tube. Mix and overlay with one drop of mineral oil. Centrifuge at 10,000 x g and 2 min. Transfer tubes immediately to the thermal cycler.

3.5.3. Cycling parameters:

PCR is performed by incubating the samples at three temperatures corresponding to the three steps (denaturation, annealing, and extension) in a cycle of amplification

	Temperature °C	Time (minutes)	Time (seconds)
Step 1	95	10	0
Step 2	6	2	0
Step 3	72	0	30
*Step 4	94	1	0
*Step 5	60	0	30
*Step 6	72	0	30
Step 7	72	0	0
Step 8	4	Pause**	

* Steps 4-6 are repeated 35 times; ** Transfer to a refrigerator (4°C)

3.6. Electrophoresis of DNA products

Agarose	Molecular biology quality
Ethidium bromide	C_{21} H_{20} N_3Br, 10 mg/mL, (Mutagen!), store at 4^0C.
TBE-buffer, (10x)	1 *M* Tris, 1 *M* Boric acid (H_3BO_3), 20 m*M* EDTA, 50 m*M* $MgCl_2$, pH: 8.1, store at 4^0C.
Sample-buffer	4 x TBE-buffer, 10% Ficoll 400 (Pharmacia), 0.4% sodium dodecyl sulphate, Pharmacia), 0.4 mg/mL Bromophenol blue, (Sigma), store at 4^oC.
100 bp DNA ladder	15 blunt-ended fragments between 100 and 1500 by in multiples of 100 bp (Gibco)

Prepare stock Tris-borate/EDTA electrophoresis buffer (10xTBE buffer), pH 8.1 store at 4^oC.

1 *M* Tris $C_4H_{11}NO_3$	121.1 gm
1 *M* H_3BO_3	61.8 gm
20 m*M* EDTA	7.44 gm
50 m*M* $MgCl_2$ x 6 H_20	10.16 gm
H_2O	to 1000 mL

Prepare a 25 mL 2% agarose gel, containing 2.5 µl of ethidium bromide in 1 x TBE buffer (use the 8 place comb). Fill the chamber with buffer solution (1 x TBE) until the top surface of the gel is submerged approximately 1 mm (about 400 mL).

Add 5 µL of sample buffer to each sample, centrifuge (10.000 x g; 20 sec).

Prepare the 100 bp DNA ladder (50 µL 100 bp ladder, 650 µl H_20, 300 µL sample buffer, mix).

Load the samples (e.g. 10 µL) and the DNA ladder (e.g. 10 µL) into the agarose gel wells; connect the electrophoresis chamber to a 60V power supply and start the run (app. 45 min.).

The separated DNA products are detected by UV illumination. Trypanosoma brucei ssp. containing a DNA sequence of 173 bp that is amplified by these TBR primers (Moser et al., 1989). Photographs of the products can be taken under UV illumination using a camera (e.g. Polaroid©).

3.7. References

KATAKURA, K., LUBINGA, C., CHITAMBO, H., TADA, Y. Detection of Trypanosoma congolense and T.brucei subspecies in cattle in Zambia by polymerase chain reaction from blood collected on a filter paper. Parasitology Research, 83: 241-245 (1997).

MASIGA, D.K., SMYTH, A.J., HAYES, P., BROMIDGE, T.J., GIBSON, W. Sensitive detection of Trypanosomes in tsetse flies by DNA amplification. International Journal for Parasitology, 22: 909-918 (1992).

MORLAIS, I., GREBAUT, P., BODO, J.M, DJOHA, S., CUNY, G., HERDER, S.. Detection and identification of Trypanosomes by polymerase chain reaction in wild tsetse flies in Cameroon. Acta Tropica, 70: 109-117 (1998).

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REIFENBERG, J.M., CUISANCE, J.D, FREZIL, J.L., CUNY, G., DUVALLET, G (1997). Comparison of the susceptibility of different Glossina species to simple and mixed infections with Trypanosoma (Nannomonas) congolense savannah and riverine forest types. Medical and Veterinary Entomology, 11: 246-252 (1997).

4. AMPLIFICATION OF *TRYPANOSOMA VIVAX* DNA

4.1. Basic equipment

Thermal Cycler
Microcentrifuges (2)
Pipettes 1-10 μL, 10-100 μL, 100-1000 μL
Freezer
Balance, accurate to 0.1 mg
PH meter
Laminar flow hood with UV option
Agarose gel apparatus, minigel size
Electrophoresis power supply
UV Transilluminator and camera

4.2. Reagents

Taq DNA Polymerase (e.g. AmpliTaq Gold©, Perkin Elmer)
Oligonucleotide primers
Deoxynucleotide triphosphates, dNTPs (dATP, dCTP, dGTP, dTTP)
PCR buffer
Mineral oil, light, sterile
Glass-distilled, sterile water (DNase and RNase free)
TBE electrophoresis buffer
Agarose
Ethidium bromide
Bromophenol blue
DNA marker (e.g. 100 bp ladder)

4.3. Supplies

Microcentrifuge tubes, 0.5 mL, with sealing, sterile
Pipette tips with sealing filter, 0.5-10 μL, l0-100 μL, 100-1000 μL
Pipette tips without filter 0.5-10 μL

4.4. Procedure-Sample preparation

The essential criteria are that the sample contains at least one intact DNA strand encompassing the region to be amplified and that impurities are sufficiently dilute so as not to inhibit polymerization. Lysing cells in a hypotonic solution or with surface-active compounds (e.g. Tritons) is a quick and effective method for preparing DNA for PCR.

(1) Mix 250 μL blood with 250 μL lysis buffer (0.32 *M* Sucrose, 0.01 *M* Tris-HCl (pH 7.5), 5 m*M* $MgCl_2$, 1% Triton X-100).

(2) Centrifuge at 13,000 x g for 20 s.

(3) Remove supernatant and add 500 μL lysis buffer; mix by vortexing.

(4) Repeat steps 2 and 3 twice.

(5) Centrifuge at 13,000 x g for 20 s., remove supernatant, and resuspend in 250 μL 1 x PCR buffer.

(6) Add 1.5 μL of proteinase-K (10 mg/mL), mix by vortexing.

(7) Incubate at 56°C for 1 h.

(8) Incubate at 95°C for 10 min to inactivate the proteinase-K.

Store frozen at -20°C.

4.5. PCR protocol

4.5.1. Preparation of the reaction mixture

Example - amplification of *T.vivax*-DNA with the primer set T.v. 80.24/T.v. 322.24; Clausen *et al*., 1998, Masake *et al*., 1997)

Reagents

10 x PCR buffer		100 m*M* Tris-HCl (pH 8.4 at 20°C), 500 m*M* KCl, (stock solution) 1% Triton X-100
100 x dNTP		stock100 m*M* dNTP´s (25 m*M* dATP, 25 m*M* dCTP, 25 m*M* dGTP, 25 m*M* dTTP)
T.v. 80.24	1 µ*M*,	5´CAGTGCTCCCGCTCGTACACGGAC-3´
T.v. 322.24	1 µM,	5´-GCACGCCACATAGCCGGGGAACAG-3´
$MgCl_2$-stock sol.		50 m*M* $MgCl_2$
Taq polymerase		5000 units/mL, Perkin Elmer, AmpliTaq Gold©
DNA samples		test samples, positive and negative controls

4.5.2. Procedure

A 25 µL reaction mixture containing 10 m*M* Tris-HCl, 50 m*M* KCL, 0.1% Triton X-100, 3 m*M* $MgCl_2$, 1 mM dNTP´s, 1 µ*M* each of the oligonucleotide primers, 1 units of Taq DNA polymerase is prepared as follows:

(a) Place 10 µL of 1/6.7 prediluted 50 m*M* $MgCl_2$ into each PCR tube,
(b) Add 5 µL of template (DNA sample),
(c) Prepare the reaction mixture: (volumes given for one sample)

2.5 µL	10 x PCR buffer,
0.18 µL	T.v. 80.24 (= 1µ*M*)
0.18 µL	T.v. 322.24 (= 1µ*M*)
0.25 µL	100 m*M* dNTP´s
6.69 µL	H_20
0.2 µL	Taq polymerase
10 µL	Total volume

(d) Transfer 10 µL of the reaction mixture to each PCR tube. Mix and overlay with one drop of mineral oil. Centrifuge at 10,000 x g and 2 min. Transfer tubes immediately to the thermal cycler.

4.5.3. Cycling parameters

PCR is performed by incubating the samples at three temperatures corresponding to the three steps (denaturation, annealing, and extension) in a cycle of amplification.

	Temperature ^{o}C	Time (minutes)	Time (seconds)
Step 1	95	15	0
Step 2	55	2	0
Step 3	72	0	30
*Step 4	94	0	90
*Step 5	55	0	90
*Step 6	72	2	0
Step 7	72	10	0
Step 8	4	Pause**	

* Steps 4-6 are repeated 29 times; ** Transfer tubes to a fridge (4°C)

4.6. Electrophoresis of DNA products

Agarose	Molecular biology quality
Ethidium bromide	C_{21} H_{20} N_3Br, 10mg/mL, (Mutagen!), store at 4^0C.
TBE-buffer, (10x)	1 *M* Tris, 1 *M* Boric acid (H_3BO_3), 20 m*M* EDTA, 50 m*M* $MgCl_2$, pH: 8.1, store at 4^0C.
Sample-buffer	4 x TBE-buffer, 10% Ficoll 400 (Pharmacia), 0.4% sodium dodecyl sulphate, Pharmacia), 0.4 mg/mL Bromophenol blue, (Sigma), store at 4^oC.
100 bp DNA ladder	15 blunt-ended fragments between 100 and 1500 by in multiples of 100 bp (Gibco)

(1) Prepare stock Tris-borate/EDTA electrophoresis buffer (10xTBE buffer), pH 8.1 store at 4^oC.

1 *M* Tris $C_4H_{11}NO_3$	121.1 gm
1 *M* H_3BO_3	61.8 gm
20 m*M* EDTA	7.44 gm
50 m*M* $MgCl_2$ x 6 H20	10.16 gm
Add H_2O	to 1000 mL

(2) Prepare a 25 mL 2% agarose gel, containing 2.5 μL of ethidium bromide in 1 x TBE buffer (use the 8 place comb). Fill the chamber with buffer solution (1 x TBE) until the top surface of the gel is submerged approximately 1 mm (about 400 mL).

(3) Add 5 μL of sample buffer to each sample, centrifuge (10,000 x g, 20 s).

(4) Prepare the 100 bp DNA ladder (50 μL 100 bp ladder, 650 μL H_20, 300 μL sample buffer, mix).

(5) Load the samples (e.g. 10 μL) and the DNA ladder (e.g. 10 μL) into the agarose gel wells; connect the electrophoresis chamber to a 60V power supply and start the run (approx. 45 min.).

(6) The separated DNA products are detected by UV illumination. *Trypanosoma brucei* ssp. containing a DNA sequence of 173 bp that is amplified by these TBR primers (Moser *et al*., 1989). Photographs of the products can be taken under UV illumination using a camera (e.g. Polaroid©).

4.7. References

CLAUSEN, P.H., WIEMANN, A., PATZELT, R., KAKAIRE, D., POETZSCH, C., PEREGRINE, A., MEHLITZ, D. Use of a PCR assay for the specific and sensitive detection of Trypanosoma spp. in naturally infected dairy cattle in peri-urban Kampala, Uganda. Ann. N.Y. Acad. Sci., 849: 21-31 (1998).

MASAKE, R.A, MAJIWA, P.A.O., MOLOO, S.K., MAKAU, J.M., NJUNGUNA, J.T., MAINA, M., KABATA, J., OLE-MOIYOI, O.K., NANTULYA, M. Sensitive and specific detection of Trypanosoma vivax using the polymerase chain reaction. Experimental Parasitology, 85: 193-205 (1997).

DNA BANK IN IAEA LABORATORY SEIBERSDORF

A. DIALLO

Animal Production Laboratory
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1. INTRODUCTION

At the first RCM of this CRP held in 2001 in Antwerp (Belgium), it was agreed to set up a DNA reference bank at the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf. The DNA should originate from Trypanosome strains that are well selected and characterized in terms of their histories. The DNA will be sequenced and the data that will be obtained, as well as DNA samples, will be made available to researchers.

1.1. DNA Samples

In 2002, a total of 27 Trypanosoma DNA samples were received at the FAO/IAEA Laboratory at Seibersdorf. They are from counterparts from: Burkina Faso, Brazil, Uganda and Colombia. Unfortunately, sixteen samples were of poor quality and could not be analysed. The remaining 11 samples were used in the studied along with 3 DNA that were extracted from Trypanosome strains available at the Animal Production Unit in the FAO/IAEA Laboratory, Seibersdorf. The QiAamp DNA mini kit from Qiagen was used for the DNA extraction (no other method was tested).

1.2. Amplification and Sequencing of 14 Trypanosoma DNA samples

All 14 samples were tested with the "pan" Trypanosoma primers provided by Mr.D'Avila from Brazil (AF/BR, CF/BR) and also with KIN primers published by Mr Desquenes et al. (Int. J. Parasitol., 2001, 31, 610-614). Those primers allow the amplification of fragment in the internal transcribed spacer 1 (ITS1) region on the DNA.

As an example of the results obtained with the different couple of primers, the electrophoretic gel of the PCR products obtained with AF/BR is presented in photo 1. As can be seen on this photo, this couple of primers allows the distinction between the different taxons of Trypanosomes which were analysed. Similar results were also obtained with the Kin primers.

The amplification products that were obtained from the different DNA samples and primer combinations (AF/BR, CF/BR, KIN1/KIN2) were cloned and sequenced. The sizes of the different PCR products according to the sequence data are summarized in Table I. The PCR product of *T.congolense* is the longest with the size at around 1030-1060 for the pair of primers AF/Br. It has a sequence of about 230 nucleotides nearby the primer Br and that is absent from the other strains. A sequence of about 95 nucleotides long is well conserved.

TABLE I. SUMMARY OF THE SIZE OF THE DIFFERENT PCR PRODUCTS ACCORDING TO THE SEQUENCE DATA.

Identification	AF/BR	CF/BR	Kin1/Kin2
T.evansi		475	
T.spp	827		
T.c.s.			533
T.c.s. (Burkina)	1030	685	533
T.c. (Burkina)	1059	712	
T.c.			749
T.b.b.	829	475	
T.b.r.	826	474	
T.b.g. (Burkina)	827	475	532
T.b.g. (Uganda)	827	477	
T.b.	827		533
T.s.	728	388	
T.v. (Burkina)	586	249	
IT.v. (ILRI)	597	259	

Comparison of the sequence data shows two main groups (Fig. 1) group T.brucei and T.evansi and the group of *T. congolense-T.vivax and T. simiae.* In that second group, *T.simiae* and T.congolense are most close related than with T.vivax.

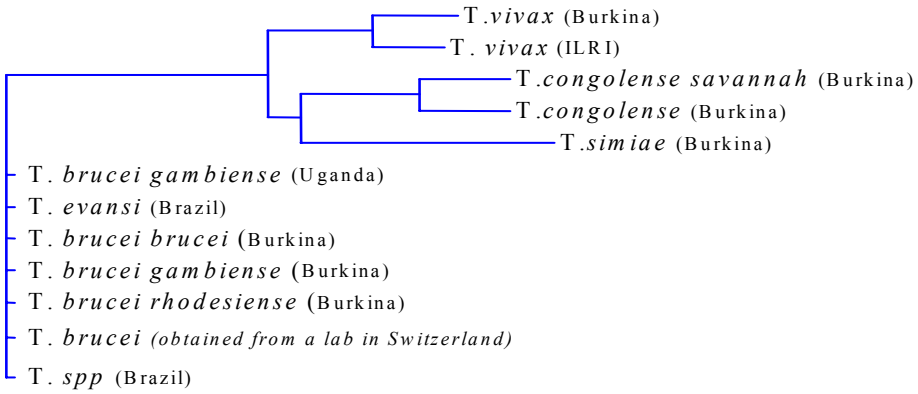

FIG. 1. Phylogenetic tree by analysing (Clustal) the sequence data of amplified ITS1 products from different Trypanosomes.

1.3. Development of a plasmid to be used as positive control in the PCR assay.

At present, the positive controls of the Trypanosome PCR assay is a sample with the actual target of the primers. Such a positive control can be a source of contamination of the test sample and false positive result. To avoid this, a plasmid was constructed with an insert corresponding to the target of the primers Kin1-Kin2 but with a deletion of about 40 nucleotides. Amplification of DNA with Kin1-Kin2 from this plasmid give a PCR product shorter than that obtained from a test sample. The two products can be differentiated easily in an electrophoresis gel (see Fig. 2). It is planned to develop such positive control for the primers AF/BR. Those plasmids could be used also by partners in a ring test or for testing the quality of primers.

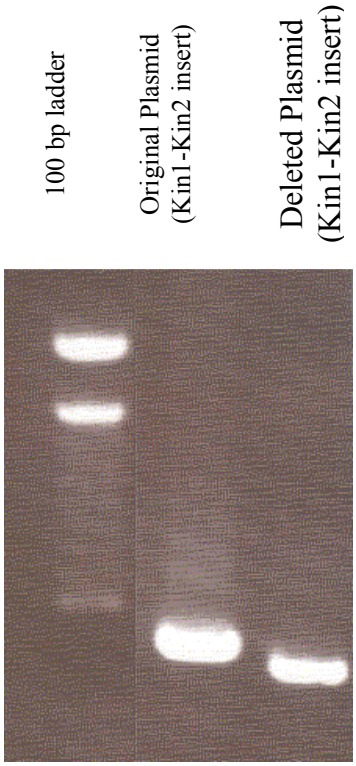

FIG. 2: Differentiation of products after amplification of plasmid DNA with *Kin1-Kin2*.

1.4. Template for Record of Trypanosoma DNA Bank

A template has been written to record the DNA that will be sent by partners to the FAO/IAEA Laboratory in Austria. When sending the material partners are requested to fill in the form. The form will be larger than this one reproduced here. The information needed is:

Trypanosoma species, laboratory code (if any), animal species from which the parasite was isolated, locality where the parasite was isolated, country of isolation, date of isolation, reference(s) if any, country and name of the laboratory which is providing the DNA and quantity of DNA provided.

Trypansome species	Laboratory code (if any)	Animal species from which isolated	Country of isolation of parasite	Locality where parasite isolated	Date of isolation	Reference (if any)	Quantity of DNA provided	Date of reception at APU

TRYPANOSOMA SPP. RING TEST PROTOCOL

G. VILJOEN

Animal Production and Health Section,
IAEA

(It is envisaged that the testing will be done annually.)

1. CRITERIA

(1) Test material will be chosen by the coordinating lab for incorporation into a proficiency panel after repeated testing by more than one analyst on multiple PCR runs on different days.

(2) Test material: suitable test material will be provided.

(3) Protocol and laboratory procedures: Each laboratory is allowed to use their own "in-house" test procedure (SOPs will nevertheless be provided by the respective laboratories for comparison and evaluation).

(4) Each PCR should be executed on two separate occasions.

(5) Test material used will be coded and sent as a blind panel to the participants. Each participating laboratory will be given a unique set of codes.

1.1. Test samples

A minimum of three samples will be included.

An unequivocal negative uninfected sample.
An unequivocal positive infected sample.
In addition, to establish sensitivity, one series of ten-fold dilutions of the positive control sample, will be included.

1.1.1. Transfer and sending of samples

(1) The co-ordinating laboratory will prepare samples and allocate codes. Samples will then be sent in a suitable form (e.g. lyophilized) as blind panels to the participating laboratories. A complete set of instructions with regards to content, reconstitution, storage and handling as well as deadlines will be included.
(2) Suitable records will be made of all samples prepared.
(3) Participants will be notified at least one month in advance (or longer depending on import regulations).
(4) Details supplied will include the projected date, method of shipment, the carrier, the air-way bill number etc.).
(5) Sample panels arriving in a questionable condition will be replaced.
(6) This activity could be the responsibility of the Seibersdorf laboratory.

1.1.2. Analysis, evaluation and recommendation

(1) Detailed records will be kept by all laboratories. The coordinating laboratory will also formulate a relatively flexible time-schedule and will store all data in a data-base
(2) The qualitative data will be scored as "positive", "negative" or "inconclusive".
(3) As a goal, at least 2/3 of the participating laboratories should obtain the same result in a proficiency test.
(4) Uninfected samples that test positive, or alternatively infected samples testing negative by more than one laboratory will be discarded.
(5) A false negative will be indicated when all dilutions from an infected source test negative
(6) A false positive will be indicated when at least one of all the dilutions from an uninfected source tests positive.
(7) PCR sensitivity will be based on number of organisms for practical reasons. This will probably only give an approximation of amount of DNA present, since DNA degradation can take place.
(8) Pass / fail criteria: a 10 fold discrepancy will be allowable.
(9) Data will be collected and summarized by the coordinator and distributed to the participating laboratories (who will be identified by code for anonymity) for discussions and further recommendations. A suitable strategy will be devised to correct any unwanted discrepancies. Data may be used for a publication.

1.2. Costs

The co-ordinating laboratory will cover the cost of preparing and distributing samples. It is understood that the individual laboratories will provide the man-power and cover the cost of test reagents used and tests performed.

STANDARDIZED PCR AND NESTED PROTOCOL

(1) TITRATIONS

Magnesium chloride concentration

Deoxyribonucleotide concentration

Primer (oligonucleotide) concentration

Template concentration

Enzyme (Taq DNA polymerase) concentration

(2) STANDARD PCR REACTION

Template (4.5 ng/µL)	1 µL
Primer 1 (3 pmol/µL, Gibco BRL)	1 µL
Primer 2 (3 pmol/µL, Gibco BRL)	1 µL
Buffer (Takara) 10 x	5 µL
dNTP's (Roche, 10 m*M*)	1 µL
Taq DNA polymerase Takara, Ex Taq, 2.5 U/µL)	1 µL
$MgCl_2$	5 µL

Water added to a final reaction volume of 50 µL

Buffer used in the PCR reaction contained no $MgCl_2$.

Template is a plasmid construct diluted to a concentration of 4.5 ng/µL

Titrations were done of the reagents separately, based on the standard PCR reaction described above.

(3) PCR REACTION PROTOCOL

94°C, 5 min

94°C, 30 s; 55°C, 30 s; 72°C, 45 s-for 30 cycles

(4) EXPECTED SIZE OF THE PRODUCT OBTAINED FROM THE PCR REACTION-1500 BP

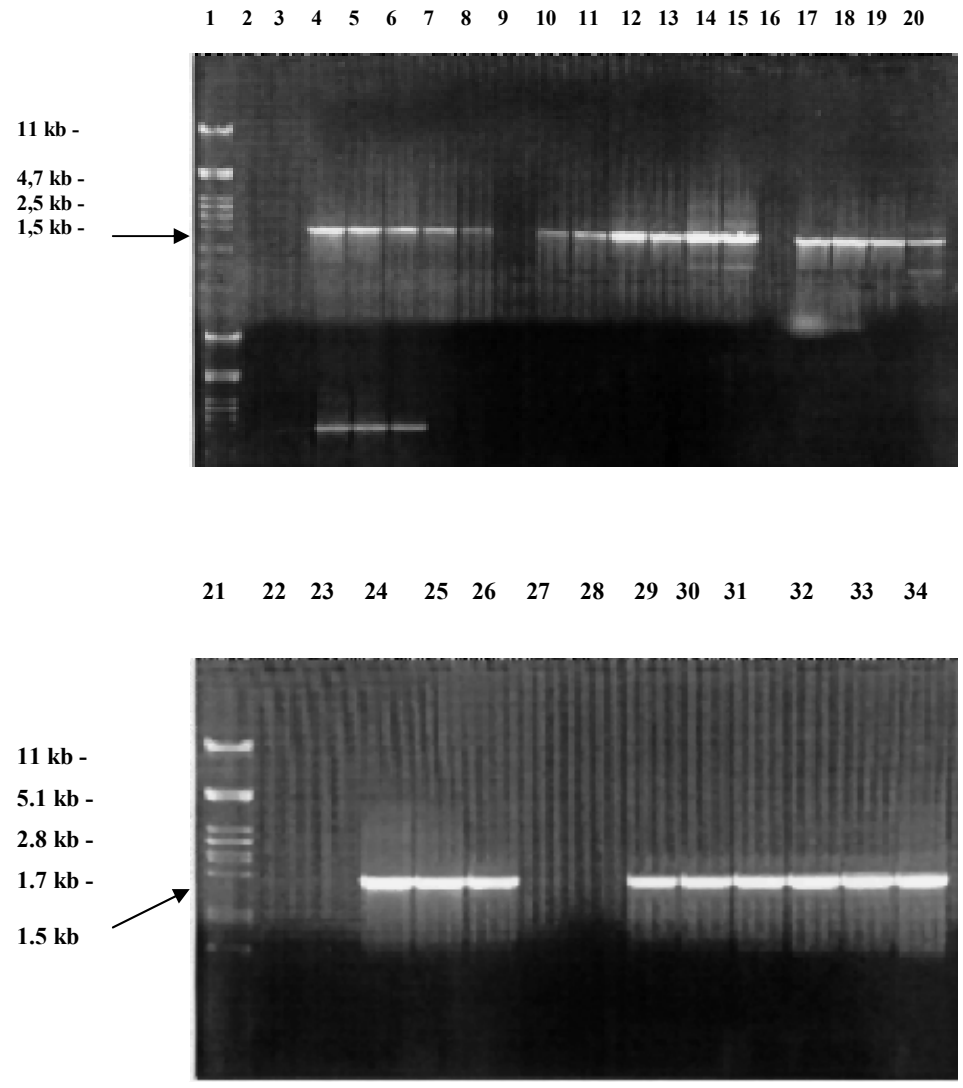

FIG. 1: Expected sizes of products indicated.

DETECTION OF PCR PRODUCTS VIA OLIGOCHROMATOGRAPHY (DIPSTICKS)

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Abstract

A concept from CORIS was developed under IAEA contract number 12851/RBF. Establishment of the dipstick test in the laboratory (proof of principle) and comparison of sensitivity and specificity with classic agarose detection was made.

1. BACKGROUND

The detection of PCR products is usually made using horizontal electrophoresis in agarose gels. This detection method is rather time consuming (+/- 1 h), a U.V. detection system is needed, and there are health and environmental risks because of the need for ethidium bromide to detect DNA in agarose (carcinogenic, disposal of EtBr). Moreover, this system is costly and not suitable for individual sample testing. To overcome these problems, a biotech company involved in molecular diagnostics, Coris Bioconcept, developed a single test format detection system, the Oligochromatography. This system has been first developed for *Toxoplasma gondii*, but could be adapted for the detection of Trypanosomal DNA. Coris Bioconcept, who has the IPR of the system, expressed their interest in collaborating to develop dipstick tests for human African Trypanosomiasis.

Oligochromatography as explained in Fig. 1 is a simple and rapid dipstick test for detection of amplified PCR products.

A complex made of PCR products, with incorporated haptens, hybridized with a specific gold conjugate probe binds to an anti-hapten antibody immobilized on the stick. This binding reaction will give rise to a collared signal.

Advantages are:

(i) hybridisation takes place at constant temperature (55°C or lower), (ii) different anti-hapten antibodies can be immobilised on one dipstick and thus allow one-step detection of different gene amplification products, (iii) the test takes only 5 min and doesn't need any specific material nor skill to be performed.

18S ribosomal RNA gene (rDNA) was chosen as the new target gene for PCR since (i) it is a multi copy gene (200 copies) and (ii) it contains both conserved and polymorphic sequences.

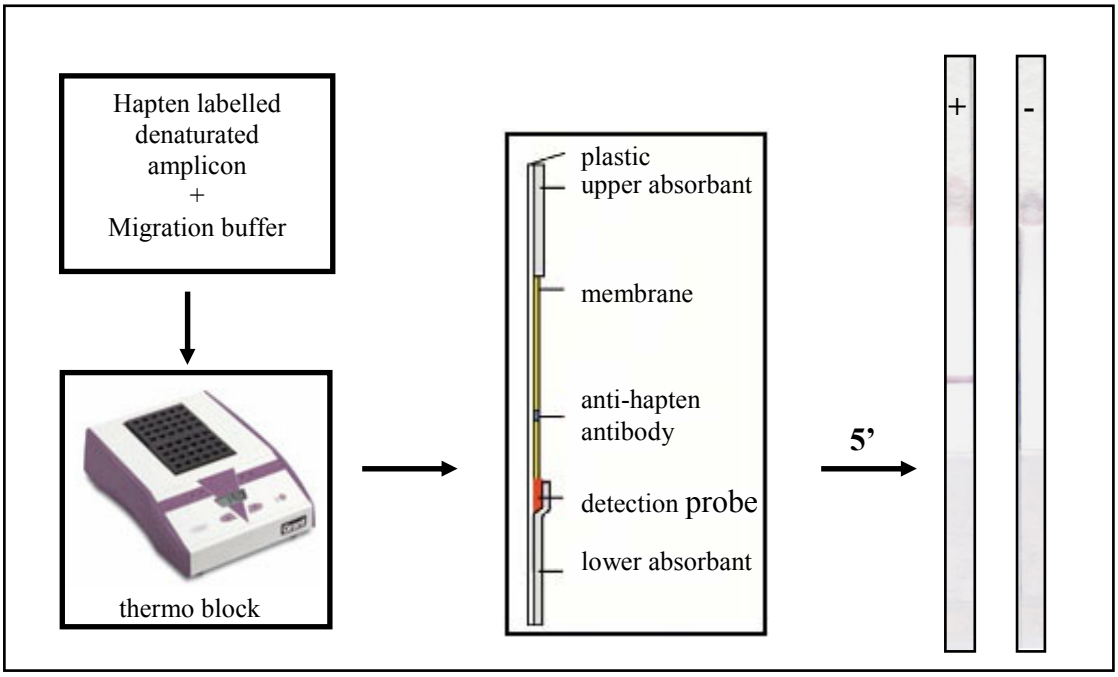


FIG.1. Overall representation of the PCR-Oligochromatography (Coris BioConceptIFG

2. OBJECTIVES OF DEVELOPMENTS THROUGH TECHNICAL CONTRACT

2.1. Development

Standard PCR protocols were adapted for use in the system: primers replaced by labelled primers and amplification conditions optimized. In first instance PCR-ISG and PCR-ITS, were used, since they seem to have the highest sensitivity. Later, more specific PCRs (SRA, TgsGP and RoTat) may be modified.

2.2. Evaluation

Use of PCR-dipstick detection test on the available DNA bank, including *T.b.brucei* (n=7), *T.b.gambiense* (n=9), T.b.rhodesiense (n=9), *T.evansi* (n=15), *T.equiperdum* (n=12), *T.congolense* (n=5) and *T.vivax* (n=4) (month 7-8). Use of PCR-dipstick detection test on available DNA from experimentally infected rabbits (n=50) and field samples (n=200).

3. RESULTS

- A Trypanosomatidae specific PCR on the 18S rRNA gene (rDNA) was developed and optimized in which the primers are situated in Trypanosomatidae conserved regions and the amplified region is polymorphic.
- A Trypanozoon specific Oligochromatography dipstick through specific probes was developed and optimized.

3.1. In silico selection of a target region in the 18S rRNA gene

In silico design of universal primers to amplify a short Trypanosomatidae specific DNA sequence by PCR. The reverse primer was biotin labelled.

Development of the PCR to amplify the Trypanosomatidae specific sequence.

Development of the PCR to amplify the specific sequences for all Trypanosomatidae.

- $MgCl_2$ concentration was optimized.
- Primers concentration was optimized.
- Annealing T was optimized
- Annealing and elongation time was optimized.
- Polymerase concentration was optimized.
- Cycle number was optimized.

Note. During optimisation of the PCR, there were problems with contamination. After sequencing this contamination was identified as the specific amplification of Bodo sp. rDNA. Bodo sp. is a free living flagellate which lives in water. This Bodo DNA was proven to be present in the QiaAMP extraction kit buffers. This problem is contained now.

3.2. Optimization results

The following PCR primers and protocol give the best results.

3.3. Primers

18S-F:	5'-CGCCAAGCTAATACATGAACCAA-3'	Tm: 66.6°C
18S-R:	5'-Biotin-TAATTTCATTCATTCGCTGGACG-3'	Tm: 66.6°C

3.4. PCR protocol 50 µL

PCR mix:	Water	
	Buffer	1 X
	$MgCl_2$	2.5 m*M*
	dNTP	200 µ*M* each
	F-primer	0.2 µ*M*
	R-primer	0.8 µ*M*
	Hot Start Taq	0.5U
	DNA	

3.5. PCR programme

94°C	15 min	
94°C	30 s	45X
58°C	30 s	
72°C	30 s	
72°C	1 min	

3.6. In silico design of a Trypanozoon specific probe

Probe:

T. brucei: 18S-PR-B TTGTGTTTACGCACTTG Tm: 49.1°C 17bp

3.7. Development of the PCR-Oligochromatography detection system with avidin immobilized on the dipstick in collaboration with Coris Bioconcept

The avidin concentrates the amplicon and only if the *Trypanozoon* amplicon is present the dipstick will show a visible band.

(1) Primer concentration in PCR was modified for optimal performance of the Oligochromatography.

(2) Primer ratio in PCR was modified for optimal performance of the Oligochromatography.

(3) Avidin concentration on dipstick was optimized.

(4) Gold conjugation on probe was optimized.

(5) Migration buffer was optimized.

3.8. Detection Limits

The detection limit of the PCR assay on human blood spiked with decreasing numbers of *T.brucei.* Comparison of detection by agarose gel and detection by oligochromatography.

(1) 10^4 parasites / 180 µL blood

(2) 10^3 parasites / 180 µL blood

(3) 10^2 parasites / 180 µL blood

(4) 10 parasites / 180 µL blood

(5) 1 parasite / 180 µL blood

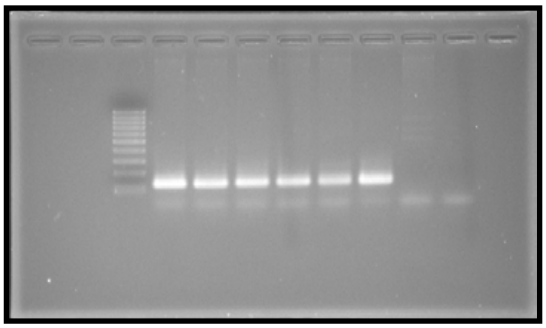
(6) Pos. control PCR *(T.b*. DNA)

(7) Neg. control extraction

(8) Neg. control PCR (H_2O)

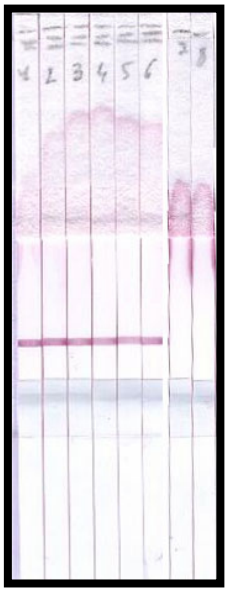
DNA extraction from the blood samples was performed using the QIAamp DNA blood kit (Qiagen, Hilden, Germany).

Detection with 2% agarose gel



Detection limit 1 parasite / 180 µL blood

Detection with dipstick



Detection limit 1 parasite / 180 µL blood

3.9. Check on the specificity of the PCR assay on non-target pathogen DNA

(1) The PCR amplifies all *Trypanozoon* (*in silico*, not yet tested experimentally).

(2) The PCR does not amplify other pathogens present in the endemic regions (*Plasmodium falciparum*, *microfilaria* and *Mycobacterium* DNA, checked experimentally).

(3) The PCR amplifies *Leishmania* which is endemic in East-Africa because this genus also belong to the *Trypanosomatidae* group [detected by electrophoresis on agarose gel].

3.9. Check of the specificity of the Oligochromatography dipstick on human blood spiked with *Trypanozoon* and non target PCR product of *Leishmania.*

The Trypanozoon Oligochromatography test detects *T.b. gambiense, T.b.rhodesiense* and in silico all the other subspecies within the *Trypanozoon* group (not tested experimentally yet) but does not detect the Leishmania PCR products.

4. CONCLUSIONS

A first prototype PCR-Oligochromatography assay for molecular diagnosis of *Trypanozoon* infections was successfully developed. The assay, so far, is specific for *Trypanozoon* and has a detection limit of 1 parasite in 180 µL of blood.

5. FUTURE WORK

To test experimentally the specificity of the 18S rDNA PCR on all the subspecies within the Trypanozoon group.

To test experimentally the specificity of the oligochromatography dipstick on all the subspecies within the Trypanozoon group.

5.1. Development of the internal controls on the rear side of the dipstick

Control for PCR

A synthetic oligonucleotide will be added to the PCR mix which will be amplified by the same primers as the target sequence. This control oligonucleotide will always be amplified if the PCR works well. On the backside of the dipstick a gold conjugated probe specific for the internal control sequence will be placed in the probe conjugate pad. The anti-hapten antibodies will be immobilized on a specified place on the dipstick. During Oligochromatography the internal control PCR product will be detected on the backside of the dipstick through hybridisation with the internal control specific probe (see Fig. 2). In comparison with the gel detection of PCR products this inclusion of the internal control for PCR is a great improvement. The internal control may also be included in a gel detection format. However the Oligochromatography detection format has the advantage that an internal control with exactly the same length as the target amplicon can be used which is obviously not the case with the gel detection format.

Control for migration

Next to the internal control for PCR an internal control for migration will also be included on the backside of the dipstick. An oligonucleotide complementary to the internal

control probe for PCR will be immobilized (see Fig. 2) on a specified place on the backside of the dipstick. During migration the gold labelled internal control probe will hybridize on this oligonucleotide which will give rise to a coloured signal. If the dipstick works properly this control line has always to be positive.

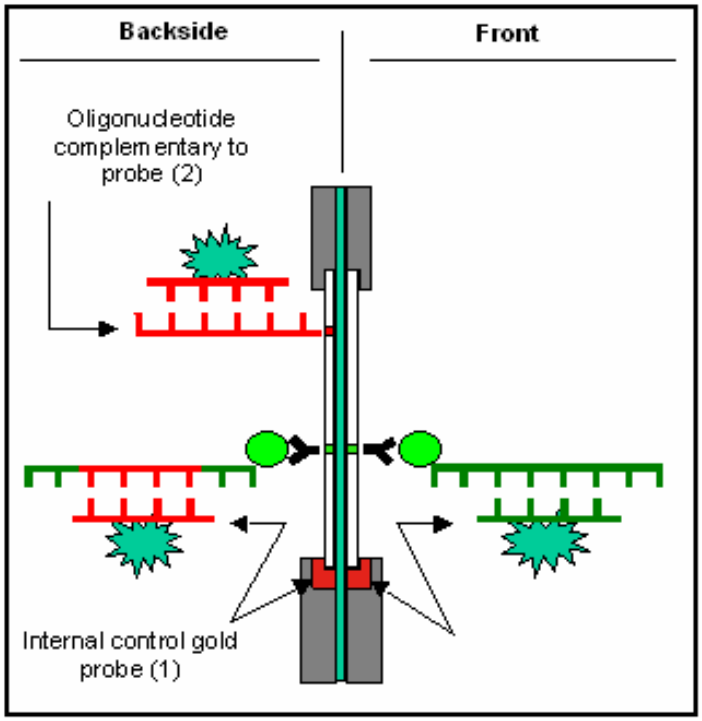


FIG. 2. Representation of the detection of the target PCR product at the front of the dipstick and internal controls for PCR (1) and for migration (2) on the rear of the dipstick.

Short laboratory (Phase I) evaluation of the prototype PCR-Oligochromatography for Trypanozoon detection

Production of sufficient prototype tests for the evaluation

Testing the diagnostic sensitivity and specificity of the prototype PCR-Oligochromatography test on available DNA from experimentally infected rabbits (n=50) and on a collection of blood samples from confirmed sleeping sickness patients (n=100) and the negative endemic controls (n=100) from R.D. Congo.

6. PERSPECTIVES

When this project is finished and a proof-of-principle evaluated prototype of the PCR-Oligochromatography for *Trypanozoon* detection is available, a Phase II evaluation can be started and managed by the IAEA in collaboration with Coris Bioconcept. If successfully evaluated a Phase III large scale evaluation can be started. Now the technique is available, the same strategy could be followed to diagnose animal Trypanosomiasis. *In silico* work showed us that the same PCR could be used to amplify *T.vivax, T.evansi, T.equiperdum, T. brucei, T.vivax* and *T.congolense* and that it will be possible to design specific probes to discriminate between the *T.congolense* group, the *T.vivax* group and the *Trypanozoon* group.

7. RING TESTING OF TRYPSTICK UNDER THE CRP

At the final RCM in Vietnam, 2005 it was decided to make a ring test to examine the performance of the Trypstick.

The reagents were supervised by F. Claes in Antwerp who also organised the transportation of the Trypsticks from the company.

Five contract or agreement holder laboratories were involved as shown in Table below as well as the laboratory in Antwerp.

	Name	Address	Info	RC number
1.	P.Solano	P. Solano Institut Pierre Richet s/c IRD, rue Fleming, 04BP 293, Abidjan 04 Côte d'Ivoire	Tel: (225) 21 35 43 70/ 21 35 70 67; Fax: (225) 21 35 40 15 E-mail: solano@ird.ci	IVC 11413
2.	J. Kangethe Kinyua	J. Kangethe Kinyua Kenya Agricultural Research Institute-Trypanosomiasis Research Center P.O. BOX 362 KIKUYU Kenya	Tel: 254 66 32960 Fax: E-mail: jkkinyuafr2001@yahoo.fr ; ketri@africaonline.co.ke	KEN 11414
3.	Viet Khong Nguyen	Viet Khong Nguyen National Institute of Veterinary Research 86 Truong Chinh, Dong Da, Hanoi, Vietnam	Tel: 0084 4 868 7642 Mobile 0913082035 Fax: 0084 4 8694082 nguykhon@fpt.vn	VIE 11420
4	J. Enyaru	J. Enyaru Livestock Health Research Institute (LIRI), P.O.Box 96, Tororo, Uganda	Tel: 226 20 97 20 53 Fax: 226 20 97 23 20 E-mail: jenyaru@hotmail.com	UGA 11418
5	P. Henning Clausen /	Peter Henning Clausen Institute of Parasitology and International Animal Health Freie Universitaet Berlin, Königsweg 67, D-14163 Berlin, Germany	Tel: +49 30 8386 2514 (office) Cell: + 49 179 862 42 00 (Cell Phone) E-mail: tropvetm@komma.zedat.fu-berlin.de	GFR 11412

Each laboratory received the following items.

- 4 boxes containing the PCR-Oligo Trypsticks (25 tests/kit, 4 kits, total: 100 tests)
- 1 box containing 23 control DNA samples for the Trypstick ring trial evaluation
- 4 boxes containing PCR-mix and polymerase, frozen
- The laboratories were told to fill in a receipt from and return it when they received the items.

A form was included to summarize data obtained in the study as shown below.

7.1. Checklist for Ring Test

For our own interpretation of the results of the *Trypanozoon*-PCR-OC ring trial, we kindly ask you to complete following checklist about the material you used during performing the tests.

(1) The PCR assay was performed in following thermocycler (please specify brand and type):

(2) The PCR assay was performed in following tubes (please specify brand and catalogue number):

3. The PCR assay was performed with ☐ heating lid

☐ mineral oil

4. The Oligochromatography assay was performed in a

☐ heating block with 13 mm tubes

☐ heating block with 1.5 mL tubes

☐ water bath with 13 mm tubes

5. The PCR mix at -20°C was only thawed one time?

☐ Yes

☐ No

If no please specify

6. I followed the protocol as described:

☐ Yes

☐ No

If no please specify

7. I encountered problems during performing the tests:

☐ Yes

☐ No

If yes please specify

8. Further remarks:

7.2. Test results 1

Please indicate + for positive result

- For negative result

Sample	*Trypanozoon* line	MC line	IPC line	Test result
Pos. control				
Neg. control				
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				

MC line: Migration control line (backside of the stick)

IPC line: Internal PCR control line (backside of the stick)

Test result: For instructions see protocol page 3

7.3. Test results 2

Please indicate + for positive result

- for negative result

Sample	*Trypanozoon* line	MC line	IPC line	Test result
Pos. control				
Neg. control				
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				

MC line: Migration control line (backside of the stick)

IPC line: Internal PCR control line (backside of the stick)

Test result: For instructions see protocol page 3

7.4. Test results 3

Please indicate + for positive result

- for negative result

Sample	*Trypanozoon* line	MC line	IPC line	Test result
Pos. control				
Neg. control				
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				

MC line: Migration control line (backside of the stick)

IPC line: Internal PCR control line (backside of the stick)

Test result: For instructions see protocol page 3

7.5. Actual results

CHECKLIST

For our own interpretation of the results of the *Trypanozoon*-PCR-OC ring trial, we kindly ask you to complete following checklist about the material you used during performing the tests.

(1) The PCR assay was performed in following thermocycler (please specify brand and type):

GeneAmP PCR Systems 9700 from Applied Biosystems Part no N8050200

(2) The PCR assay was performed in following tubes (please specify brand and catalogue number):

GeneAmP autoclaved reaction tubes with caps,part no N801-0612

(3) The PCR assay was performed with √ heating lid

☐ mineral oil

(4) 4. The Oligochromatography assay was performed in a

☐ heating block with 13 mm tubes

☐ heating block with 1.5 mL tubes

√ water bath with 13 mm tubes

(5) The PCR mix at -20°C was only thawed one time?

√ Yes

☐ No

If no please specify

(6) I followed the protocol as described:

√ Yes

☐ No

If no please specify

(7) I encountered problems during performing the tests:

☐ Yes

√ No

If yes please specify

(8) Further remarks:

The protocol was simple and straight forward to follow, with few manipulations to carry out.

TEST RESULTS 1

Laboratory: Livestock health Research Institute (LIRI), Tororo, Uganda

Executor: Dr. John Enyaru

Date: 09-02-2006

Please indicate + for positive result

- for negative result

Sample	***Trypanozoon* line**	**MC line**	**IPC line**	**Test result**
Pos. control	+	+	-	+
Neg. control	-	+	+	-
1	+	+	-	+
2	-	+	+	-
3	-	+	+	-
4	-	+	+	-
5	-	+	+	-
6	-	+	+	-
7	+	+	-	+
8	+	+	-	+
9	-	+	+	-
10	-	+	+	-
11	+	+	-	+
12	-	+	+	-
13	+	+	-	+
14	+	+	-	+
15	-	+	+	-
16	-	+	+	-
17	+	+	-	+
18	-	+	+	-
19	+	+	-	+
20	-	+	+	-
21	+	+	-	+

MC line: Migration control line (backside of the stick)

IPC line: Internal PCR control line (backside of the stick)

Test result: For instructions see protocol page 3

TEST RESULTS 2

Laboratory: Livestock health Research Institute (LIRI), Tororo, Uganda

Executor: Dr. John Enyaru

Date: 09-02-2006

Please indicate + for positive result

- for negative result

Sample	*Trypanozoon* line	MC line	IPC line	Test result
Pos. control	+	+	-	+
Neg. control	-	+	+	-
1	+	+	-	+
2	-	+	+	-
3	-	+	+	-
4	-	+	+	-
5	-	+	+	-
6	-	+	+	-
7	+	+	-	+
8	+	+	-	+
9	-	+	+	-
10	-	+	+	-
11	+	+	-	+
12	-	+	+	-
13	+	+	-	+
14	+	+	-	+
15	-	+	+	-
16	-	+	+	-
17	+	+	-	+
18	-	+	+	-
19	+	+	-	+
20	-	+	+	-
21	+	+	-	+

MC line: Migration control line (backside of the stick)

IPC line: Internal PCR control line (backside of the stick)

Test result: For instructions see protocol page 3

TEST RESULTS 3

Laboratory: Livestock health Research Institute (LIRI), Tororo, Uganda

Executor: Dr. John Enyaru

Date: 09-02-2006

Please indicate + for positive result

- for negative result

Sample	***Trypanozoon* line**	**MC line**	**IPC line**	**Test result**
Pos. control	+	+	-	+
Neg. control	-	+	+	-
1	+	+	-	+
2	-	+	+	-
3	-	+	+	-
4	-	+	+	-
5	-	+	+	-
6	-	+	+	-
7	+	+	-	+
8	+	+	-	+
9	-	+	+	-
10	-	+	+	-
11	+	+	-	+
12	-	+	+	-
13	+	+	-	+
14	+	+	-	+
15	-	+	+	-
16	-	+	+	-
17	+	+	-	+
18	-	+	+	-
19	+	+	-	+
20	-	+	+	-
21	+	+	-	+

MC line: Migration control line (backside of the stick)

IPC line: Internal PCR control line (backside of the stick)

Test result: For instructions see protocol page 3

8. OVERALL LABORATORY RESULTS

A sample set of 23 samples consisting of a positive (*T.b.brucei* DNA) and negative (human DNA) control and 21 coded "blind" identical DNA samples was sent to all participating laboratories together with the necessary standardized test reagents, a test report sheet, and a standard operating procedure (SOP), so the only sources of variability between the laboratories are the manipulator and the PCR thermocycler. Each laboratory received sufficient materials to perform PCR-Oligochromatography in triplicate for each sample.

The blind samples included a two-fold serial dilution series of *T.b.brucei* control DNA (7 samples) to evaluate the analytical sensitivity of the assay, the five different (sub)-species of *Trypanozoon* (*T.b.brucei*, *T.b.gambiense*, *T.b.rhodesiense*, *T.evansi*, and *T.equiperdum*), and nine non-*Trypanozoon* DNA samples to assess the analytical specificity of the diagnostic test (Table I).

DNA was extracted using the QIAamp DNA mini kit (Qiagen, Germany) according to manufacturer's manual and was quantified with a Nanodrop (Isogen, Belgium). 200 µL of each sample was sent on dry ice to each participant by express courier. The PCR-Oligochromatography protocol was performed as described by Deborggraeve et al. (2006).

The results of this multicenter trial were analysed using to the formulae described by Vandervoet et al. (2001). The advantage of these formulae is that they can be used to evaluate qualitative rather than quantitative data.

Two main parameters were analysed *in casu* the accordance (ACC) or intra-laboratory repeatability which is defined as the percentage chance of finding the same result for two identical DNA samples analysed in the same laboratory under standard operating conditions (independent from whether the result is correct or not), and the concordance (CON) or inter-laboratory reproducibility which is defined as the percentage chance of finding the same result for two identical samples analysed in different laboratories under standardized conditions. Confidence intervals were calculated via Monte-Carlo / Markov model with 5.000 iterations (ref).

Statistical evaluation from the data set, with 95% confidence intervals (CI) gave following results: an accordance of 88.7% (CI 84.4-92.5%), and a concordance of 88.1% (CI 84.3 – 92.3%). These data and their distribution are presented in Figs 1A and 1B respectively. Note that the results from lab 5 were excluded from this calculation due to multiple positive results in the negative sample population, possibly due to cross-contamination or errors during test performance. Thus, the final analysis was performed on the data from the 5 remaining laboratories.

The analytical sensitivity of the PCR-Oligochromatography assay was 2.5 fg of DNA per PCR reaction in 4 out of 5 laboratories (excluding the results from laboratory 5) and 20 fg DNA in laboratory 3. This means that in all laboratories evaluated, the assay can detect up to 1 parasite per reaction, since we assume that the genome of one Trypanosome is 0.2 pg. The target sequence of the assay is a multicopy 18S sequence which explains the higher analytical sensitivity on extracted DNA samples.

The assay was developed in such way to be *Trypanozoon* specific. Results from this trial show that this is indeed the case, except for some occasional false positive results (Table

8.1. Results for accuracy and concordance are satisfactory

This study shows that it is feasible to organize a trial in different continents (and including developing countries). To our knowledge, this is the first multicenter collaborative trial to be performed for diagnostic tests for human or animal Trypanosomiasis.

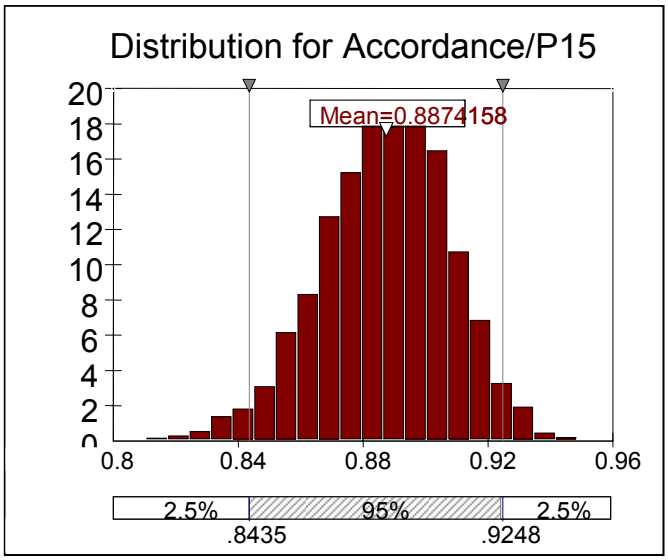
The results show that (i) PCR-oligochromatography may serve as diagnostic test for HAT, after a further phase III evaluation, (ii) this statistical approach may be used in the future to analyze other newly developed tests for HAT or animal Trypanosomiasis.

TABLE I. LABORATORY RESULTS IN THE COLLABORATIVE TRIAL OF THE PCR-OLIGOCHROMATOGRAPY ASSAY (3 REPETITIONS PER LABORATORY)

Sample	Expected	Lab 1			Lab 2			Lab 3			Lab 4			Lab 5			Lab 6		
	Result	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
T.b.brucei AnTat 2.2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Human DNA	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
T.b.brucei 2.5 fg	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1
T.b.brucei 5 fg	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	1	1	1	1
T.b.brucei 10 fg	1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	1
T.b.brucei 20 fg	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
T.b.brucei 80 fg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T.b.brucei 320 fg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T.b.brucei 1280 fg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T.b.gambiense LiTat 1.3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T.b.rhodesiense AnTat 25.1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T.evansi RoTat 1.2	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1
T.equiperdum OVI	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
T.b.gambiense AnTat 9.1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1
T.congolense TRT 17	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
T.vivax ILRAD 700	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Theileria parva	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Leishmania	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
Plasmodium	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0
Schistosoma	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Bovine DNA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Trypanosoma cruzi	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0
Trypanosoma rangeli	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0

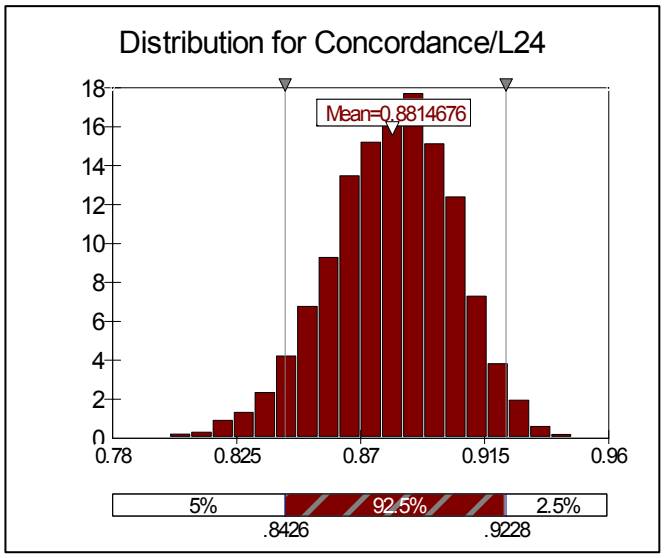
8.2. The accordance

Intra-laboratory repeatability was 88.7% (CI 84.4-92.5%)



8.3. The concordance

Inter-laboratory “reproducibility was 88.1% (CI 84.3-92.3%)



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