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SEROLOGICAL AND MOLECULAR DIAGNOSTIC TOOLS WITH THEIR ADVANTAGES AND DISADVANTAGES OF ANIMAL TRYPANOSOMOSIS: A REVIEW

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ABSTRACT

Animal trypanosomosis is caused by trypanosome species such as a *T. conglonse, T. vivax* and *T. b. brucei* and Mechanically transmitted trypanosomes *T. evansi, T. vivax* and *T. equiperdum*. The most important trypanosomes in terms of economic loss in domestic livestock are the tsetse transmitted species. The disease is manifested by different clinical signs and symptoms Apart from clinical signs, demonstration of the trypanosome in body fluid/tissue is of great value during chronic infection and in early phase the number of parasites in blood is too low to be detected. To avoid such problems in diagnosis of animal trypanosomosis techniques based on serology and molecular tools like CFT, Trypanolysis, CATT, Ab-ELISA and Ag-ELISA have been developed. More over PCR diagnosis of trypanosomosis is the widely used technique. The species specific PCR are labor intensive and expensive to perform to diagnose the about 10 trypanosomes species affecting animals. Therefore recently PCR based on internal transcribed spacer of ribosomal DNA is highly sensitive and specific, and cost effective as it allows detection of all trypanosomes in single PCR. Serological tests have a problem, they don't discriminate between past and current infection since antibodies can persist in circulation for long even after the infection has been cleared. Molecular diagnostic tests are more sensitive and specific. They detect current infection since they target DNA of the pathogen. Molecular amplification tests amplify DNA of the infecting pathogen to produce millions or billions of copies of the target DNA hence increasing the sensitivity of the test. The use of pathogen specific oligonucleotide primers and probes ensures increased specificity. However, molecular tests are expensive, time consuming, require sophisticated laboratories and expensive equipment, skilled labor, electricity, reagents, and may not be performed in the field settings. Now a days researches are geared towards development of cheap, rapid, easy to use, sensitive, specific, penside /field based diagnostics.

KEYWORDS: Animal, Diagnostic, Molecular, Serological, Trypanosomosis

INTRODUCTION

Animal trypanosomosis, caused by wider number of trypanosome species and carried with higher prevalence by a greater number of Glossina species, is invariably the greater epidemic across the African continent with direct economic consequences.[1]

Trypanosomosis is among the well-known constraints to livestock production in Africa as it causes a serious and often fatal disease of livestock mainly in the rural poor community and rightfully considered as a root cause of poverty in the continent.[2]

The most important trypanosomes in terms of economic loss in livestock are the tsetse-transmitted species such as *T. congolense*, *T. vivax* and *T. brucei*.[3] Closely related *T. brucei* subspecies, *T. b. rhodesiense* and *T. b. gambiense*, cause human sleeping sickness. It was estimated that about 50 million people[4] and 48 million cattle[5] are at risk of contracting trypanosomosis.

The disease is considered to be the most important in cattle, but it can also cause serious loss in camels, horses, sheep, goats and pigs.[6] Various researchers indicate that about 10 million square Km of sub –Sahara Africa is infested with tsetse flies, extending through the 38 countries.[7] It is estimates that some 45-60 million cattle live under tsetse transmitted trypanosomosis risk in that tsetse infested areas. The number of cattle per square km area is those tsetse infested areas is estimated to be 8-9

while tsetse in free areas is 14.4. This implies that 62% of total potential for increasing cattle production had the AAT has been controlled.[8]

Trypanosomosis has serious impacts on the individual animal and national development endeavors. On an individual basis, untreated animal or human trypanosomosis will lead, at best, to a chronic debilitating condition and at worst, to death. Furthermore, animals infected with trypanosomes are reported to have reproductive disorders[9] and it can cause severe losses on production performance in cattle and water buffalo.[10]

The economic loss due to AAT is estimated to be 2 billion us dollar per year. Animal trypanosomosis can be diagnosed based on parasitological examination of specimen, serological and molecular techniques. These diagnostic tests vary in sensitivity and specifically detecting the trypanosomes. For control and prevention of the disease, accurate diagnosis of animal trypanosomosis deems necessary.[11] Thus, the objectives of this review paper are to assess the existing serological and molecular diagnostic tools of animal trypanosomosis and to review the advantages and disadvantages associated with these diagnostic tools.

TAXONOMY OF TRYPANOSOMES

Scientifically trypanosomes are Classified under phylum protozoa, Sub phylum Sarcomastigophora Super class Mastigophora Class Zoomagophora Order Kinetoplastidae Sub order Trypanosomatina Family Trypanosomatidae Genera Trypanosoma Major sections Stercorarian and Salivarian.[12] Species of Trypanosoma infecting mammals grouped in to two distinct sections (Table 1). These are Stercorarian and Salivarian based on their site of development in their vector and on mode of transmission.[13,14] The salivarian to which the causative agents of African Trypanosomes are grouped is characterized by anterior station development and inocculative transmission. By virtue of variable Surface Glycoprotein (VSG) genes, they are the only Trypanosoma to exhibit Antigenic Variation. There are 4 sub-genera in this section (Table 2). These are: Duttonela, Nannomonas, Trypanozoo and pycomonas.[3]

Table 1: Sub-genera of salivarian section of Trypanosomes.

Sub genera	Species included	Development in vectors	Morphological characteristics
Vivax (duttonella)	*T. vivax* *T. uniforn*	Development in tsetse occurs only in the proboscis Mechanically transmission by biting flies	Mono morphic Free flagellum present Kinetoplast larger and usually terminal
Congolense (Nannomonas)	*T. congolense* *T. siminiae*	Development in the tsetse ; Mid gut and proboscis	Small forms usually no free flagellum, kinetoplast medium sized and marginally located
Brucei (Trypanozoon)	*T. brucei* *T.b. gambiense* *T.b. rhodesiense* *T.b. evansi* *T.b. equiperdum*	Development in tsetse fly; mid gut and salivary gland Mechanical transmission for *T.evasi* Sexual transmission for *T.equiperdum*	Pleomorphic ,slender, intermediate, Stumpy Kinetoplast small and sub terminal but invisible with light microscopefor *T. equiperdum* undulating membrane conspicuous , posterior end ; taper to A point except stumpy forms.

Source:[15]

MORPHOLOGY AND GENOME OF AFRICAN ANIMAL TRYPANOSOME

A sound knowledge of the basic features of the various trypanosomes enables the identification of each species and so the exact cause of the disease. Once the basic features possessed by all trypanosomes are appreciated, the diagnostic differences can be recognized and the species identified.[12]

Trypanosomes are unicellular, flagellated organisms which live in the blood or tissue fluids of their vertebrate hosts. Trypanosomes have a body varying in different species from 12-30μm long, an anterior and posterior end, a kinetoplast, a nucleus and sometimes some granules (Fig. 1). Reproduction is by a process of division to produce two daughter cells (binary fission). The location of these organelles is used in differentiation between the species. Trypanosomes move by twisting their body into S shape. The main power of this movement comes from flagellum (anterior end).The speed of movement is very variable: *T. vivax* moves quickly across the field of microscope, *T. brucei* may move considerable distance but less lively and *T. congolense* is active but confined to the same area.[12]

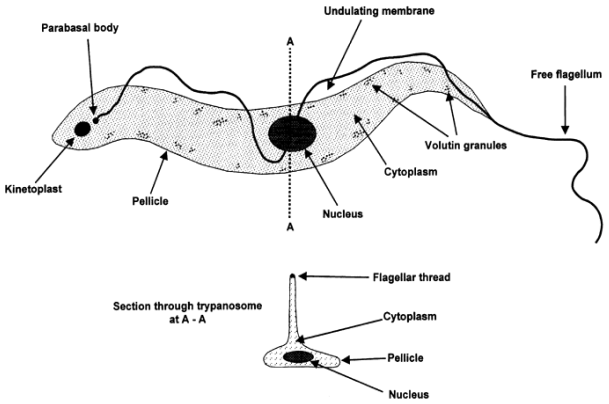

Figure 1: A diagrammatic illustration of the fundamental features of a trypanosome (trypomastigotes) as seen in a stained preparation made from the blood of an infected animal.
Source:[12]

Kinetoplastids are eukaryotes and hence exhibit conventional features such as the presence of nucleus delimited by a nuclear membrane and organelles such as ER, the Golgi apparatus, the endo/exocytosis system, the mitochondrion and others. However, many of these organelles exhibit specific and sometimes extreme features often found only in the Kinetoplastids. These include the presence of kinetoplast DNA, flagellum and flagellar pocket, unique gene regulation, RNA editing, and the presence of the glycosomes.[16]

DIAGNOSIS OF ANIMAL TRYPANOSOMOSIS

The diagnosis of African Trypanosomosis is more or less the same. It includes applications of clinical signs, parasitological tests, serological tests and molecular techniques. Various levels of accuracy are required in this diagnosis that enable us species level identification. Of course simple presence of pathogenic trypanosomes can be sufficient for a treatment decision, while *Trypanosoma species*, subspecies, type or even isolate identification can be required for medical, sanitary, taxonomic epidemiological or research purposes.[17]

Identification of trypanosomes has been based on microscopic observation (such as morphology, morphometric and motility of the parasites in host tissues), host range, geographical distribution, and predilection sites in the cyclical vector and also by the ability of the parasites to grow *in vivo* or *in vitro*.[13] However those methods are not accurate enough for species and sub species level identification. Moreover their specificity and sensitivity is also questionable. According to,[18] using such a method is laborious and detection of the parasite requires presence of large amount of the parasite in the sample. Moreover the visual examination doesn't allow us identification below subgenus level and also it fails to detect mixed infection and immature stages. Thanks to molecular biology the limits of these old methods for sensitivity and specificity is now solved. Here below are summary of the existing diagnostic tools of trypanosomosis.[18]

1. Clinical Signs and Symptoms

The African trypanosomosis is generally characterized by chronic infection; they are mostly clinically manifested by Anemia which is progressive, weakness, intermittent fever and emaciation.[14,19] But those clinical signs are not specific to the disease. According to[20] surra can be diagnosed by its clinical signs, but they are not sufficient for specific diagnosis. Dourine is characterized by edematous lesions, anemia and progressive emaciation[21,22] which can help us for tentative diagnosis and requires farther diagnosis to confirm it. The clinical signs of African trypanosomosis can only be used for tentative diagnosis prior to treatment and prior to sample collection for research.[20]

2. Parasitological methods

Different approaches can be employed to detects trypanosome species from blood samples or vector tissue including such as hematocrit centrifugation techniques (HCT),[23] Mini anion exchange centrifugation techniques (mACT) and Xenodiagnosis.[23]

The isolation of the parasite from blood of infected host is the only true gold standard diagnosis test. However, those techniques are insensitive, mainly because of the periodical occurrence of parasitemia in the host. In addition, the tests themselves have inherent weakness that limits their sensitivity.[24] Techniques of parasite detection such as direct microscopy, animal inoculation and concentration techniques can't always detect current infections because of the fluctuating level of parasitemia, particularly during the chronic stage of infection.[24]

3. Serological Diagnosis

Recognition of the invaluable contribution of serological methods to the control of diseases of viral and bacterial etiologies, has led to considerable research into the development of immunological methods for the diagnosis of trypanosomosis.[12] The different serological tests applied for diagnosis of trypanosomosis are as indicated below:

3.1. Complement fixation test

The complement fixation test (CFT) was used extensively and exclusively in North America in the successful campaign for the eradication of equine dourine (*T. equiperdum* infection), in which trypanosomes are rarely demonstrable in blood or body fluids. Although it has been compared favorably with the indirect fluorescent antibody test in the diagnosis of *T. congolense* infections in cattle, this test suffers problems in reagent preparation, standardization and ant-complementary activity in sera and has not found a useful role in the diagnosis of bovine trypanosomosis. The indirect haemagglutination (IHA) test has also found some use in the diagnosis of *T. evansi* infections but is

known to be unreliable for detecting *T. vivax* infection.[16]

CFT was performed according to the Manual of Standards for Diagnostic Tests and Vaccines. The Trypanosoma antigen was manufactured by LTD SME ''Biocentre'' (Russia). Test sera were screened in serial dilutions in phosphate buffered saline (PBS). A sample is considered positive when the titer is 5 or higher HCFT was performed. The Trypanosoma antigen used in this test was the same as was used in CFT. Sera were screened in serial dilutions in PBS. A sample was considered positive when the end titer was 5 or higher.[25]

3.2. Indirect fluorescent antibody test

The development of the indirect fluorescent antibody test (IFAT) a primary binding assay and thus one in which the antibody–antigen reaction is measured directly, was a significant step forward in the detection of anti-trypanosomal antibodies. In this test, blood films from infected animals with high parasitaemia are fixed and used as a source of trypanosomal antigens to which anti-trypanosomal antibodies in test sera may bind specifically. Bound antibodies are visualized using anti-host species immunoglobulin (e.g. anti-bovine immunoglobulin) conjugated to a fluorescent dye, which may be observed using an ultraviolet microscope. The IFAT has been shown to be both sensitive and specific in the detection of bovine anti-trypanosomal antibodies.[26] Although a degree of cross-reactivity between *T. brucei*, *T. congolense* and *T. vivax* means that the IFAT is not reliably species specific, cross reactivity is not complete and thus all three antigens must be used for maximum efficiency. Other disadvantages of IFAT are the requirement of an expensive ultraviolet microscope, the subjectivity of the interpretation of the results and the lack of quantification of the antibody respond.[27]

3.3*. Card agglutination trypanosomosis test*

The card agglutination trypanosomosis test (CATT), which has found wide spread application in the diagnosis of *T. gambiense* sleeping sickness. The antigens originate from particular variable antigenic types (VATs) of *T. gambiense* that are highly conserved across the range of this species, and thus the majority of infected individuals develop antibodies that cause visible agglutination when whole blood or serum is mixed with the antigen on a card. Although this test has been adapted for use for diagnosis of *T. evansi* in animals, it is less likely to be applicable to *T. gambiense.* Another simple antibody detection method that is particularly suitable for field use in Africa *T. congolense* or *T. vivax* than the *Trypanozoon* species is important because of the difficulty in identifying suitable VATs in these species.[28]

The CATT/*T. evansi* is a direct card agglutination test which uses formaldehyde fixed; freeze-dried trypanosomes of *T. evansi* VAT RoTat 1.2 stained with Coomassie blue. Test sera were screened in serial dilutions in phosphate buffered saline (PBS). A sample was considered positive when the end titer was 4 or higher. The principle is that infection with tryponomosis results in production of antibodies against the surface antigens of the parasite. These antibodies can be demonstrated in serum or in plasma of infected animals. RoTat 1.2. Predominant VAT (Variable antigen types) or VSG species Specific to *T. evansi*. The problem is that there is a *T. evansi* strain (type B) when do not express RoTat 1.2 VSG and there is a cross reaction by T. equiperdum. But as a principle it can be used, if species specific Antigen is identified for any of the *Trypanosoma species* example: CAT/*T.gambianse* has been tried and failed in Nigerian sample.[25]

3.4. Latex/T. evansi

It is a rapid indirect agglutination test, in which the antigen consists of purified VSG of VAT RoTat 1.2.covalently coupled to latex particles.[29] It is failure lays on the same reason for CATT/*T. evansi*; LATEX *T. b gambianse*[30] has been developed based on combination of 3 VSG as Antigen.[25]

3.5. Immune Trypanolysis (TL)

Immunotrypanolysis is performed according to[31] with *T. evansi* VAT RoTat 1. 2 or with *T. b. brucei* VAT AnTat 1.1 latter serves as one type control for the anti RoTat 1.2 positives sera. This VAT is never expressed by *T. evansi*. AnTat 1.1 is VAT exclusive for T. brucei. RoTat a VAT that appears early after infection and it is express by many if not all *T. evansi* seroderms. TL is a test for antibody detection using dive blood stream trypanosomes.[32]

3.6. Enzyme-Linked Immunosorbent Assay

The Enzyme-Linked Immunosorbent Assay *(*ELISA), another primary binding assay, was developed in the early 1970s and has since, in various forms, become one of the most widely used techniques in biomedical science. In the ELISA, in its original form, antigens are immobilized by passive adsorption on to a solid-phase and frequently 96 well polystyrene microtitre plate, and detected using specific antibody labeled with an enzyme that catalyses the conversion of a colourless substrate to a visible coloured product. In practical terms the attraction of the ELISA is two fold. Firstly, the test may be performed without specialized equipment, so that it may be adapted to inexpensive versions for use in the field or under-resourced laboratories. Secondly, with suitable equipment various degrees of automation are possible, allowing a high through put of test samples in suitably equipped laboratories.[28] Moreover, the ELISA gives quantitative responses that are directly (or indirectly) related to the level of analyze in the sample, making it particularly amenable to quantitative epidemiological studies.[28]

Antibody-detection enzyme-linked immunosorbent assay (Ab-ELISA): The indirect ELISA was adapted for a number of protozoan diseases, including trypanosomosis[33] and it was shown to be capable of

detecting specific antibodies in trypanosome-infected cattle.[26] It was also shown to detect more serologically positive cattle than the IFAT. As with the IFAT, cross-reactivity between the three major tsetse-transmitted trypanosome species occurs in the indirect ELISA using crude antigen preparations, and sera must be screened against all three antigens for optimum sensitivity.[26,28] The fractionation of trypanosomal antigens by column chromatography has been claimed to increase the species specificity of antibody detection by ELISA,[33] but this approach has yet to see widespread application. Similarly, although the genes for a number of candidate trypanosome antigens have been cloned, no suitable recombinant products are widely available for detection of species- or subtype-specific anti-trypanosomal antibodies.[33]

As with most serological tests for disease diagnosis, a single positive serological result cannot be used to demonstrate the presence of active infection, since antibody frequently persists far longer than does the infectious agent within the host. In cattle, this has been shown to be the case for up to 6 and possibly as long as 13 months following clearance of trypanosome infection.[28] This has sometimes been viewed as a major drawback to the usefulness of the Ab-ELISA for bovine trypanosomosis and this would indeed be a serious disadvantage were the assay to be used as a basis for diagnosis and treatment of individual animals.[34]

In the last decade (1990–2000) interest in the Ab-ELISA for bovine trypanosomosis has focused on its use as an epidemiological tool for mapping and quantifying trypanosomosis prevalence and risk. In this context, the persistence of antibody may be regarded as advantageous, since the Ab-ELISA will provide information on the aggregated level of trypanosome challenge over a prolonged period prior to the survey. The technique has recently found use in many regions of sub- Saharan Africa, for example, under a Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA) Coordinated Research Programme,[35] and there is an increasing body of data attesting to its reliability and robustness. While most workers continue to use crude trypanosomal antigens, modifications to the original method include the use of dried blood spots on filter papers rather than conventional serum samples,[36] thus obviating the requirement for cold chain facilities, the adoption of rigorous quality assurance based on the use of reference sera,[36] the use of heat/detergent-denatured antigen and the use of ELISA plates pre-coated with antigen.[37]

Antigen-detection enzyme-linked immunosorbent assay (Ag-ELISA): Demonstration of the parasite itself rather than antibodies is necessary for the confirmation of active infection, though it is widely recognized that the conventional parasitological techniques are relatively insensitive. Modifications of the ELISA have enabled the technique to be used for the detection of antigens, which may be a better indicator of active infection than the detection of antibodies. The double-sandwich ELISA method was shown by Rae and Luckins (1984) to detect antigens in animals within 10–14 days of infection with *T. congolense* and *T. evansi*, and these antigens were shown to disappear within 21 days of trypanocidal drug treatment. In this ELISA, polyclonal antibodies raised against crude trypanosomal antigen preparations were used to coat microtitre plates, and antigen present in test sera bound to the antibody. The bound antigen was then detected using the same antibody conjugated with enzyme and a suitable substrate.[38]

Antigen-detection enzyme-linked immunosorbent assay for the tsetse-transmitted trypanosomiasis were developed at the International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya, using the sandwich ELISA methodology, but incorporated trypanosome species-specific monoclonal antibodies (Mabs), which reacted with determinants of *T. brucei*, *T. congolense* or *T. vivax* (Nantulya *et al.*, 1987). The aim of the development of these tests was to increase the sensitivity of diagnosis, in both the analytical sense (i.e. a smaller quantity of the analyte – trypanosome – can be detected) and in the epidemiological sense (i.e. a greater proportion of infected animals react in the test). In addition, it was hoped to maximize the specificity of the tests (i.e. to minimize the number of false positive results). In practice this was equated to lack of cross-reactivity to other protozoon pathogens,[39] and lack of cross-reactivity of each species-specific test to the other two trypanosome species.[39]

3.7. Monoclonal Antibody Specificity

A number of ostensibly species-specific Mabs directed against salivarian trypanosomes are shown in Table 3. Of these, some have been tested in IFAT, some in Ag-ELISA using procyclic Lysates, some in Ag- ELISA on samples derived from experimental infections of cattle and goats, some with and some without treatment, and some in Ag-ELISA on bovine field samples. Ag- ELISA results from various species, including cattle, pigs, monkeys and humans, are reported in numerous publications without mentioning the precise identity of the Mabs used. Clearly, it is difficult to assess the performance of an immunoassay technique in which there is such variability and uncertainty regarding the primary immunological reagents.[40]

3.8. Antigen recognition

The earliest groups of anti-trypanosomal Mabs to be described for use in Ag-ELISA[39] were shown by IFAT to react with molecules on the plasma membrane of live and formaldehyde-fixed homologous procyclic forms, but not bloodstream forms. In the same work, ELISA reactions were obtained with supernatants of procyclic lysates, procyclic culture supernatants and, significantly, lysates of bloodstream forms prepared from stocks isolated in various countries. This work suggested that the antigens detected were water soluble, present in more

than one stage of the life cycle, and relatively conserved over the parasites' geographical ranges. However, with regard to the bloodstream forms, neither the details of the species, number and origins of the trypanosome isolates tested, nor which Mabs they reacted with were described in detail.[40]

Table 2: Monoclonal antibodies to trypanosome invariant antigen used in IFAT and Ag-ELISA.

Mab identification	Immunogen	Mab class	Subgroup Specificity
TB7/8.1.48[b]	*T. b. brucei* LUMP 427 pt	IgM	*Trypanozoon*
TB7/8/13.12[a]	*T. b. brucei* LUMP 427 pt	IgM	*Trypanozoon*
TR7/47.34.16[a,c,e,f]	*T. b. rhodesiense* B704 pt	IgM	*Trypanozoon*
TC3/17.1.13[a]	*T. congolense* STIB 212 pt	IgG1	*Nannomonas*
TC6/25.25.4[b]	*T. congolense* STIB 212 pt	IgG3	*Nannomonas*
TC40/31.15.45[c]	*T. congolense* KILIFI/83/IL/9pt	IgM	*Nannomonas*
TC39/30.38.16d,[e]	*T. congolense* bf	IgM	*Nannomonas*
TV8/8.33.42b,[c]	*T. vivax* IL 1392 pt	IgG3	*Duttonella*
TV8/8.5.38[a]	*T. vivax* IL 1392 pt	IgM	*Duttonella*
TV27/9.45.15[e]	*T. vivax bf*	IgG1?	*Duttonella*

[a]Reactivity tested in IFAT but not Ag-ELISA against procyclic lysates , [b]Reactivity tested in IFAT and Ag-ELISA against procyclic lysates, [c]Reactivity tested in Ag-ELISA against infected cattle sera, [d]Used in Ag-ELISA for *T. congolense* in goats and cattle, [e]Used in Ag-ELISA for field diagnosis in cattle at Nguruman, [f]Used in Ag-ELISA for *T. brucei* infections in cattle bf, bloodstream forms; pt, procyclic trypomastigotes.
Source:[40]

The introduction of the Ag-ELISA for diagnosis of bovine trypanosomiasis was nothing if not controversial. Highly polarized views on the effectiveness of the technique ranged from the Ag-ELISA being regarded as 100% sensitive and specific on the one hand, to it being regarded as worthless on the other. These views were debated in numerous flora, notably the biennial meetings of the International Scientific Council for Trypanosomiasis Research and Control (ISCTRC), often heatedly and with little scientific basis to many of the arguments used. The reality probably lay somewhere between these two extremes.[39]

Although a large body of work was conducted using the Ag-ELISA, very few of these studies were designed so as to allow an impartial and objective evaluation of its usefulness. Moreover, frequent changes in the Mabs used in the Ag-ELISA and in the assay protocol itself added to the confusion, resulting in capricious and often unexplainable results. Ultimately, definitive double blind trials were conducted at the International Livestock Research Institute (ILRI), Nairobi, on *T.congolense* and *T. vivax* experimentally infected cattle, which formally demonstrated that the version of the Ag-ELISA in use at that time was insufficiently sensitive to be of diagnostic value. Moreover, significant cross-reactivity was demonstrated between the *T. brucei* Ag-ELISA and sera from *T. congolense*- infected cattle.[41]

The outcome of this was that by the late 1990s both ILRI and the Joint FAO/IAEA Division abandoned work on the Ag-ELISA. It is worth noting that the ELISA and related immunoassay techniques are widely applied and accepted in other areas of biomedical science for the detection and quantification of antigens, hormones and drugs and that perhaps in the fullness of time this approach can be re-evaluated in the context of diagnosis of African animal trypanosomiasis. Once laboratory-based ELISAs are developed, pen-side versions of these immunoassays should be technically feasible and would have the advantage of providing immediate results on which therapeutic decisions could be based. However, even if they were to be developed, they would be unlikely to become available in tsetse-infested areas of Africa in the foreseeable future for economic reasons, since even in the developed world the prohibitive expense of pen-side immunoassays generally precludes their routine use on farm animals.[41]

4. Molecular Methods for Detecting Trypanosomes

Due to the low sensitivity and /or low specificity of parasitological and serological techniques a wide range of molecular techniques, have found increased application to the diagnosis of trypanosomosis. Molecular methods based on the detection and amplification of nucleic acids (DNA and RNA) certainly has the technical potential to achieve them.[42]

The detection and identification of trypanosomes by molecular means should be based upon stable, parasite-specific genetic characteristics specific to that parasite that can with stand environmental influences exerted by either the host or the vector. These markers should be able to reveal the presence of the trypanosome irrespective of the developmental stage of the parasite at the time of identification. They also have significant and reliable advantage on the epidemiological study of the disease such as tsetse blood meal identification based on analysis of vertebrate hosts DNA obtained from the Glossina spps gut.[43,44] According to[45] DNA techniques are much faster and more reliable ways to determine host species than serological techniques. A molecular marker can be used for identification of reservoirs of AAT and/

or HAT. The method can identify host blood after several days post feeding by tsetse flies.[43,46]

Regions of highly found within the genomes of the different Trypanosome species are unique cyclical, multicopy DNA sequences as demonstrated by[47] with the identification of a 177 BP repeat of which more than 10,000 copies existed within the haploid genome of *T. brucei*. These regions were first demonstrated as providing ideal targets for the PCR in 1989.[48] Since then a great deal of work has been carried out to optimize protocols for the continual identification of these sites by specific PCR primers. Both DNA hybridization and PCR have been applied to great effect to characterize specific trypanosome species infections within both host and vector, with probes often being used to validate PCR amplifications and to confirm the identity of the amplified targets.[46]

Recently the development of molecular techniques such as restriction enzyme sequencing, synthesis of the DNA, DNA probing and polymerase chain reaction (PCR) has had considerable input in to trypanosome identification, characterization and diagnosis with better accuracy and reliability at various taxonomic levels. Polymerase chain reaction (PCR) has allowed the in vitro amplification of specific DNA sequences.[17]

In Deoxyribonucleic Acid based diagnosis of trypanosomes there are a number of molecular tools so far developed. These molecular tools could be based on the knowledge of specific DNA sequence or it could be possible to identify the trypanosome species without prior knowledge of the sequence of the parasite. Some of the molecular tools identified in molecular characterization and identification of trypanosomes include RFLP (Restriction Fragment Length Polymorphism) analysis,[49] Genome finger printing,[50] analysis of repetitive DNA[51] and analysis of repetitive kDNA.[52] The PCR based approaches such as: Mini satellite DNA analysis,[53] AFLP (Amplified Fragment Length Polymorphism),[51] MEGA (Multiplex endonuclease genotyping),[55] MGE-PCR (mobile genetic element-PCR), SSR-PCR (Simple Sequence Repeat-PCR)[56] and RAPD (Random Amplification of Polymorphic DNA)[57] are few to mention. For the analysis of the genetic variation with in and among species of Trypanosoma, recently: florescence in situ hybridization (FISH) with peptide nucleic acid (PNA) probes[57] and Loop mediated isothermal amplification have been developed.[58]

4.1.DNA-probe hybridization

Probe is a radio labeled DNA molecule carrying a foreign DNA sequence used in hybridization to detect DNA homologous to the fragment in the sample to be tasted. A DNA probe is a known DNA sequence which can be obtained by cloning or by PCR with labeled nucleotides (enzymes, or isotopes). It depends on a small sequence of nucleic acid (oligonucleotides) that has been labeled with a radioactive isotope or dye (e.g. enzyme) to locate a complimentary nucleotide sequence or gene. It entails exposing a denatured DNA sample fixed on nitro-cellulose to a labeled DNA probe under specific salt and temperature conditions. If the complimentary DNA sequence is present in the sample, the probes will bind to it and remain on the nitro-cellulose where they can be visualized with substrate or film.[58]

Deoxyribonucleic Acid probes have been developed for the main pathogenic trypanosomes.[60] This is not sufficient for trypanosome detection in mouthpart of the vectors (hematophagous fly) or in host blood, when the parasitemia is low. The kinetoplast DNA (kDNA) minicircles of trypanosomes have also been used as a source of DNA probes for their specific identification. The extent to which the minicircles drift in nucleotide sequence is unknown; however, the nucleotides at the origin of replication of the minicircles are conserved in all the Kinetoplastida where minicircles exist. The kDNA minicircles or sequences have therefore been exploited as a means of identifying the Trypanosomatids by PCR. In particular, two strains *T. evansi* (typical type and the camel strain type) of have been identified on the basis of differences in their kDNA minicircles sequences. A major disadvantage of using kDNA minicircles in the detection of trypanosomes is that they vary in sequence and proportion among different trypanosomes; furthermore, there are some non-tsetse-transmitted trypanosomes that lack kDNA and thus would not be detected by probes based upon the minicircles sequences.[61]

4.2.Polymerase Chain Reaction

When using PCR to identify multiple copy segments of DNA, the presence of parasite DNA equivalent to one trypanosome in 10 ml of host blood can be detected.[62] This is due to the very high abundance of target sequence within the genome, only a very small percentage of which is required for successful detection. Furthermore, in experimental studies, the PCR can detect trypanosomes in cattle as early as 5 days after an infective tsetse bite.[62]

It is an enzymatic process by which a specific region of DNA that lies between two regions of oligonucleotide primers is replicated repeatedly to yield several million copies (approximately 270 million times) of a particular sequence.[62] Primers are short (at least, 18-30 bases), single stranded DNA molecules that are complimentary to the ends of defined sequences of a DNA template.[59]

The *Polymerase Chain Reaction* (PCR) technique exploits thermostable DNA polymerases such as Taq polymerase which synthesizes a new strand of DNA template by coping an original DNA template.[59,64] The template primer mixture is subjected to repeated cycles of heating to separate the double stranded DNA and cooling in the presence of nucleotide and other thermostable DNA polymerase enzymes.[59] The

resulting PCR product can be evaluated (visualized) on agarose gel or polyacrylamide gel, after staining with ethidium bromide and exposing it under ultraviolet ray.[17]

The molecular markers and the type of primers used in PCR diagnosis of African Trypanosomosis is as summarized in Table 4 below.[17,18]

Table 3: Primers used for PCR amplification of trypanosome DNA.

Specificity	Code	Primer sequence	Fragment length
T. congolense (forest)	TCF1	5'-GGACACGCCAGAAGTACTT-3'	350bp
	TCF2	5'-GTTCTCGCACCAAATCCAAC-3'	
T. congolense(savanah)		5'-TCGAGCGAGAACGGGCACTTT GCGA-3'	341
	TCN2	5'TTAGGGACAAACAAATCCCGCACA-3'	
T. congolense (kilif)	TCK1	5'-GTGCCCAAATTTGAAGTGAT-3'	294
	TCK2	5'-ACTCAAAATCGTGCACCTCG-3'	
T. simiae	IL1	5'-CGACTCCGGGCGACCGT-3'	600
	IL2	5'-CATGCGGCGGACCGTGG-3'	
T. godfreyi	DDG1	5'CTGAGGCTGAACAGCGACTC-3'	
	DDG2	5'-GGCGTATTGGCATAGCGTAC-3'	
T. brucei		5'-GAATATTAAACAATGCGCAG-3'	164
	TBR2	5'-CCATTTATTAGCTTTGTTGC-3'	
T. vivax		5'-CTGAGTGCTCCATGTGCCAC-3'	150
	TVW2	5'-CCACCAGAACACCAACCTGA-3'	

Source:[18]

Internal transcribed spacers (ITS) of ribosomal DNA (rDNA) towards a single PCR: In most of sub Saharan countries there are about ten pathogenic trypanosomes occur and overlap in most of the tsetse belt[65] such that multiple infections of both host and vector is 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 *T. vivax, T. congolense savannah, congolense kilifi, T. congolense forest* and *T. brucei spp*. Furthermore three PCRs for suids (*T. simiae, T. godfreyi* and *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 a single PCR have been discouraging due to lower sensitivity compared to individual species-specific tests. Therefore, multi-specific diagnosis of trypanosomosis is required. Nuclear DNA bears the genes coding for the ribosomal RNA; the ribosomal DNA cistern genes occur in multiple copies in tandem arrays. They are made of transcriptional units (TU) separated by non-transcribed spacers (NTS). The TU is Ade of a 18s ribosomal subunit, internal transcribed spacer 1 (ITS-1), 5.85 ribosomal subunits, ITS-2, 28S ribosomal subunit, etc.[65]

The *Internal transcribed spacers (*ITS-1) is usually 300-800bp in length, and has a variable length depending on the Kinetoplastida species, but is presumed to be constant within a species. Preliminary studies have demonstrated that "KIN 1 and 2" primers, amplifying the ITS-1 of Kinetoplastidea specifically, give different size products in Leishmania and Trypanosoma (Mc Laughlin *et al*., 1996) Further evaluation has indicated that the following Trypanosoma species can be identified through a single PCR (even in the case of mixed species-specific DNA): *T. vivax, T. theileri, T. simiae, Trypanozoon, T. congolense savannah, T. congolense forest and T. congolense Kilifi*; it is noteworthy that these primers allows the detection and identification of *T. theileri*, a non-pathogenic trypanosome of cattle for which specific primers had never been described.[17]

SENSITIVITY AND SPECIFICITY OF POLYMERASE CHAIN REACTION

A recent study demonstrated that a single PCR restriction fragment length polymorphism (RFLP) assay can be used to characterize all important bovine trypanosome species. This was the first report a sensitive pan-trypanosoma PCR assay amplifying all species including *T. vivax* to a comparable extent, using a single primer pair. Restriction enzyme analysis using Msp1 and Eco571 gave a clear distinction between *T. congolense*, *T. brucei*, *T. vivax* and *T. theileri*. Several subgroups within the *T. congolense* group could be distinguished using this method, although no difference could be detected between the species belonging to the subgenus Trypanozoon further evaluated this method using mixed infection with *T. b. brucei*, *T. theileri*, *T. congolense* and *T. vivax.*[66]

The result showed that all mixed infections gave clear profiles that could be easily differentiated except in the case of *T. theileri* and *T. congolense* mixed infections where the *T. theileri* band was concealed by *T. congolense* profile. By reducing the cost of PCR diagnosis, this technique would allow a greater number of field samples to be tested in the epidemiological studies and/or would increase the variety of trypanosome

species that could be detected. Similarly PCR has allowed an increase in the specificity of diagnosis in vectors such as tsetse flies. Direct determination of the trypanosome species-type in the vector or host is likely to provide a more reliable estimate of parasite prevalence since no selection is exerted in the ability of the parasite to grow either in culture or in animals.[66]

Many natural trypanosome infections of either tsetse or livestock are due to more than one trypanosome species.[67] Precise evaluation of such infections by PCR requires that the oligonucleotide primers for PCR retain their specificities under standard assay conditions. Species specific DNA probes have been shown to detect simultaneous infection of cattle with *T. vivax*, *T. brucei*, and *T. congolense* when conventional methods are revealed only single infection.[67] PCR coupled with DNA probe hybridization could prove to be highly sensitive tool for the diagnosis and assessment of the therapeutic efficacy and disease progress especially in the chronic trypanosomosis. With regards to the sensitivity, PCR allows detection of single specific sequence of DNA; consequently, a single parasite (1 pg of DNA) or even fewer can be detected when using satellite DNA.[67]

This very sensitivity also brings a very high risk of false positive results since a very small amount of other biological materials can contaminate the sample. The sensitivity threshold of the trypanosome detection by PCR generally ranges from 1 to 20 parasite/ml of blood, depending on the technique used. Below this level of parasitemia, PCR cannot detect the infection. It is not a problem of sensitivity; it is only due to the fact that there is no DNA in the sample investigated when the parasitemia is so low. To increase the sensitivity, it should then be necessary to increase the volume of samples processed and the concentration through the parasitological techniques described.[17]

CONCLUSION AND RECOMMENDATIONS

Animal trypanosomosis is one of the most devastating animal diseases which severely affected the production and productivity of animals mainly in sub-Saharan Africa. There are a number of trypanosome species affecting different animal species. The disease is manifested by array of clinical signs and symptoms, thus not reliable to make diagnosis. Apart from clinical signs, demonstration of the trypanosome in body fluid/tissue is of great value, however during chronic infection and in early phase the number of parasites in blood is too low to be detected. To circumvent such problems in diagnosis of animal trypanosomosis techniques based on serology and molecular tools have been developed. There are a number of serological tests including CFT, Trypanolysis, CATT, Ab-ELISA and Ag-ELISA. Each of these serological test have there pros and cons. Furthermore PCR diagnosis of trypanosomosis is the widely used technique. The species specific PCR are labor intensive and expensive to perform to diagnose the about 10 trypanosomes species affecting animals. Therefore recently PCR based on internal transcribed spacer of ribosomal DNA is highly sensitive and specific, and cost effective as it allows detection of all trypanosomes in single PCR. Therefore, accurate diagnosis of animal trypanosomosis should be done using the most sensitive and specific diagnostic tools ,veterinary laboratories need to be equipped with serological and molecular diagnostic tools for proper identification of animal disease in general and trypanosomosis in particular and appropriate training should be provided to the professionals to use such high-tech diagnostic aids.

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