Soft Options for Effective Diagnosis of African Animal Trypanosomiasis: A Review

[1]Dabo, N. T., & *[1, 2]Maigari A. K.
[1]Department of Biological Sciences,
Bayero University,
Kano.
[2]Kano State Liaison Office,
Nigerian Institute for Trypanosomiasis (and Onchocerciasis) Research,
Infectious Diseases Hospital (IDH),
France Road, Kano.
*akabirum@gmail.com

ABSTRACT
African Animal Trypanosomiasis (AAT) still remains a major obstacle to sustainable livestock development. However, the type of diagnostic test used for the detection of infections caused by AAT varies according to the epidemiological characteristics of the disease and the strategy for control. The clinical signs are not pathognomonic and the standard techniques for the detection of trypanosomes are not sufficiently sensitive. It can thus be seen that despite several improvements in the techniques for trypanosome detection, a high proportion of infections still go undetected as the majority of infections are chronic and often aparasitaemic. This diagnostic challenge has led to the development of alternative methods, for instance molecular techniques, because they are extremely sensitive and better diagnostic tools. This write – up therefore, underscores the relevance of various diagnostic techniques in the epidemiological study of African Animal Trypanosomiasis. It is concluded that, these newer techniques can allow the study of the phylogeny and diversity of trypanosomes that would help in devising appropriate control measures to limit the economic losses arising from the disease.

Key Words: *African Animal Trypanosomiasis, Trypanosomes, Diagnosis*

INTRODUCTION

Background to the Study

African Animal Trypanosomiasis (AAT) is one of the major haemoparasitic diseases of domestic animals (Senait *et al.*, 2016; Deken, 2011).The menace of AAT still constitutes a major obstacle to food security in spite of previous attempts towards chemotherapy and tsetse control (Nakayima, 2016; Senait *et al.*, 2016).The tsetse-transmitted form of the disease is endemic throughout the tropical regions of Africa where the vector is prevalent (Senait *et al.*, 2016; Akoda *et al.*, 2009).

The clinical manifestation of trypanosomiasis in animals is influenced by the host as well as the trypanosome species and "strain" (WHO, 2013; Osório, 2008). In general, the disease is characterized by the intermittent presence of parasites in the blood and intermittent fever (WHO, 2012; 2013). Anemia usually develops in infected animals, and this is followed by weight loss, loss of body condition, infertility and abortion, reduced productivity and, often, high mortality (Mbaya *et al.*, 2012; WHO, 2012; 2013; FAO, 2007). Animals which survive often remain infected for several months or years, exhibiting a low level of fluctuating parasitaemia which serves as a reservoir for the disease (WHO, 2012; 2013; Ezeani *et al*., 2008)).

AAT in livestock is caused by *Trypanosoma congolense* (OIE, 2013; WOAH, 2012), *T. vivax* (OIE, 2013; FSPH, 2009) and *T. brucei* (OIE, 2013; Olukunle *et al., 2010;* Antia *et al*., 2009). The host preferences of each trypanosome species may differ, but *T congolense, T. vivax* and *T. brucei* have a wide host range among domesticated animals (Senait *et al*., 2016; FSPH, 2009). Similarly, some species of trypanosomes like *T. simiae, T. godfreyi* and *T. suis* occur in pigs (FSPH, 2009). However, although *T. simiae* appears to be most important in pigs (FSPH, 2009), this trypanosome has also been detected using PCR in camels and horses (Senait *et al*., 2016).

AAT is also encountered outside the tsetse fly belt, where the most important pathogenic trypanosome species, *T. vivax* and *T. evansi*, are transmitted mechanically by biting flies (Senait *et al*., 2016), while *T. equiperdum* is transmitted sexually by horses and donkeys (WHO, 2012; FSPH, 2009). The principal domestic animals affected by *T. evansi* are camels, pigs, water buffaloes and cattle (FSPH, 2009).

Trypanosomiasis is often a chronic disease in susceptible animals. The morbidity rate is high, and many untreated animals infected with *T. vivax, T. brucei* or *T. congolense* eventually die (Senait *et al*., 2016). In cattle infected with some strains, the mortality rate can reach 50-100% within months after exposure, particularly when poor nutrition or other factors contribute to debilitation (Chanie *et al*., 2013).

The considerable economic and social repercussions make control of this disease a priority operation for the development of a large part of the African continent (Senait *et al*., 2016; Chanie *et al*., 2013). Moreover, owing to the varied clinical manifestations of AAT, diagnosis cannot be based on clinical signs alone (OIE, 2013). Therefore, laboratory confirmation of the parasites is an absolute necessity. The standard laboratory method for diagnosis of trypanosomiasis is to demonstrate and identify trypanosomes in the blood of the infected animal (Nakayima, 2016). There are several techniques for parasite detection, which include direct microscopy, concentration techniques, animal inoculation, serology and molecular techniques (Nakayima, 2016). However, direct demonstration of trypanosomes in the infected animal gives conclusive proof of infection (Nahla *et al.*, 2011; Ezeani *et al.*, 2008), but, due to the limitations of parasitological diagnosis (Yusuf *et al.*, 2015; Gibson, 2007; Adams *et al.*, 2006; Desquesnes, 2001), extensive research has been invested into alternative techniques that provide indirect evidence of infection. This will go along line towards effective epidemiological studies of AAT that will help in designing appropriate control strategies to limit economic losses arising from the disease.

OPTIONS FOR THE DIAGNOSIS OF AAT

Generally, diagnoses for AAT are classified into: clinical, microscopy, immunological and molecular diagnoses (Nakayima, 2016).

Clinical diagnosis

AAT is characterized by severe anaemia, loss of production, pyrexia, lacrimation, pallor of the mucus membrane, weight loss, infertility and abortion, with death occurring in some animals during the acute phase of the disease (Maigari *et al.*, 2015a; FAO, 2007; IAEA, 2007). Animals which survive often remain infected for several months or years, exhibiting a low level of fluctuating parasitaemia which serves as a reservoir for the disease (IAEA, 2007). However, the clinical manifestation of AAT is influenced by the host as well as the trypanosome species and "strain" (FAO, 2007). Thus, owing to these varied clinical manifestations, diagnosis of trypanosomiasis cannot be based on clinical signs alone. Laboratory confirmation of AAT therefore, becomes an absolute necessity.

Microscopy

Microscopy entails demonstration of parasites based on standard trypanosome detection methods as developed by Woo (1969) and their modifications. The methods include wet mount, animal inoculation, thick/thin smears, haematocrit centrifugation techniques (HCT), buffy coat methods (BCM) and mini-anion chromatography (Nakayima, 2016).

Thick smear is used for detection but not speciation based on parasite morphology whereas thin smear is used for speciation based on parasite morphology (Nakayima, 2016). Similarly, Wet film is used for speciation based on parasite motility. *T. vivax* moves/swims very fast across the microscope field. *T. brucei* actively moves but in one position. *T. congolense* attaches onto Red Blood Cells (RBCs) and just purves in one position (Nakayima, 2016).

The sensitivity of direct microscopic examination was improved through concentration of the parasites by centrifugation. When whole blood is spun in a haematocrit centrifuge, the trypanosomes are concentrated at the buffy coat. Examination of the buffy coat is thus more sensitive than examination of blood films (Camara *et al.*, 2010).

Improvement in the sensitivity of the concentration methods can be achieved if red blood cells are removed from the blood sample prior to centrifugation. The red blood cells are removed by lysis either by hypotonic shock or be removed from the test blood sample by diethyl amino-ethyl (DEAE) anion exchange chromatography (Chappuis *et al.*, 2005). The miniature anion-exchange chromatography technique (MAECT) as it is known, is widely used to diagnose *T. gambiense* infections in man (Camara *et al.*, 2010; Chappuis *et al.*, 2005) but is yet to be established as a routine test for diagnosis of AAT due to its more cumbersome procedure (Nakayima, 2016).

Blood from suspect animals can also be inoculated into susceptible laboratory animals, usually mice or rats. This technique is more sensitive than direct microscopic examination of the blood sample (Nakayima, 2016). Animal inoculation has the added advantage that trypanosome isolates can be collected for other studies in the laboratory. This, however, is not a practical technique because diagnosis is not immediate (Yusuf *et al.*, 2015). In addition, the cost of maintaining the animals makes the method prohibitively expensive for routine diagnosis, especially in the field (Nakayima, 2016). Furthermore, some trypanosome isolates, notably East African *T. vivax*, *T. simiae* and, to a lesser extent, *T. congolense*, do not infect laboratory rodents (Yusuf *et al.*, 2015). Also, the intermittent parasitaemia (Maigari *et al.*, 2015a, b; Salim *et al.*, 2011; Ezeani *et al*., 2008) may preclude detection of the parasites even in acute infections (Maigari *et al.*, 2015a, b; Ezeani *et al.*, 2008). These drawbacks have necessitated the development of alternative methods of diagnosis.

Immunodiagnostic Techniques

There are many reports of the significance of immunodiagnosis of trypanosomes, but most of them do not play an integral part in control programs. This is because most of these are based on the detection of immune responses of animals to the infection (OIE, 2013; Ezeani *et al*., 2008).

Antibody Detection Tests

Antibody detection techniques include complement fixation test (CFT) that has been used in the diagnosis of *T. equiperdum* (Lucking, 1992), which causes dourine in equines, and indirect fluorescent antibody test (IFAT) which was used in herd diagnosis of trypanosomes (OIE, 2013; Lucking, 1992). The IFAT has proven to be both sensitive and specific in detecting trypanosomal antibodies (Lucking, 1992), although there is cross-reactivity between the trypanosome species (Lucking, 1992), requiring sophisticated microscopy. Moreover, in addition to its being expensive its subjectivity can make comparison of results quite difficult (Nakayima, 2016). However, modifications in the preparation of antigens have

provided antigens which are stable even at 4°C using methods such as fixation of the parasites in acetone and formalin (Nakayima, 2016; Lucking, 1992). This equally allows trypanosomes to be kept in suspension until required and ability to discriminate between different trypanosome species (Nakayima, 2016).

Card agglutination test for trypanosomiasis (CATT), the simplest for *T. evansi*, has also been used (Ezeani *et al*., 2008; Eisler *et al*., 2004). CATT uses the formalin fixed variable antigen types of *T. evansi* that are used in the agglutination test (Eisler *et al*., 2004). However, the major draw backs of this method is that, when antibodies are detected, they do not distinguish between current and past infections (Ezeani *et al*., 2008), in addition to cross-reactions among trypanosome species (Eisler *et al*., 2004).

Enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays (RIA) have also been used. ELISA has particularly been used for epidemiological surveys to detect trypanosome antibodies (Ezeani *et al*., 2008). However, the detection techniques involve use of either whole parasite or crude parasite lysate as the antigen which are not often standardized (Ezeani *et al*., 2008). It can be varied to use blood spotted filter papers thus bypassing the use of cold chain facilities (Ezeani *et al*., 2008). However, tests using crude sonicated trypanosomal extracts showed that the ELISA had a sensitivity and specificity similar to the IFAT (Ezeani *et al*., 2008). Thus, the introduction of ELISA was a major breakthrough in the field of immunodiagnosis (OIE, 2013). This is because ELISA requires simple equipment, the technique is straight-forward and sensitive, and can be used for large scale screening of samples. However, a major limitation of ELISA in its application as a routine diagnostic test is the nature of the antigens used in the assay (Nakayima, 2016). The antigen is usually a crude trypanosome lysate, the quality of which is ill-defined. This makes the test difficult to standardize with regard to specificity and sensitivity (Nakayima, 2016).

Antigen Detection Tests

The use of assays to detect trypanosome-specific antigens in the blood of infected animals has been advocated as an alternative approach to antibody detection. Enzyme immunoassays can detect the circulating antigens of *T. congolense*, *T. vivax* and *T. brucei* in blood of infected animals (Goto *et al*., 2011). Similarly, Latex Agglutination Test (LAT) has been used specifically for *T. evansi* (Nakayima, 2016). Hence, the demonstration of trypanosome antigens is an indicator of active infection if an animal has not been recently treated for the disease and thus, is equivalent to parasitological diagnosis.

Many studies have shown that, Antigen ELISA technique may give false negative results even in parasitologically proven cases (Nguyen *et al*., 2012; Goto *et al*., 2011). This occurs in sera from acute or early phase of infection and has been observed in *T. congolense*, *T. vivax* and *T. brucei* infections in cattle and goats (Goto *et al*., 2011). The monoclonal antibody used in antigen ELISA is directed at an internal or somatic unsecreted antigen that is only released after trypanosome lysis (Nguyen *et al*., 2012; Goto *et al*., 2011). Thus, before the first parasitaemic peak, the test can give negative results due to absence or low levels of antigens in blood (Nguyen *et al*., 2012). It is, therefore, important to combine antigen detection ELISA with the parasitological techniques for effective diagnosis of Trypanosomiasis (Nakayima, 2016).

Currently, there is a promising use of recombinant antigens to improve on the available trypanosome cell lysate to detect antibodies (Nakayima, 2016). A tube-ELISA, as opposed to a microtitre plate-ELISA, has since been developed (Nguyen *et al*., 2012). This test gave similar results (Nguyen *et al*., 2012), thus providing, for the first time, a potentially suitable test for immunodiagnosis of individual animals in the field (Nguyen *et al*., 2012).

Molecular Diagnostic Methods

The difficulty of morphological identification is compounded by the disappearance of the few useful distinguishing features during trypanosome development in the vector. Thus, it has been clear that more than just morphological examination is needed for identification of trypanosomes.

Trends in DNA based Methods for the diagnosis of Trypanosomiasis

DNA-based identification methods tap into the intrinsic genetic identity of the organism provides an unchanged genetic signature is particularly useful for parasites where individual life cycle stages may show little resemblance to each other (Gibson, 2009). Therefore, DNA-based methods have a wider range of applicability and efficacy in epidemiological studies than morphological examination alone, and are now indispensable tools for the study of trypanosomiasis because of the improved sensitivity and specificity (Gibson, 2009).

Molecular detection techniques have been developed for the diagnosis of infections with African trypanosomes in humans, animals and tsetse flies (OIE, 2013). For instance, Polymerase chain reaction (PCR) first performed in 1983 now has various primer sets available that can amplify different trypanosome subgenus, species and types (Yusuf *et al.*, 2015; Gibson, 2007, 2009; Adams *et al.*, 2006, 2008; Desquesnes and Dávila, 2002; ; Desquesnes, 2001). Additionally, species-specific probes are now available to identify trypanosome to sub species level (Gibson, 2009).

PCR can detect infection as early as 5 days following an infective tsetse bite (Masake *et al.*, 2002). Moreover, using the quantitative PCR confers an additional advantage of identification as well as establishing the parasite burden (Zarlenga and Higgins, 2001).

Size variation among trypanosome species in the internal transcribed spacer (ITS-1) of the ribosomal RNA (rRNA) locus provided the necessary discriminatory power (Cox *et al*., 2005; Njiru *et al*., 2005; Desquesnes *et al*., 2001) and this methodology has been widely applied to identification of both tsetse midgut and livestock blood samples (Cox *et al*., 2005; Njiru *et al*., 2005; Adams *et al*., 2006).

In the ITS-1 generic PCR test, the PCR primers flank the ITS-1 spacer, which is variable in both length and sequence; the primers are chosen in the 18S and 58S rRNA genes such that they match all trypanosome species of choice (Adams *et al*., 2006). The size of the single PCR fragment amplified from a trypanosome sample is then measured by gel electrophoresis against a DNA size marker and compared with known values from reference species (Adams *et al*., 2006; Gibson, 2009).

Moreover, ITS-1 PCR will fail to discriminate species where amplified fragments cannot be resolved by size and this can have major consequences.

Fluorescent fragment length barcoding (FFLB) relies on size polymorphisms in multiple small regions of the 18S and 28S rRNA genes detected by analysis on an automated sequencer, so that differences as small as a single base pair can be measured; gene fragments are amplified by PCR using fluorescently-labeled primers in conserved regions of the rRNA genes that flank regions of variable size, allowing detection of PCR products by fluorescence (Hamilton *et al*., 2008). Although, this technique requires more 28Sα rRNA 58SrRNA 18S rRNA ITS-1 RNA locus showing locations of 18S, 58S and 28S rRNA genes and ITS-1 PCR primers specialized equipment and reagents (Gibson, 2009) and gains are made not only in accuracy but also in the capacity for high throughput, with the possibility of analyzing 96 samples simultaneously within a day or two (Gibson, 2009).

Alignment of the 18S and 28S rRNA genes from the tsetse transmitted trypanosomes quickly identified regions of similarity and variability, allowing the positioning of suitable PCR primers (Gibson, 2009). The sizes of amplified fragments were predicted from the database sequences and subsequently confirmed in reference trypanosome isolates, providing each

known trypanosome species with a recognizable barcode of fragment sizes. Any non-matching barcode found among field isolates would represent an unknown genotype that could only be investigated further by sequence analysis.

Prospects of High Throughput Molecular Techniques

Newer molecular techniques are being developed namely: Loop-mediated isothermal amplification (LAMP) (Bhattacharya *et al*., 2002) and nucleic acid sequence-based amplification (NASBA) (Van der Meide *et al*., 2005).

LAMP involves the amplification of the target sequence at a constant temperature of 60-65°C using either two or three sets of primers and a Blast DNA polymerase with high strand displacement activity in addition to a replication activity (Thekisoe *et al*., 2007a; Bhattacharya *et al*., 2002). LAMP is a simple (using water bath/heating block), rapid (1h amplification) highly sensitive and specific in addition, cost effective molecular technique (Thekisoe *et al*., 2007a). It is now increasingly being explored in the detection of various infectious diseases such as viral (Pham *et al*., 2005) tuberculosis (Geojith *et al*., 2011) malaria, (Poon *et al*., 2006) and Trypanosomosis (Njiru *et al*., 2008a) and has the potential to replace conventional gene amplification methods once it is validated (Thekisoe *et al*., 2007a, b).

On the other hand, NASBA (Mugasa *et al*., 2008, 2009, 2014; Van der Meide *et al*., 2005) is a novel amplification technique with comparable sensitivity and simplicity to LAMP which can be combined with oligochromatography (NASBA-OC) (Mugasa *et al*., 2014). Both are isothermal tests, easy to perform, and yield results within a relatively short time, without the need for specialized heating equipment or complicated downstream detection of the products, such as the use of the ethidium bromide stained agarose gel and the expensive real-time equipment (Mugasa *et al*., 2008).

Mugasa *et al*. (2008) reported that, the major difference between LAMP and NASBA is that, NASBA amplifies RNA while LAMP amplifies DNA as starting material. In addition, targeted genomic regions are different (18S RNA versus RIME: repetitive insertion mobile element). From this angle, there could be differences in sensitivity arising from available template copies or even stability of the targeted nucleic acid (Mugasa *et al*., 2014). LAMP detects DNA for which the targeted RIME occurs 500 copies per haploid genome (Bhattacharya *et al*., 2002), while NASBA detects 18S rRNA that could have more than 10,000 copies floating in the cytoplasm (Van der Meide *et al*. 2005). Thus, the higher abundance of 18S rRNA (at least 20 times more) could explain the higher sensitivity reported.

CONCLUSION

AAT is still a major impediment to sustainable livestock development and has contributed negatively to food security in Nigeria. Disease diagnosis may be based on the clinical signs and symptoms, by demonstration of the causative organism or by reactions to diagnostic tests. However, the type of diagnostic test used for the detection of infections caused by AAT will vary according to the epidemiological characteristics of the disease and the strategy for control. This diagnostic challenge has had significant improvements although a high proportion of infections still remain undetected. Consequently, alternative methods of diagnosis have been developed which can give a measure of relatedness and subsequently allow the study of the phylogeny of trypanosomes that would culminate in devising appropriate control measures to limit the economic losses arising from the disease.

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