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The diagnosis of trypanosome infections: applications of novel technology for reducing disease risk

Picozzi, K.[1], Tilley, A.[1], Fèvre, E.M.[1], Coleman, P.G.[1], Magona, J.W.[2], Odiit, M.[2], Eisler, M.C[3] and Welburn S.C.[1*]

[1]Centre for Tropical Veterinary Medicine, Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Easter Bush, Roslin, Midlothian, Scotland, EH25 9RG, UK [2]Livestock Health Research Institute, PO Box 96, Tororo, Uganda [3]University of Glasgow Veterinary School, Bearsden Road, Glasgow, Scotland

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Reliable DNA based methodologies to determine prevalence of trypanosome species in domestic livestock have been available for over 10 years. Despite this, they are rarely used to generate baseline data for control operations for these diseases in the field. Rather, such operations tend to rely on data which can be generated using low technology methods such as direct observation of parasites by light microscopy. Here we show the pitfalls of relying on such low tech methodology which, although simple in its application, can provide inaccurate and inadequate data on which to base control methodologies. Our analysis of 61 cattle selected for trypanosome carrier status by either microscopy, low PCV or poor condition score, showed that 90% were infected with trypanosomes while 84% of the total were infected with *T. brucei*. Diagnosis by PCR on buffy coat preparations on Whatman® FTA® matrices was the most sensitive methodology relative to the gold standard, whereas microscopy was the least sensitive.

Keywords: sleeping sickness, human African trypanosomiasis, epidemiology, chemotherapy, PCR.

INTRODUCTION

Human African trypanosomiasis (HAT) or sleeping sickness, a disease thought to have been largely conquered during the 1960's, has re-emerged as a serious public health problem over large swathes of sub-Saharan Africa (Welburn et al., 2001). It has been estimated that 300,000–500,000 people are currently infected and 100,000 deaths are caused each year by the disease (Cattand et al., 2001). HAT is strictly a problem for rural communities in Africa since it is dependent for transmission on tsetse. The disease persists in areas which have suffered most acutely from general breakdown of infrastructure, including health care (Louis, 2001).

The cause of human sleeping sickness in eastern Africa is *Trypanosoma brucei rhodesiense*, a zoonotic parasite, which is mainly a non-pathogenic parasite of livestock and wild bovids (Heisch et al., 1958; Onyango et al., 1966) but which results in sleeping sickness when transmitted to humans. Control of *T. b. rhodesiense* HAT is complicated by the fact that *T.b. rhodesiense* co-exists in domestic livestock with the morphologically identical *Trypanosoma brucei brucei* which is not pathogenic to humans. Furthermore, in the regions where HAT prevails, several other trypanosome species, including *T. vivax* and *T. congolense,* are prevalent which affect the health of cattle and other livestock. Taken as a group, the trypanosomiases of livestock are responsible for severe losses in the agricultural sector (Kristjanson, 1999), and together with the human disease burden imposed by human sleeping sickness (Odiit et al., personal communications), the trypanosomiases more generally form a very significant group of parasitic infections.

Quantifying the risk presented to rural peoples by *T.b. rhodesiense* infected livestock and wild animals is essential in determining the extent to which control activities are required and the control methodology which is appropriate. Of particular importance to public health is this new possibility of rapidly identifying animals that are serving as reservoirs for human infective trypanosomes. The risk of spreading sleeping sickness is closely related to the movement of cattle populations, and the screening and treatment of livestock to prevent disease spread has been advocated (Fèvre et al., 2001). This was until recently

*Corresponding author; tel: +44 131 650 6228; fax: + 44 131 7348; e-mail: sue.welburn@ed.ac.uk

impossible, as there was no means of safely differentiating this human infective parasite from morphologically identical *T. b. brucei*. Recently, however, it was observed that a strain of *T. b. rhodesiense* belonging to the Busoga zymodeme (noted for its persistence over time in southeast Uganda) had a gene (serum resistance associated gene, *SRA*), co-expressed with the variant antigen gene, which was shown experimentally to confer human serum resistance (Xong et al., 1998). This gene has been shown to unequivocally differentiate *T. b. brucei* from *T. b. rhodesiense* in southeast Uganda. *SRA* has been found in all human isolates of *T. b. rhodesiense* identified in southeast Uganda to date and moreover can confirm the human infectivity status of trypanosomes isolated from animals. *SRA* is not present in those samples previously designated as *T. b. brucei* by restriction fragment length polymorphism analysis (Welburn et al., 2001b; Welburn and Odiit, 2002).

Although these molecular diagnostic tools are proving extremely useful for elucidation of sleeping sickness and animal trypanosomiasis epidemiology, they are also of practical use in terms of better targeting drug use in animals to improve animal health. Perhaps most importantly, the molecular tools allow the identification of individual reservoir hosts carrying human-infective *T. b. rhodesiense* parasites. Despite this, however, more traditional technologies are still in widespread use, particularly microscopy-based diagnosis (reviewed in Uilenberg, 1998).

In this paper, we compare different methodologies used to prepare parasite material in the field for the eventual assessment of trypanosome-infection status of the animals, and thus for the risk posed by those animals to disease in cattle-keeping populations. The more accurate the diagnosis of infections can be, the more targeted interventions can become and the greater the reduction in the risk of human disease. Selective treatment of infected animals in this way results in a holistic approach to disease control, concurrently combating livestock and human disease agents, and resulting in an overall improvement in the livestock and human health of rural populations.

METHODS

Sampling

In 2001, as part of a large survey of trypanosome infections in the cattle population in the sleeping sickness focus in Soroti district, eastern Uganda (Fevre et al., 2001), 150 animals were subjected to clinical examination and screened for trypanosomes by two standard field methods, the Buffy Coat technique, BCT (Murray et. al., 1977) and using the haematocrit centrifugation technique, HCT (Woo, 1970). Animals were selected for the study according to either a positive identification of carrier status by microscopy, a low PCV or a poor observed condition score (Nicholson & Butterworth, 1986) following Duvallet et al. (1999) who showed that 50% of parasitologically negative animals with a haematocrit value under 25% were subsequently identified as trypanosome positive using PCR. Ten millilitres of blood was withdrawn into a heparinised vaccutainer from the jugular vein [Solano et al. (2002) showed heparin was less inhibitory to PCR reactions than EDTA].

Sample preparation for PCR in the field

Three methods of sample preparation were assessed for sensitivity analysis for PCR determination of *T. brucei*: direct DNA preparation using DNAzol; application of whole blood to Whatman FTA cards; application of buffy coat preparations to FTA cards.

Direct DNA preparation

DNA was directly isolated from 1ml of bovine blood using DNAzol® BD Reagent (Chomczynski et al., 1997). DNAzol "is a complete, non-toxic and ready to use reagent for the isolation of genomic DNA from various biological sources". Whole blood was mixed at a ratio of 1:2 with DNAzol ® BD prior to the addition of isopropanol. The sample was shaken vigorously and left at room temperature for 5 minutes. Precipitated DNA was isolated by centrifugation at 6000 g for 6 minutes and stored at -20 C prior to further processing.

Whole blood application to FTA® Cards

One hundred microlitres of whole blood was applied directly onto FTA® Cards (Whatman®) which were allowed to dry thoroughly prior to storage at room temperature.

Buffy coat preparations onto FTA Cards

One ml whole blood was centrifuged at 10,000 g for 5 minutes. 100 l of buffy coat was withdrawn and applied directly on the FTA® Cards.

Preparation of DNA for PCR analysis

***DNAzol® BD lysate*:** The DNA pellet was washed with 0.5ml of reagent buffer, mixed to ensure complete dispersal, and centrifuged at 2000g for 4 minutes. The supernatant was removed and the pellet washed with 1ml of 95% ethanol. DNA was recovered by

centrifugation and solubilised in 66 l of 8mM NaOH, the solution neutralised after 5 minutes with the addition of 33 l of HEPES.

FTA® Card: Two mm discs were cut from the sample-saturated cards and prepared according to the manufacturers instructions. Briefly, the discs were washed twice in FTA purification reagent to remove any PCR inhibitors from the sample, and traces of FTA buffer were removed by two further washes in TE buffer. The discs were air dried, then transferred to PCR tubes where they were used to seed the amplification reaction.

Trypanosome species specific PCR

DNA prepared using DNAzol, and bound to FTA matrix (from blood of buffy coat source) were assessed for the presence of the trypanosomes which may be present in domestic cattle; namely Trypanozoon, Duttonella and Nannomonas (four types of *Trypanosoma congolense*). The primers used were supplied by Sigma (see Table 1).

PCR reaction conditions and amplification protocols

Standard PCR amplifications were carried out in 25 l reactions mixtures containing the final concentrations, 10 mM TrisHCL pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 m of each of the 4 deoxynucleoside triphosphates and 1 Unit of *REDTaq* DNA polymerase (Sigma) . Genomic template preparations of 1 l DNAzol purified DNA or 1x2mm FTA card discs were amplified. Specific PCR conditions in terms of primer concentrations and amplification programmes are

Table 1. Primer sequences and amplification product size.

Trypanosome species/subgroup	Primer sequence	Product size
Trypanozoon Artama et al*.*, 1992	TBR1 dCGA ATG AAT AAT AAA CAA TGC GCA GT TBR 2 dAGA ACC ATT TAT TAG CTT TGT TGC	177 bp
Kabiri et al*.*, 1999	Museq 1 dGCG TTA GCA GCA GCT GCA GCT GGG Museq 2 dCCT CCT CGG ATA TTT TCC GCA CCC	286bp
T. vivax Masake et al*.*, 1997	ILO1264 dCAG CTC GGC GAA GGC CAC TTC GCT GGG GTG ILO1265 dTCG CTA CCA CAG TCG CAA TCG TCG TCT CAA GG	400 bp
Masake et al, 1994	TVW A dGTG CTC CAT GTG CCA CGT TG TVW B dCAT ATG GTC TGG GAG CGG GT	175bp
T. congolense Savannah Masiga et al*.*, 1992	TCS 1 dCGA GAA CGG GCA CTT TGC GA TCS 2 dGGA CAA AGA AAT CCC GCA CA	316 bp
T. congolense Forest Masiga et al*.*, 1992.	TCF 1 dGGA CAC GCC AGA AGG TAC TT TCF 2 dGTT CTC GCA CCA AAT CCA AC	350 bp
T. congolense Kilifi Masiga et al*.*, 1992.	TCK 1 dGTG CCC AAA TTT GAA GTG AT TCK 2 dACT CAA AAT CGT GCA CCT CG	294 bp
T. congolense Tsavo Majiwa et al*.*,1993.	ILO 893 dGTC CTG CCA CCG AGT ATG C ILO 892 dCGA GCA TGC AGG ATG GCC G	450 bp

Table 2. Species specific PCR conditions and programmes.

Trypanosome species	Primer identification	PCR primer concentration	Amplification conditions
Trypanozoon	TBR 1/2	0.2 M 0.4 M	(30 cycles) 94 C for 45 s, 60 C for 60 s, 72 C for 30 s. (30 cycles) 94 C for 45 s, 55 C for 60 s, 72 C for 30 s.
Trypanozoon	Museq 1/2	0.4 M	(30 cycles) 94 C for 60 s, 60 C for 60 s, 72 C for 60 s.
T. vivax	ILO 1264/5	2 M	(30 cycles) 94 C for 60 s, 55 C for 120 s, 72 C for 120 s.
T. vivax	TVW A/B	1 M	(30 cycles) 94 C for 45 sec, 60 C for 1 min, 72 C for 30 s.
T. congolense Savannah	TCS 1/2	2 M	(30 cycles) 94 C for 60 s, 55 C for 120 s, 72 C for 120 s.
T. congolense Forest	TCF 1/2	2 M	(30 cycles) 94 C for 60 s, 55 C for 120 s, 72 C for 120 s.
T. congolense Kilifi	TCK 1/2	2 M	(30 cycles) 94 C for 60 s, 55 C for 120 s, 72 C for 120 s.
T. congolense Tsavo	ILO 892/3	2 M	(30 cycles) 94 C for 60 s, 60 C for 120 s, 74 C for 30 s

listed on Table 2, a final elongation step of 5 minutes was incorporated into each programme. PCR products were separated by electrophoreses in a 1.5% (w/v) agarose gel containing 0.5 g/ml ethidium bromide and visualised by ultraviolet light.

Defining any trypanosome species infection

It was assumed that each test was 100% specific (i.e. identification by microscopy or detection or amplification of DNA primers was indicative of trypanosome presence) . Therefore, the gold standard, or true infection status of each of the 61 cows, was defined as positive if at least one of the four diagnostic tests were positive.

Defining *T. brucei* species infection

Because of the difficulty of distinguishing trypanosome species with microscopy, the gold standard, or true *T. brucei* species infection status for each of the 61 cows, was defined as positive if a least one of the three DNA tests were positive.

Statistical analysis

For each of the four tests, the sensitivity (and 95% confidence intervals) was calculated relative to the gold standard status, for both infection with any trypanosome species and infection with *T. brucei* species. Differences between the sensitivity of the different tests were examined using the ^{2} test. The kappa statistic (and 95% confidence intervals), which gives a measurement of the degree of agreement between each of the four tests and the gold standard, was also calculated, again for infection with any trypanosome infection and infection with *T. brucei* species. The value of kappa lies between 0 and 1, with 1 meaning perfect agreement between tests and 0 meaning that the association is no better than expected from chance alone. All statistical analyses were performed in SPSS (release 10.0.5).

RESULTS

A total of 61 cows were diagnosed using the four tests. A summary of the results is shown in Table 3. Overall, 55 (90.1%) of the cows had a trypanosome infection with 51 infected with *T. brucei* species. The sensitivities of the four different tests, for any trypanosome infection and *T. brucei* species specifically, relative to the gold standards (defined above) are shown in Figure 1. Statistical comparisons of the test sensitivities are shown in Tables 4a (for any trypanosome species detection) and 4b (for *T. brucei* species detection) . The measurements of agreement between each of the four diagnostic tests and the defined gold standard are shown in Figure 2.

Table 3. Summary of positive diagnostic results.

	Microscopy	DNAzol	Cards-blood	Cards-buffy coat	"Gold standard"
Any trypanosome species	21	36	38	53	55
T. brucei s.l.	13*	31	22	47	51

*includes one cow that was identified as positive for *T. brucei* species with microscopy but negative on all three PCR tests. The cow was shown by PCR to have a *T. vivax* infection.

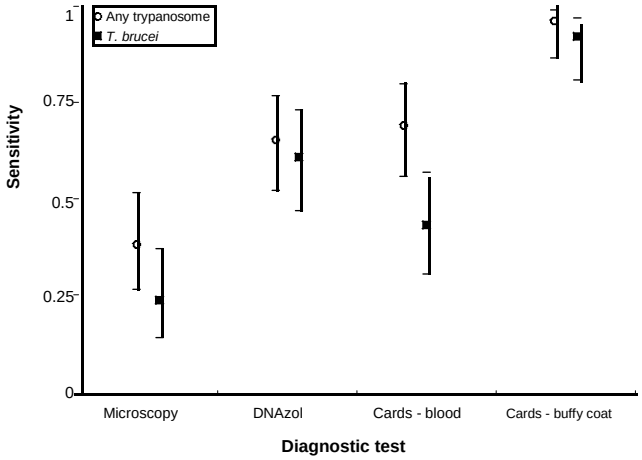

Figure 1. Sensitivity of the four diagnostic tests relative to the gold standard.

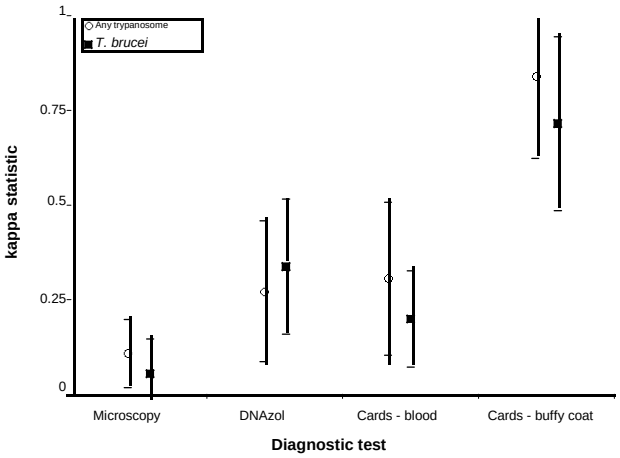

Figure 2. Measure of agreement (kappa statistic) between each of the four diagnostic tests and the gold standard.

DISCUSSION

Molecular methods for the species-specific identification of trypanosomes have been available for nearly 10 years (Masake et al., 1994, 1997; Majiwa et al. 1993, 1994). Despite this, their application in disease control and research programmes in the field has been limited to a handful of studies on cattle like that of Clausen et al. (1998) on naturally infected dairy cattle in peri- urban Kampala, Solano et al. (1999) in Burkina Faso and Mugittu et al. (2001) in Tanzania. The more traditional methods involving microscopy have prevailed in most field studies, despite the fact that it has been suspected for some time that these classical methods are missing a large proportion of the infections present in livestock, due to the presence of trypanosome-positive DNA signals from cross-checked cattle deemed aparasitaemic by these methods (Majiwa et al., 1994). The principal problem with microscopy is one of sensitivity.

The application of DNA techniques provides much improved levels of sensitivity, such as the possibility of detection of individual organisms in samples of whole blood. Microscopy remains useful particularly as it can be carried out directly at the field level and gives immediate results. The original molecular protocols were not immediately applicable to conditions in the field, due to the amount of processing required and the time involved prior to the testing of the samples themselves. However, recent developments in methods of extracting and/or preserving DNA have changed this, making sample collection with a view to diagnosis by PCR more realistic at the field level.

In this study, we compared the diagnostic sensitivity of classical parasitological examination by microscopy against DNA methodologies – PCR on blood samples treated with DNAzol and PCR on blood and buffy coat samples collected on FTA cards, to evaluate which methods provides the greatest sensitivity for trypanosome identification and species diagnosis. The direct preparation of DNA using DNAzol direct purification (Chomczynski et al., 1997) uses guanidine thiocyanate and a detergent mixture. The recent development of the Whatman® FTA® matrix offers

Table 4a. Comparison of the test sensitivities for any trypanosome species-detection using the ^{2} test.

	Microscopy	**DNAzol**	**Cards-blood**	**Cards-buffy coat**
Microscopy	_	^{2}=7.14 *P*=0.008	^{2}=9.36 *P*=0.002	^{2}=39.68 *P*<0.001
DNAzol		_	^{2}=0.04 Not Significant	^{2}=15.07 *P*<0.001
Cards - blood			_	^{2}=12.47 *P*<0.001
Cards-buffy coat				_

Table 4b. Comparison of the test sensitivities for *T. brucei* species detection using the ^{2} test.

	Microscopy	**DNAzol**	**Cards-blood**	**Cards-buffy coat**
Microscopy	_	^{2}=13.03 *P*<0.001	^{2}=3.57 Not Significant	^{2}=46.48 *P*<0.001
DNAzol		_	^{2}=2.51 Not Significant	^{2}=12.26 *P*<0.001
Cards - blood			_	^{2}=25.8 *P*<0.001
Cards-buffy coat				_

significant advantages for sample collection for the preservation of DNA (Hsiao et al., 1999). These FTA matrices have been successfully tested for whole blood storage and malaria diagnosis by PCR (Zhong et al., 2001) and Dobbs et al. (2002) found that tumour cells stored on an FTA matrix performed as well in PCR as freshly extracted DNA in over 95% of cases. Samples stored on this matrix have the added advantage of a 10-year shelf life at room temperature.

Here, we have shown that PCR following application of buffy coat to the FTA matrix is the most sensitive diagnostic test for all trypanosome species-specific PCR reactions currently available. There was no significant difference in the sensitivity of PCR on whole blood applied to the FTA matrix and whole blood DNA extraction with DNAzol for all trypanosomes, and all these methods are a significant improvement on the sensitivity offered by the standard HCT and BCT microscopy methods for all trypanosomes. However, the results show that PCR on whole blood on the FTA matrix is not significantly different from microscopy for *T. brucei* alone, suggesting that, for the accurate determination of *T. brucei* prevalence in Zebu cattle, a concentration step (preparation of buffy coats) is necessary.

Given that in this region (Soroti, eastern Uganda) 40% of cattle with *T. brucei* infections were also positive for the human serum resistance gene (Welburn et al., 2001b), sensitive and accurate detection of *T. brucei* infections in cattle is essential for planning control programmes for human African trypanosomiasis. These findings are consistent with earlier studies of Clausen et al. (1998) who demonstrated that the detection rate by PCR was two times higher than the detection rate with parasitological techniques, and that of Solano et al. (1999) who showed that PCR on buffy coat samples for diagnosis is more sensitive than on whole blood extracted from filter papers using Chelex, as the concentration of trypanosomes is higher in the buffy coat. Furthermore, Mugittu et al. (2001) found that trypanosomes could be detected in 43% of parasitologically negative cattle (62 samples) using PCR.

Given these improved tools, it becomes apparent that there are far more trypanosome-infected cattle circulating in the herds in tsetse infested regions than previously thought, and that control programmes which have been designed around prevalence information gained from microscopy studies may have under-estimated the degree of the problem. In terms of animal herd and human health, the aim of such programmes is the control of parasites in the reservoir of infection. When the extent of this reservoir is not properly accounted for due to the poor sensitivity of the tools used, the resulting interventions will not be adequate. Screening of animals is a necessary step to assessing, and ultimately reducing, the risk of outbreaks of the trypanosomiases in previously unaffected areas (Enyaru, 1999; Fèvre et al., 2002).

Sustainable control programmes must also consider ways of eliminating or limiting the spread of the trypanosome reservoir of disease through domestic livestock populations. One solution would be to treat cattle at point of sale in the markets. Controlling the reservoir of parasites of all species is essential (Welburn and Odiit, 2002) while bearing in mind the longer-term problems that large-scale chemotherapy of cattle may pose in terms of trypanocidal drug resistance (Barrett, 2001).

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