Challenges in the Diagnostic Performance of Parasitological and Molecular Tests in the Detection of African Trypanosomiasis in Cattle in Mambwe District in Eastern Zambia

Gloria Mulenga (mmukuka2000@yahoo.com)
Kakumbi tsetse and trypanosomiasis research station https://orcid.org/0000-0002-3611-5016
Boniface Namangala
The University of Zambia
Kalinga Chilongo
Department of Veterinary services
Chrisborn Mubamba
Department of Veterinary services
Kyoko Hayashida
Hokkaido University: Hokkaido Daigaku
Lars Henning
James Cook University Division of Tropical Health and Medicine
Bruce Gummow
James Cook University Division of Tropical Health and Medicine

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Abstract

Background: Trypanosomiasis is a Neglected Tropical Disease with serious health and economic implications. Disease eradication and control programs rely on active case detection through mass population screening. Screening tools and techniques therefore need to be adequately sensitive, practically quick to perform, and affordable. This study compared the field application of the polymerase chain reaction, the loop mediated isothermal amplification technique, and microscopy, in the detection of trypanosomes in cattle blood in Mambwe district in eastern Zambia.

Methods: Blood samples were collected from 227 cattle into three heparinised micro capillary tubes, and tested for trypanosomiasis infection using microscopy, ITS-PCR and RIME-LAMP. The comparative diagnostic performance of each of the methods was evaluated using the chi-square test, kappa test and receive operator curves.

Results: Microscopy on buffy coat detected 17 cases (n=227), by thin smears detected 26 cases (n=227), and by thick smears detected 28 cases (n=227). In total, microscopy detected 40 cases (n=227). ITS-PCR- on blood spots stored on filter paper detected 47 cases (n=227), ITS-PCR- on blood spots stored on FTA cards detected 83 cases (n=227) and RIME-LAMP-FTA detected 18 cases (n = 131). Using microscopy as gold standard, sensitivity and specificity of ITS-PCR was compared. ITS-PCR-FTA had a better specificity and sensitivity (SE=77.5%; SP=72.2%; k = 0.35) than ITS-PCR-FP (SP = 88%; SE = 60%; kappa = 0.45). Prevalence of *Trypanosoma brucei* s.l. was higher on RIME-LAMP-FTA (18/131) than ITS-PCR-FTA (19/227).

Conclusion: Our results are not perfect but are a good illustration of the current diagnostic challenges in rural Africa. Findings showed that none of the diagnostic tests could be taken as having performed better than the others and that each of the tests offered some advantages and limitations. In endemic rural areas of Africa, the use of PCR and LAMP requires specialised staff, laboratory supplies and infrastructure which is often not available. For this reason, microscopy remains the most practical option for field diagnosis of trypanosomes but understanding its limitations is critical particularly when applied for surveillance purposes.

Background

Tsetse-transmitted trypanosomiasis, caused by protozoan parasites of the genus *Trypanosoma*, affects both man and animals. While *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei* s.l. cause nagana or Animal African Trypanosomiasis (AAT) in livestock, the two sub species of *T. brucei* s.l.: *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* are responsible for Human African Trypanosomiasis (HAT) commonly known as 'sleeping sickness'. Countries affected by nagana have continued to suffer from economic losses in millions of dollars (1–3).

Historically, microscopy has been traditionally regarded as the gold standard in detecting the presence of trypanosomes. Microscopic examination of buffy boat and wet blood films, as well as thin and thick blood smears stained with Giemsa, are the most common methods used in Africa for trypanosome detection. Microscopy is considered a good diagnostic method because it is simple, cheap and can also simultaneously detect other haemoparasites (microfilaria and *Plasmodium* spp.) (4). However, microscopy has very low sensitivity especially in detecting early infections that are associated with low parasitaemia (5–7).

Molecular techniques such as the polymerase chain reaction (PCR) have significantly improved the level of sensitivity and accuracy in trypanosome diagnosis, in comparison to the traditional parasitological methods. However, most remote areas of Africa have not had the resources to facilitate the use of such molecular techniques (8, 9). Molecular tests have the ability to facilitate differentiation of trypanosome species and subspecies, e.g. through use of specific primers (10–13). Internal Transcribed Spacers (ITS)-PCR can be used for the detection of both AAT and HAT, but its use in the field is limited by high costs and the need for highly trained personnel (14, 15).

The invention of the repetitive insertion mobile element-loop-mediated isothermal amplification (RIME-LAMP) method a decade ago has given new impetus to development of point-of-care diagnostic tests, based on amplification of pathogen DNA - a technology that has been the precinct of well-developed laboratories (13, 16). RIME-LAMP is a simple diagnostic test that amplifies the RIME sequence of the *Trypanozoon* subgenus group, and is reportedly highly sensitive, and specific (17–19). The advantage of RIME-LAMP over ITS-PCR is that it is less expensive and quicker to carry out. Furthermore, the sensitivity of RIME-LAMP is reported to be equal to or higher than that of the ITS-PCR that targets the same gene (16, 17).

Understanding the capabilities of each diagnostic technique is key to quick and accurate detection of trypanosomiasis in samples and is critical to disease control and eradication. Unfortunately, in most rural settings of Africa, poor detection of trypanosome infections has occurred due to the poor understanding of the limitations of diagnostic tests used. Against this background, this study compared the diagnostic performance of microscopy, ITS-PCR, and RIME-LAMP in detecting trypanosomes.

Methods

Study area

The study was undertaken in Mambwe district in the Eastern Province in Zambia, from February to April 2019. The district was purposively selected, considering that most of the district is highly tsetse infested and with a high prevalence of bovine trypanosomiasis (20). Located along the Luangwa river basin, the district covers an area of 4,480 km^2 and is home to the South Luangwa National park. With a human population of 92,445 belonging to 18,489 households, most of the local community rely on tourism and small-scale farming for their livelihoods (Zambia Central Statistical Office (CSO), 2015).

Study design and sample size

The study compared three diagnostic techniques (RIME-LAMP, ITS-PCR, and microscopy) for the detection of bovine trypanosomiasis under field conditions. This was done through establishment of prevalence of trypanosome species in 227 cattle among a sample of cattle-owning small-scale farmers in selected parts of Mambwe district – i.e. located in tsetse-infested parts of the district close to the South Luangwa National Park. The cattle farmers were selected largely based on willingness to participate in the study. Oral/written informed consents were provided.

Sample collection

From each animal, blood was drawn into three micro capillary tubes containing heparin (anticoagulant) after puncturing the ear vein of the animal with a blood lancet. One capillary tube was sealed with crista seal for on-site examination by buffy coat. Blood from the second capillary tube was used to make thin and thick smears for analysis on microscopy later at the laboratory (4). About 50 µl of blood from the third capillary tube was applied onto a well labelled Whatman FTA Classic Card (GE Healthcare, Madison, WI, USA; CAT No. WB120203 Lot No. 15146046) and on Whatman® No. 1 filter paper (GE Healthcare). After air drying, both filter paper and FTA card samples were separately packed in zip locked storage bags containing silica gel and transported to the laboratory for further processing with ITS-PCR and RIME-LAMP (21).

Application of diagnostic tests

Microscopy: On site microscopic examination was conducted for cattle blood stored in heparinized capillary tubes. The sealed capillary tubes were spun in a microhematocrit centrifuge for five minutes at 10,000 rpm, after which packed cell volumes (PCVs) were determined using a PCV reader. Buffy coat from each sample was then placed on a microscopic slip with cover slip and examined on site at ·400 magnification for the presence of motile trypanosomes. After staining with 10% of Giemsa solution at the laboratory, thin and thick smears collected from cattle samples were also examined for the presence of trypanosomes (22, 23).

DNA extraction from Whatman® No. 1 filter paper: DNA from stored blood spots was extracted using the Tris-EDTA buffer technique. Discs of about 6 mm diameter were cut from each blood spot and placed in labelled 1.5 ml sterile tubes. About 66 µl of TE buffer (10 mM Tris-HCl pH 8.0 and 0.1 mM EDTA in distilled water) was added to each tube and incubated at 50^{o}C for 15 minutes. The discs were then pressed gently at the bottom of the tube using a new rod for each tube and heated at 97^{o}C for another 15 minutes to eluate the DNA. The tubes were then spun down at 10,000 rpm for 1 minute (24)

DNA extraction from FTA cards: DNA was extracted from the stored blood spots using the Chelex method. A disc of about 6 mm diameter from each blood spot was placed in a labelled sterile 1.5 ml sterile tube. About 200 µl of Whatman purification reagent was used to wash each disc for 15 minutes, after which the solution was carefully decanted. The discs were then washed twice with 200 µl of 1 · concentrated TE buffer for 15 minutes after which the solutions were decanted gently. A separate rod for each sample was used during decanting to make sure that the discs did not flow over with the solutions. The discs were then left to air dry for an hour after which 100 µl of 5% (w/v) Chelex-100 (Sigma-Aldrich Japan, Tokyo, Japan) in distilled water solution was added and mixed thoroughly. The discs containing Chelex solution were finally incubated at 90^{o}C for 30 minutes to elute DNA. The eluted DNA was stored at 4^{o}C for use within 12 hours and at -20^{o}C for long-term storage (21)

RIME-LAMP: The test was performed for cattle blood spots stored on FTA cards using dried (vitrificated) master mix containing RIME primer for *T. brucei* s.l. detection. The test tubes were produced in house according to the previously reported procedure (19). About 23 µl of reaction buffer was then added to the tubes followed by 2 µl of DNA eluate from cattle blood spots on FTA cards. The tubes were then carefully closed and turned upside down for 2 minutes and mixed several times to dissolve the reagents. The reaction mixture was then incubated at 63^{o}C for 45 minutes after which positivity was determined through the confirmation of the fluorescent in the reaction mixtures observed by LED detector (17, 19).

ITS ITS-PCR: ITS-PCR was undertaken in 25 µl reaction mixtures containing primers AITS-F: CGGAAGTTCACCGATATTGC and AITS-R: AGGAAGCCAAGTCATCCATC (25), One Taq 2 · master mix (New England BioLabs, Ipswich, MA, USA), nuclease free water and 5 µl of extracted DNA sample. For the detection of *T.brucei rhodesience*, SRA F (5'-ATAGTGACAAGATGCGTACTCAACGC-3') and SRA R (5'-AATGTGTTCGAGTACTTCGGCACGCT-3') were used-procured from Inqaba Biotec (Pretoria, South Africa). Thermocycler amplification conditions were at 94^{o}C for 5 minutes, 94^{o}C for 40 seconds, 58^{o}C for 40 seconds, 72^{o}C for 90 minutes and 72^{o}C for 5 minutes for 40 cycles. ITS-PCR targets the internal transcribed spacer 1 (ITS1) of the small ribosomal subunit (100–200 copies per genome), producing different sized products for different trypanosome species (11, 15, 25). ITS-PCR products were separated by electrophoresis (95 volts for 60 minutes) in a 2% (w/v) agarose gel containing ethidium bromide. Separated products were then visualized under ultraviolet light in a transilluminator. Known positive controls of *T. congolense* (560–705 bp), *T. vivax* (226–238 bp) and *T. brucei* (415–431 bp) and a negative control were included in each reaction. All samples positive for *T. brucei* were subjected to a multiplex ITS-PCR using serum resistant-associated antigen (SRA) targeting primer for the detection of *T. b. rhodesiense.*

Data analysis

Statistical analyses were performed in SPSS version 26 (IBM Corporation., 2019). Trypanosomiasis prevalence determined by microscopy (buffy coat, thin smears, and thick smears) was taken as the gold standard. The prevalence determined by the ITS-PCR were compared against this gold standard and the sensitivity and specificity were calculated on this basis. Chi-square test was used to determine statistical significance between the tests. For expected values less than 5, the Fisher's exact test was used. P values less than 0.05 were considered statistically significant. Impact of diagnostic test performance was estimated using positive and negative predictive values for each test. The usefulness and benefits between the tests were measured using the Receive operator curve (ROC) while Kappa coefficient measured agreements and accuracy between tests. Area under the receive operator curve (AUC-ROC) scores were used to distinguish between a perfect and worthless test. AUC scores were classified as follows: excellent (0.90-1), good (0.80–0.90), fair (0.70–0.80), poor (0.60–0.70) and worthless (0.50–0.60). Kappa values were classified as follows: values ≤ 0 indicated no agreement, slight agreement (0.01-.20), fair (0.21–0.40), moderate (0.41–0.60), considerable (0.61–0.80) and perfect (0.81-1.00).

Results

Microscopic examination of trypanosome infection on buffy coat detected 17/227 cases (7.5%), thin smears detected 26/227 cases (11.5%) while thick smears detected 28/227 cases (12.3%). Combined microscopy using these three microscopic techniques in parallel recorded a total of 40/227 cases (17.6%). (Table 1). RIME-LAMP-FTA detected 18 cases (13.7%, n = 131).

Table 1
Cases of trypanosomes detected in cattle blood

Lab test Technique		n	No. of cases detected
Microscopy	Buffy coat	227	17
	Thin smear	227	26
	Thick smear	227	28
	Combined microscopy	227	40
ITS-PCR	FP	227	47
	FTA	227	83
RIME-LAMP	FTA	131	18

Out of the 227 cattle blood samples screened using ITS-PCR, the overall prevalence of trypanosomiasis from blood spots stored and transported on FP was 20.7% (47/227) while a 36.6% (83/227) prevalence was recorded from blood spots stored and transported on FTA cards. Mean Packed Cell Volume (PCV) for trypanosome positive samples was 34.21 (95% CI = 33.21–35.22) while that for negative samples was 35.21 (95% CI = 34.21–36.22).

Table 2
Prevalence of trypanosome species in cattle (n = 227) by ITS-PCR

Trypanosome species	ITS-PCR-FP	Sample prevalence %	Confidence Interval at 95%	ITS-PCR-FTA	Sample prevalence %	Confidence Interval at 95%
T. congolense	7	3.1	0.8–5.3	14	6.2	3.0-9.3
T. vivax	39	17.2	12.3–22.1	50	22.0	16.6–27.4
T. brucei	1	0.4	-0.4-1.3	19	8.4	4.8–12.0
T. b. rhodesiense	0	-	-	3	1.3	-0.2-2.8
Mixed	1	0.4	-0.4-1.3	9	4.0	1.4–6.5
TOTAL	**47**	**20.7**	**15.4–26.0**	**83**	**36.6**	**30.3–42.8**

Diagnostic accuracy, sensitivity and specificity of ITS-PCR on blood spots stored on filter paper FP (ITS-PCR-FP) and ITS-PCR on blood spots stored on FTA cards (ITS-PCR-FTA) was determined using Microscopy as the gold standard as shown in Tables 3 and 4 respectively. Agreement between the tests was measured using the Kappa test. The results of comparison of ITS-PCR using FTA and FP as collection methods is shown in Table 5.

Table 3
Comparison between Microscopy (gold standard) and ITS-PCR-Filter paper

		Buffy coat			Thin smear			Thick smear			Combined Microscopy		
		Positive	Negative	TOTAL	Positive	Negative	TOTAL	Positive	Negative	TOTAL	Positive	Negative	TOTAL
ITS-PCR-FP	Positive	9 (TP)	38 (FP)	47	15	32	47	22	25	47	24	23	47
	Negative	8 (FN)	172 (TN)	180	11	169	180	6	174	180	16	164	180
	TOTAL	**17**	**210**	**227**	**26**	**201**	**227**	**28**	**199**	**227**	**40**	**187**	**227**
PPV		19.1			31.9			46.8			51.1		
NPV		95.6			93.9			96.7			91.1		
SE		52.9			57.7			78.6			60.0		
SP		81.9			84.1			87.4			87.7		
PLR		2.93			3.62			6.25			4.88		
NLR		0.57			0.5			0.25			0.46		
Accuracy		0.8			0.81			0.86			0.83		
kappa		0.19			0.31			0.51			0.45		
P-Value		0.01			0.00			0.00			0.00		

TP, True Positive; TN, True Negative; FP, False Positive; FN, False Negative

SE = Sensitivity = TP/(TP + FN)

SP = Specificity = TN/ (TN + FP)

PPV = Positive Predictive Value = TP/(TP + FP)

NPV = Negative Predictive Value = TN/(TN + FN)

PLR = Positive Likelihood Ratio = Sensitivity/(1-Specificity)

NLR = Negative Likelihood Ratio = (1-Sensitivity)/Specificity

Accuracy = TP + TN/TOTAL

Kappa test value (k)

Table 4
Comparison of Microscopy (gold standard) and ITS-PCR-FTA

		Buffy coat			Thin smear			Thick smear			Combined Microscopy		
		Positive	Negative	TOTAL	Positive	Negative	TOTAL	Positive	Negative	TOTAL	Positive	Negative	TOTAL
ITS-PCR-FTA	Positive	13	70	83	19	64	83	24	59	83	31	52	83
	Negative	4	140	144	7	137	144	4	140	144	9	135	144
	TOTAL	**17**	**210**	**227**	**26**	**210**	**227**	**28**	**199**	**227**	**40**	**187**	**227**
PPV		15.7			22.9			28.9			37.4		
NPV		97.2			95.1			97.2			93.8		
SE		76.5			73.1			85.7			77.5		
SP		66.7			68.2			70.4			72.2		
PLR		2.29			2.3			2.9			2.79		
NLR		0.35			0.4			0.2			0.31		
Accuracy		0.67			0.69			0.72			0.73		
kappa		0.15			0.21			0.30			0.35		
P-Value		0.01			0.00			0.00			0.00		

Table 5
Comparison of ITS-PCR-FTA and ITS-PCR-FP

		ITS-PCR-FTA			Accuracy	kappa	P value
		Positive	**Negative**	**Total**			
ITS-PCR-FP	Positive	30	17	**47**	**0.69**	**0.27**	**0.00**
	Negative	53	127	**180**			
	Total	**83**	**144**	**227**			

For the detection of *T. brucei* s.l., RIME-LAMP was used to compare trypanosome detection with ITS-PCR (Table 6).

Table 6
Comparison of RIME-LAMP-FTA with ITS-PCR for the detection of *T. brucei* s.l.

		RIME-LAMP-FTA			PPV	NPV	SE	SP	PLR	NLR	Accuracy	kappa
		Positive	Negative	**Total**								
ITS-PCR FP	Positive	1	0	1	**5.6**	**100**	**100**	**86.9**	**7.7**	**0.0**	**0.87**	0.09
	Negative	17	113	130								
	Total	**18**	**113**	**131**								
ITS-PCR FTA	Positive	13	6	19	**72.2**	**94.7**	**68.4**	**95.5**	**15.3**	**0.3**	**0.92**	0.65
	Negative	5	107	112								
	Total	**18**	**113**	**131**								

Receiver operating characteristic (ROC) curves (Fig. 1) were used to compare sensitivity and specificity across a range of values and area under the ROC curve was used to measure test performance. The curves show the usefulness of ITS-PCR and its ability to detect trypanosomes when compared with buffy coat (ROC 1), thin smear (ROC 2) thick smear (ROC 3) and combined microscopy (ROC 4) while Fig. 2 illustrates the abilities of ITS-PCR and RIME-LAMP to detect *T. brucei* s.l.

Area under the receive operator curve (AUC-ROC) scores for ITS-PCR-FP, ITS-PCR-FTA and RIME-LAMP were as shown in Table 7. The higher the AUC score, the better the test at distinguishing diseased from non-diseased individuals.

Table 7
Areas under the ROC Curves shown in Figs. 1 and 2

Reference	Test Result Variable(s)	AUC	Std. Error[a]	P-Value	AUC 95% Confidence Interval		Test performance relative to reference
					Lower Bound	Upper Bound	
(1) **Buffy coat**	ITS-PCR-FP	.674	.075	.020	.528	.821	Poor
ROC 1	ITS-PCR-FTA	.716	.063	.001	.592	.839	Fair
(2) **Thin smear**	ITS-PCR-FP	.709	.060	.001	.591	.827	Fair
ROC 2	ITS-PCR-FTA	.706	.054	.000	.601	.812	Fair
(3) **Thick smear**	ITS-PCR-FP	.830	.047	.000	.738	.922	Good
ROC 3	ITS-PCR-FTA	.780	.044	.000	.695	.866	Good
(4) **Combined Microscopy**	ITS-PCR-FP	.739	.049	.000	.643	.834	Fair
ROC 4	ITS-PCR-FTA	.748	.043	.000	.665	.832	Fair
(5) **RIME-LAMP**	ITS-PCR-FP	.528	.076	.715	.379	.677	worthless
Figure 4	ITS-PCR-FTA	.835	.064	.000	.708	.961	Good

Discussion

Our study showed that prevalence can be under-estimated by single microscopy technique, as compared to combined microscopy methods and combined molecular techniques. Differences and discrepancies in the number of cases detected from the three microscopy tests may be attributed to remote conditions under which these tests were conducted, low parasitaemia of trypanosome species, operator expertise and time during which observations were made. The buffy coat which is considered to be more sensitive than thick and thin smears (26) but in this case detected the least number of trypanosomes. Low case detection on buffy coat is a very common scenario as most field conditions are not favourable to allow for thorough screening of samples as compared to laboratory screening where operators take time to thoroughly screen the samples. Factors that may have negatively affected case detection on buffy coat, could include (among others), poor quality of capillary tubes, and high temperatures prevalent in the study area which could have led to increased prospects for diminished motility and/or death of trypanosomes before examiners could observe trypanosome movement in the buffy coat.

To validate available molecular diagnostic techniques for AAT, ITS-PCR and RIME-LAMP were employed using blood spots that were stored and transported on FTA cards and normal filter paper. ITS-PCR using blood spots stored and transported on normal filter paper (ITS-PCR-FP) detected (47/227; 95% CI: 15.4–26.0) while ITS-PCR using blood spots stored and transported on FTA cards (ITS-PCR-FTA) detected a higher number of trypanosome infections (83/227; 95% CI: 30.3–42.8). This result suggested that blood spots collected and stored on FTA paper are more reliable in determining trypanosomiasis prevalence than blood spots collected and stored on common filter paper (Chi-square p-value < 0.01). Such results may be attributed to the fact that FTA paper, unlike common filter paper has the ability to protect DNA from degradation (21, 26).

Unfortunately, due to costs attached to the use of FTA cards, their use may be limited as they may not be readily available by most researchers in trypanosomiasis endemic areas of Africa. Comparative analysis between the use of FTA and FP for blood sample storage and ITS-PCR analysis further indicated a fair agreement between the two techniques (kappa = 0.27) and greater probability in detecting trypanosomes. Our data show that both techniques could be useful in the detection of African trypanosomiasis considering that transportation of whole blood samples for ITS-PCR analysis may not be feasible under remote field conditions. Our study has demonstrated the convenience on the use of dry blood samples in areas with limited refrigeration facilities. Dry blood samples could be practically collected from selected animals and stored on FTA cards or filter papers on a regular basis for onward analysis at diagnostic centres (6, 27). Both FTA cards and filter paper may however, inhibit ITS-PCR making it less accurate compared to if DNA was extracted directly from whole blood samples which could explain why microscopically positive samples tested negative on PCR (26).

Our results showed a significant improvement in the number of *T. brucei* s.l. detected on RIME-LAMP (18/131) as compared to ITS-PCR (19/227) supporting the notion that RIME-LAMP is more sensitive and specific at detecting *T. brucei* s.l. than ITS-PCR. ITS-PCR on the other hand is more useful for studying trypanosomes that causes trypanosomiasis in cattle (26). Our findings also showed that filter paper is not good for transporting blood spots for HAT detection as seen in the one case of *T. brucei* s.l. detected on ITS-PCR-FP. Comparative analysis with respect to blood sample collection techniques employed in our study, showed poor agreement of ITS-PCR-FP with RIME-LAMP (k = 0.09) in detecting *Trypanozoon* as compared to ITS-PCR-FTA and RIME-LAMP which showed good agreement (k = 0.65) which is consistent with findings from other studies (19, 28).

When ITS-PCR-FTA was compared to microscopy, results indicated a gradual increase in both sensitivity and specificity with single microscopy tests reporting the lowest sensitivity and specificity: buffy coat (SE = 76.5%; SP = 66.7%; k = 0.15), thin smear (SE = 73.1%; SP = 68.2%; k = 0.21), thick smear (SE = 85.7%; SP = 70.4%; k = 0.30) as compared to combined microscopy tests (SE = 77.5%; SP = 72.2%; k = 0.35) which had a relatively higher sensitivity and specificity. This pattern of gradual increase in the ability of the tests to correctly determine diseased and non-diseased cases was also observed for microscopy and ITS-PCR-FP comparisons. Such results indicate the need for combining buffy coat, thin and thick smear techniques when considering microscopy for trypanosomiasis case detection in remote areas of Africa due to limitations attached to the use of molecular tests.

Although microscopy was used as gold standard in this study, there were positive cases that could be missed. Microscopy is more prone to human error because it is a manual test thus its inability to detect most positive cases. If ITS-PCR-FTA is used as gold standard with respect to combined microscopy, we observed a different scenario were positive and negative predictive values of 77.5% and 72.2% respectively became sensitivity and specificity of 37.4% and 93.8% respectively. In this scenario, ITS-PCR considerably improved case detection and demonstrated how its use may impact positively on trypanosomiasis prevalence estimations.

Using the “rule-in” and “rule-out” test as described by Florkwoski (29), results showed that ITS-PCR-FTA (kappa = 0.18) (high NPV and high sensitivity) was a better test for identifying diseased cattle than ITS-PCR-FP (kappa = 0.30). AUC-ROC scores for ITS-PCR-FP (0.7), ITS-PCR-FTA (0.8) and RIME-LAMP-FTA (0.7) were however, within the acceptable range of 0.7 to 0.9 indicating that both ITS-PCR and RIME-LAMP were significant in trypanosome case diagnosis (18, 20). Nevertheless, the robustness, simplicity, and ability to quickly read and visualize results has made RIME-LAMP a more popular diagnostic tool in Africa instead of ITS-PCR which requires a lot of instrumentation and expertise to achieve required amplification (13, 17). Although the technology required to perform RIME-LAMP exist in most developing countries, its use has still been limited due to non-availability and lack of production of dried ready to use master mix containing RIME primers. This study was not immune to this challenge resulting in only 131 out of 227 blood spots being tested using RIME-LAMP. Despite their affordability, RIME-LAMP kits could not be sourced locally or from regional suppliers that supply most molecular reagents. All PCR reagents on the other hand were available from regional suppliers though at an expensive price. Since Zambia does not produce any molecular reagents, importation and transportation costs also posed a challenge. The use of both ITS-PCR and RIME-LAMP is therefore still limited as most rural laboratories have not yet transitioned to the use of molecular techniques for point of care diagnosis of African trypanosomiasis and other zoonotic diseases.

Findings from this study brought out limitations that come with the use of the existing tests for African trypanosomiasis in rural areas of Africa i.e. Microscopy, ITS-PCR and RIME-LAMP, which may have crucial clinical and epidemiological implications (15, 30, 31).

Although previous studies suggested that *T. congolense* is the main cause of AAT and anaemia in Eastern and Southern Africa (6, 32–34), data from the current study demonstrate that *T. vivax* (ITS-PCR-FP; 39/227 and ITS-PCR-FTA; 50/227) which is less virulent than *T. congolense* was responsible for trypanosomiasis in most of the sampled cattle and that anaemia was not an indicator for trypanosome infection. Our mean PCV for trypanosome negative

samples was slightly higher mean PCV (35.21) for positive samples. Anaemia in cattle is usually associated with the virulence of circulating trypanosomes (34.21). Secondly, ITS-PCR has previously been reported as being better at detecting *T. vivax* infections as compared to other trypanosome species (8, 15). High prevalence of *T. vivax* infections may also suggest that trypanosomiasis transmission within sites included in this study could be mechanical by other blood sucking insects prevalent in the area other rather than by tsetse flies. Finally, the detection of the human infective trypanosomes -*T. b. rhodesiense* from cattle blood samples analysed in this study 3/227 (1.3%; 95% CI: -0.2-2.8), highlights the risks that communities living in tsetse-infested areas may be facing in contracting sleeping sickness (1). Cattle may be potential sources of sleeping sickness when humans get bitten by tsetse after the fly has taken a blood meal from an infected animal (35–38). Our results support the need for a more holistic approach in the control of trypanosomiasis with a focus on the control of the disease in domestic animal reservoirs.

Conclusions

The study illustrates current challenges with AAT diagnosis using molecular and microscopy techniques in rural areas and the need for innovation in the area of diagnostics. However, considering that trypanosomiasis is prevalent in remote rural areas where access to diagnostic facilities is limited, FTA cards and filter papers should be considered for collecting, storing and transportation of blood samples for analysis using ITS-ITS-PCR and RIME-LAMP where collection of whole blood is not feasible. Currently used diagnostic tests have their own advantages and limitations. ITS-PCR is a better screening test of trypanosomes causing Nagana while RIME-LAMP is a better test for the detection of *T. brucei* s.l. However, their use may be limited and not practical in remote rural areas of Africa were trypanosomiasis is endemic. Microscopy could, therefore, be used for diagnosis but as a combination of the three commonly used techniques; buffy coat, thin and thick smears. Microscopy remains the most practical option for diagnosis of trypanosomes in the field but understanding its limitations is critical when using it for surveillance purposes.

Abbreviations

PCR: Polymerase chain reaction; RIME-LAMP: Repetitive insertion mobile element-loop-mediated isothermal amplification; AAT: Animal African trypanosomiasis; HAT: Human African trypanosomiasis; ITS: Internal transcribed spacers; FP: Filter paper; PCV: Packed cell volume; NPV: Negative predictive value; PPV: Positive predictive value; AUC: Area under curve; ROC: Receive operator curve; DNA: Deoxyribonucleic acid.

Declarations

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Availability of supporting data

Not applicable.

Author Contributions

GM and BG developed, conceptualized and drafted the manuscript. BG, BN and LH edited the manuscript, supervised and facilitated finances for the project. KC, KH and BN facilitated smooth operations of both field and laboratory work. CM helped in data collection. All authors reviewed, read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Human and animal ethical clearances obtained from James Cook University (H7226 and A2498), Zambian Ethics Committee (Ref. No. 2018-Oct-001) and research approval from Zambia National Health Research Authority. Individual farmer consent was obtained.

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests to disclose.

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Figures

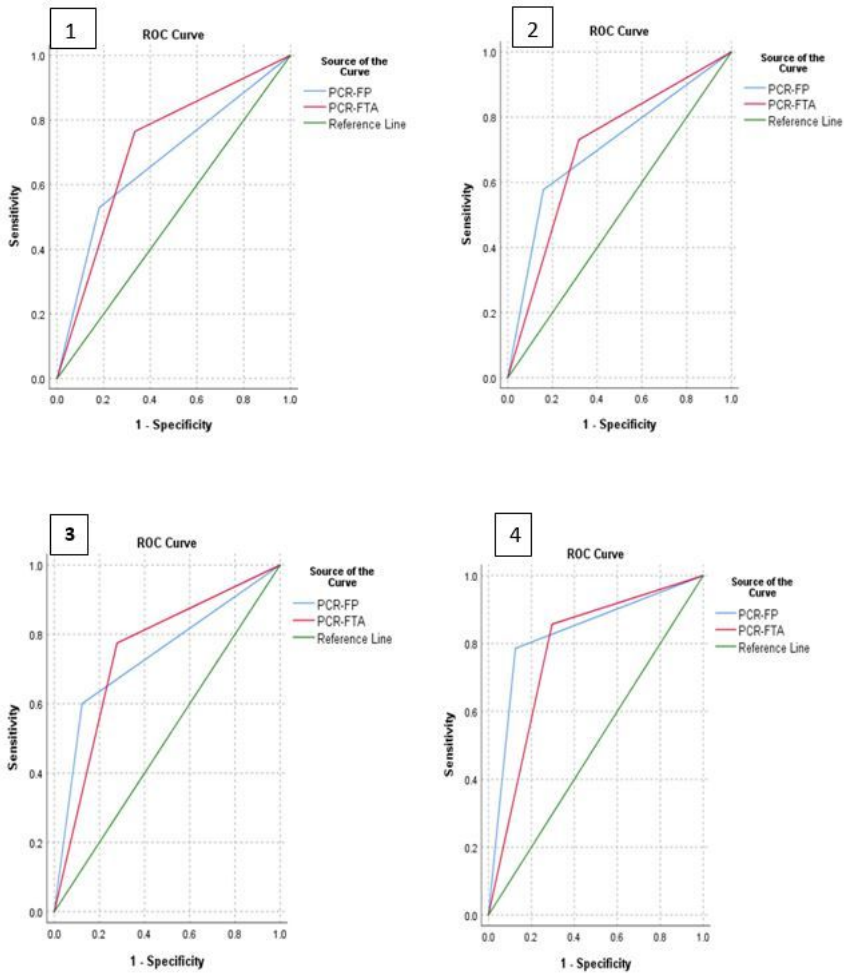
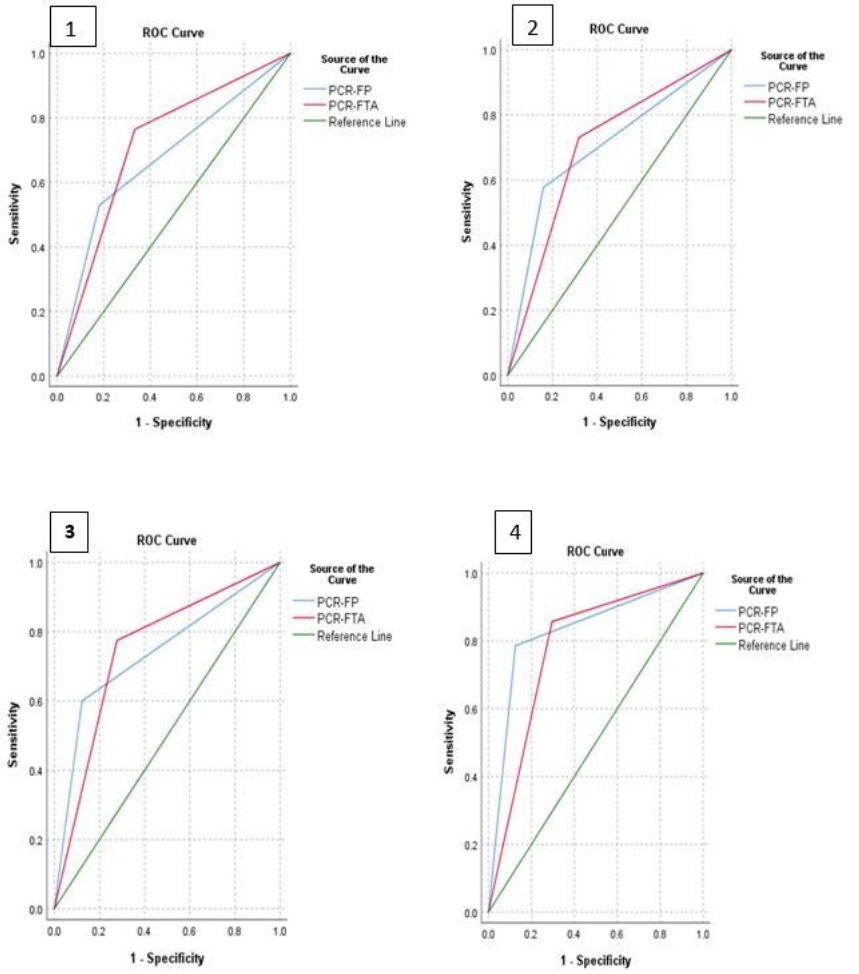


Figure 1

Receive Operator Curves illustrating diagnostic abilities of laboratory tests used. 1- ITS-PCR compared to buffy coat 2- ITS-PCR compared to thin smear 3- ITS-PCR compared to think smear 4- ITS-PCR compared to combined microscopy



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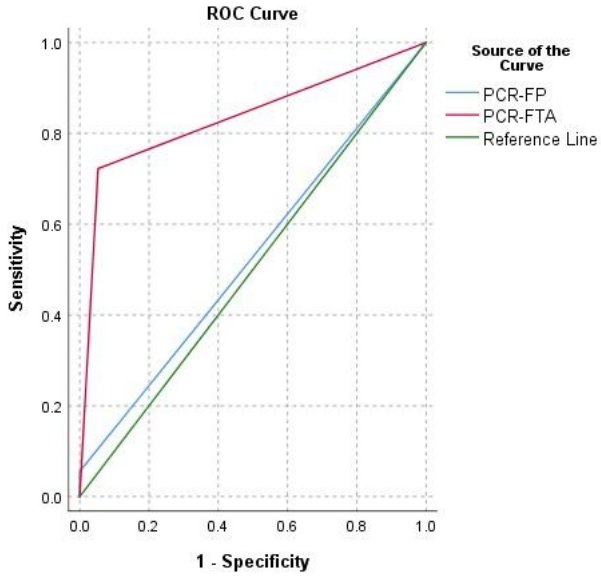
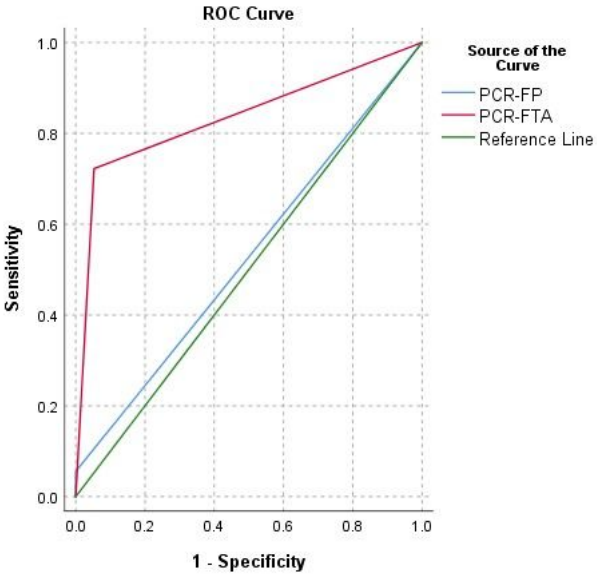


Figure 2

Receive operator curve illustrating diagnostic ability of ITS-PCR when compared to RIME-LAMP



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