Detection of *Trypanosoma brucei* DNA in faeces of experimentally-infected cattle

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1. BACKGROUND AND OBJECTIVES

Species of *Trypanosoma* transmitted by the tsetse fly (*Glossina*) vector are responsible for clinically significant diseases in both human and animal populations. Although significant advances have been made in the control of human African trypanosomiasis (HAT), with the disease targeted for elimination by 2030 [1], animal African trypanosomiasis (AAT) remains a disease of significant economic burden in sub-Saharan Africa. Current AAT surveillance tools suffer from poor sensitivity and specificity [2,3], with serological methods also requiring animal restraint and blood collection by trained personnel. Molecular tools have greatly improved the sensitivity and specificity of AAT surveillance, however high cost and limited field applicability remain a barrier for use in affected regions [2]. Faecal sampling is an option for more accessible sample collection and screening. Successful detection of blood-borne protozoan DNA in human and wildlife faeces has been demonstrated previously, including *Plasmodium sp.*, *Leishmania sp.* and *Trypanosoma sp.* [4–9]. However detection of *Trypanosoma* DNA in livestock faeces has not yet been explored. **Therefore, this proof-of-principle study set out to determine whether it is possible to detect DNA of an AAT pathogen (*T. brucei*) in the faeces of experimentally-infected cattle, and how these results compare to screening matched blood samples over the course of a ten-week longitudinal study**.

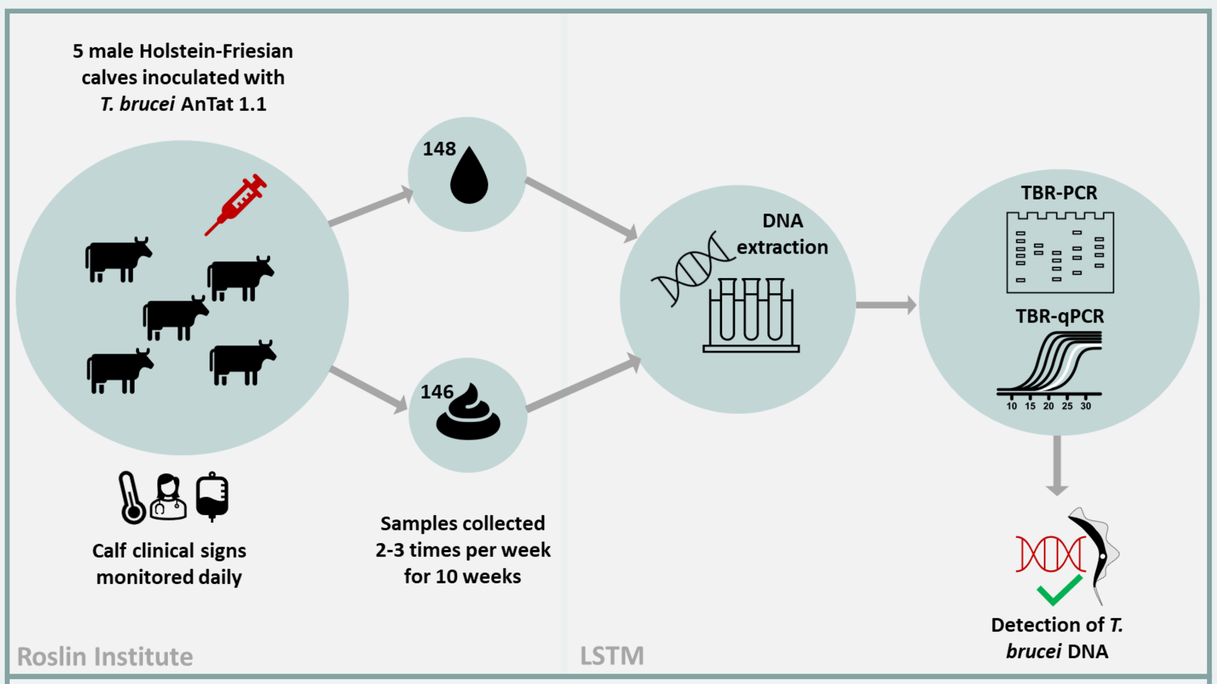

Figure 1: A flow diagram showing the experimental framework for the study.

2. METHODS

Experimental Infections: Experimental infections were carried out at Roslin Institute (Home Office Project License number PE854F3FC, Roslin Institute Animal Welfare Ethical Review Board number L447) for the purposes of a separate project, however the opportunity was taken to collect environmental samples or utilise surplus sample material. The infection study and sample collection was carried out as indicated in Fig.1, with samples linked to individual calf ID where possible. A total of 146 faecal samples stored in RNA*Later*™ (12 pre-inoculation, 134 post-inoculation) and 148 jugular blood samples (10 pre-inoculation, 138 post-inoculation) were collected and stored at -80ºC.

DNA Extraction: Faecal samples (150µg) were processed using Quick-DNA Fecal/Soil Microbe DNA Miniprep kit (Zymo Research). Blood samples (70µL) were processed using Dneasy 96 Blood and Tissue kits (QIAGEN).

PCR and qPCR: PCR reactions were carried out using MyTaq™ Red Mix (Meridian Bioscience) and Applied Biosystems™ Veriti™ thermal cycler (Life Technologies). All qPCR reactions were carried out using Bio-Rad SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories). Thermocycling, fluorescence detection and data capture was carried out using a Mic and micPCR v.2.9.0 software (Bio Molecular Systems). For qPCR, a novel hydrolysis probe-based assay was designed based on *T. brucei* s-I minicircle DNA (accession number K00392.1) using Primer3. The PCR and qPCR primers used are detailed in Table 1.

TBR-PCR Product Sequencing: Following gel electrophoresis, TBR-PCR target products (Fig.6) from 4 faecal samples were excised and purified using an Exo-CIP™ Rapid PCR Cleanup Kit (New England Biolabs). Sanger sequencing was performed by Source BioScience Ltd. Sequence clean-up and alignments were performed in BioEdit v7.2. Resultant sequences were subject to BLAST™ nucleotide analysis (National Centre for Biotechnology Information).

Oligo Name	Sequence (5' - 3')	Source
TBR_PCR_F	CGAATGAATATTAAACAATGCGCAGT	[10]
TBR_PCR_R	AGAACCATTTATTAGCTTTGTTGC	
TBR_QPCR_F	CGCAGTTAACGCTATTATACACA	This study
TBR_QPCR_R	CATTAAACACTAAAGAACAGCGT	
TBR_QPCR_P	FAM-TGTGCAACATTAAATACAAGTGTGT-ZEN	

Table 1 (above): Primers used in the study

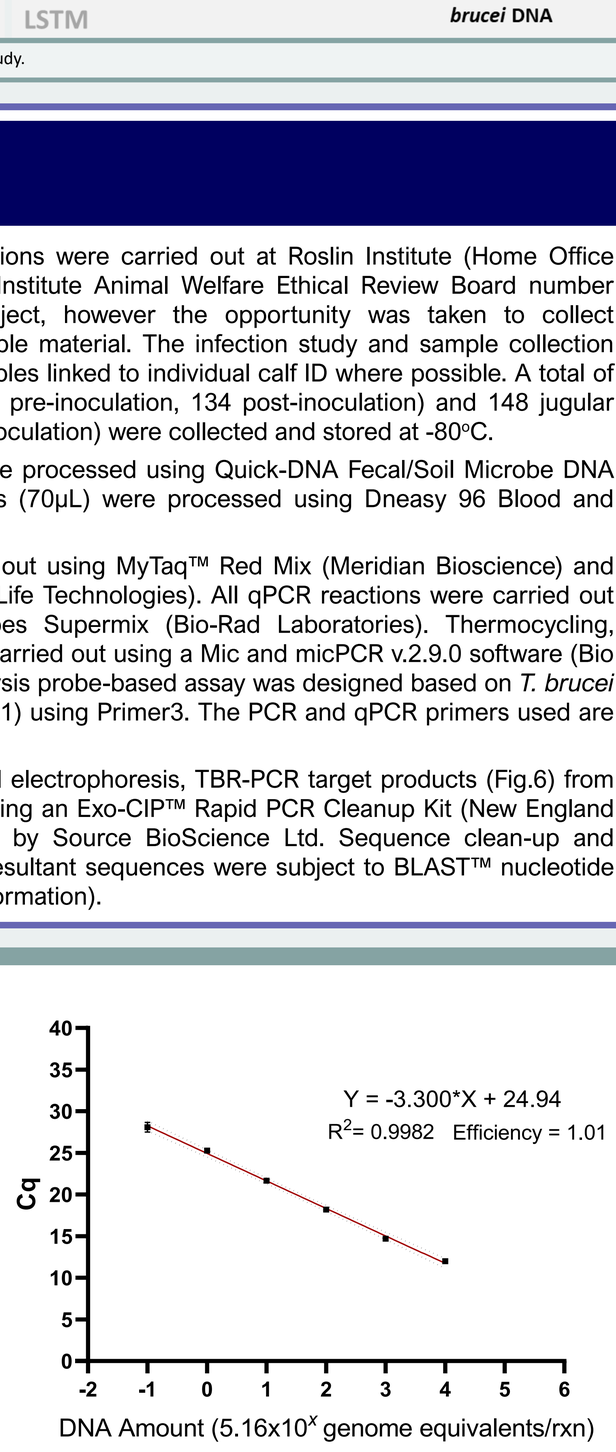

Figure 2 (right): Linear regression analysis on mean Cq values obtained from optimised TBR-qPCR assay against 10-fold dilution series of 1ng/µL to 10fg/µL target DNA. Assays were performed in triplicate. Error bars = standard deviation.

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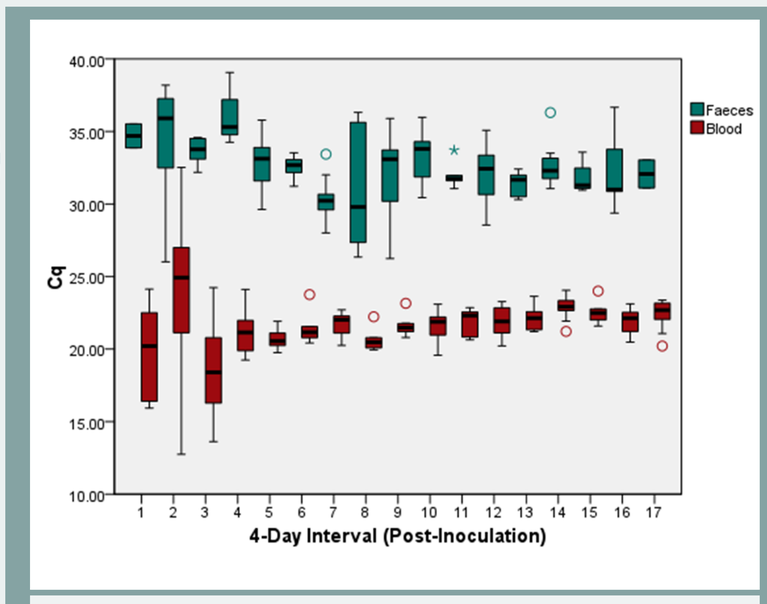

Figure 3: A box plot chart displaying Cq values obtained from screening blood and faecal samples with TBR-qPCR over consecutive 4-day intervals. Error bars represent range.

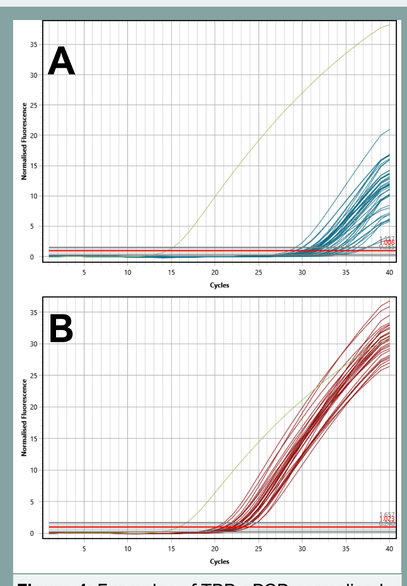

Figure 4: Examples of TBR-qPCR normalised amplification plots obtained from screening 46 faecal samples (A) and 46 blood samples (B). Positive control (*T. brucei* M249) in lime green.

3. RESULTS

Detection of T. brucei DNA in faecal and blood samples: Target DNA was successfully detected in 85% (n=114) of post-inoculation faecal samples by TBR-qPCR and 50% (n=67) by TBR-PCR. Target DNA was detected in faecal samples collected between 4 days post-inoculation (dpi) to 66 dpi by both TBR-qPCR and TBR-PCR. In post-inoculation blood samples, target DNA was detected in 100% (n=138) of samples by TBR-qPCR. No amplification was recorded in pre-inoculation blood (n=10) or faecal (n=12) samples by PCR or qPCR.

Quantification and relationship of T. brucei DNA in faecal and blood samples: Cq values obtained from faecal samples were consistently higher (mean=32.54, ±2.53 SD) than for blood samples (mean=21.54, ±2.36 SD)(Fig.3). Lowest Cq values (faecal=Cq 26.01, blood=Cq 12.75) were obtained from samples taken from the same calf at 5 dpi. Linear regression analysis revealed a weak yet statistically significant positive relationship ($p=0.0354$, $R^2=0.06345$) between Cq values obtained from matched blood and faecal samples (Fig.5).

Confirmation of T. brucei DNA detection: Amplification of *T. brucei* target DNA was confirmed by Sanger sequencing of 4 target PCR products in faecal samples. BLAST™ analysis revealed significant homology (mean percentage identity of 91.59% ±2.834 SD) to *T. brucei* s-I minicircle DNA (accession number K00392.1).

TBR-qPCR assay analytical sensitivity and specificity: The optimised TBR-qPCR assay (Fig. 2) had 95% limit-of-detection of 10fg/µL target genomic DNA (approximately 0.5 genome equivalents per reaction). Analytical specificity testing of the optimised TBR-qPCR assay revealed successful amplification in 9/9 target DNA samples (*T. brucei* M249, *T. b. rhodesiense* Z212, *T. b. gambiense* ELIANE). However low-level amplification was also recorded in 9/21 non-target *Trypanosoma* DNA samples (3/3 *T. vivax*, 3/3 *T. godfreyi*, 2/3 *T. simiae* and 1/3 *T. congolense kalifi*).

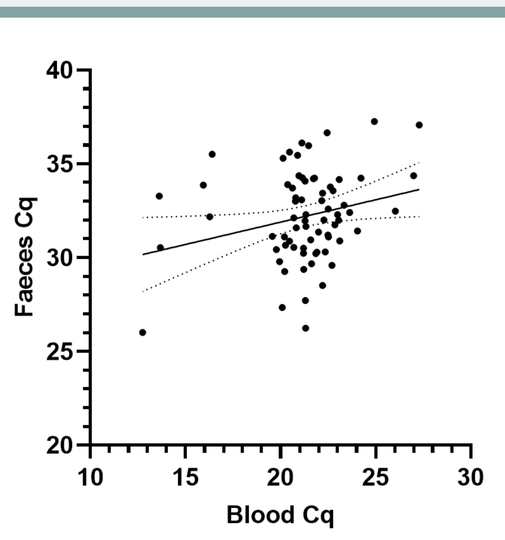

Figure 5: Linear regression analysis between individual calf matched blood and faecal Cq values (n=70).

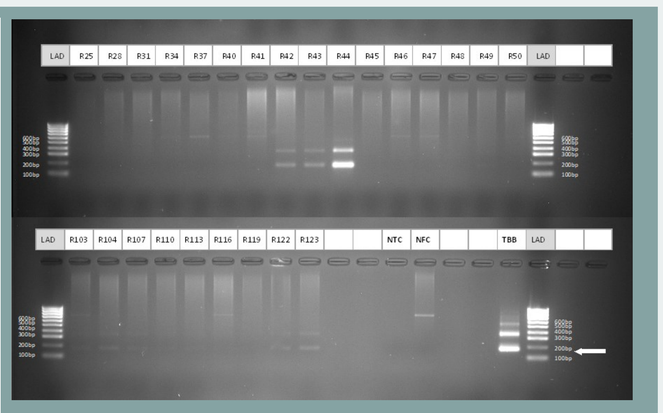

Figure 6: An image displaying results of products resulting from TBR-PCR screening on DNA extracted from 25 post-inoculation cattle faecal samples. LAD = 100bp molecular ladder, NTC = no-template control (nuclease-free water), NFC = negative faeces control. TBB = *T. brucei* M249 DNA 1ng/µL (positive control). A white arrow in the lower right corner indicates the position of diagnostic 173bp TBR-PCR target product, visible in positive control in addition to several samples.

4. CONCLUSIONS

- **These results confirm, for the first time, the ability to consistently detect *Trypanosoma* DNA from the faeces of infected cattle over a 10-week infection period.**
- There is a significant but weak association between quantity of *T. brucei* DNA in blood and faeces. Although the method by which the DNA enters the faeces remains unknown, it may be linked to blood.
- Although highly sensitive, this TBR-qPCR assay may not suitable for field use due to cross-reactivity with DNA from other *Trypanosoma sp.* Other genetic targets should be explored for detection and identification of *Trypanosoma* DNA.
- This study showcases interdisciplinary collaboration and being able to utilise *in vivo* research animals for several parallel studies, thereby reducing the number of animals used in research.
- **With refinement of DNA extraction and molecular detection methods, this technique could be used as an AAT or HAT surveillance tool in both livestock and wildlife mammalian species.**

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