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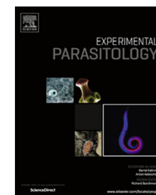

Sensitivity testing of trypanosome detection by PCR from whole blood samples using manual and automated DNA extraction methods

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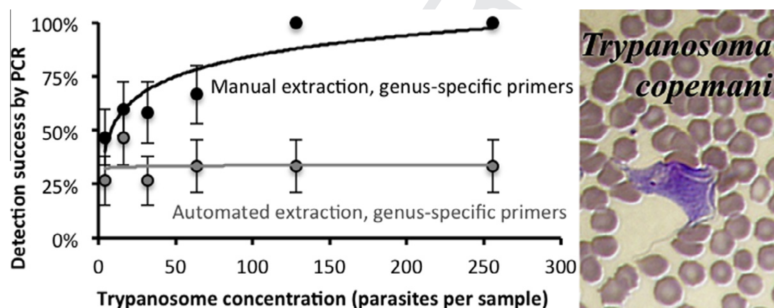
HIGHLIGHTS

- Knowing the detection sensitivity of techniques used to detect pathogens is important.
- We tested the trypanosome detection in replicates of blood samples of known content.
- Automated and manual extraction methods and different primer sets were compared.
- Manual extraction outperformed automated extraction methods.
- We emphasise the importance of testing laboratory procedures to avoid false negatives.

GRAPHICAL ABSTRACT



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ABSTRACT

Automated extraction of DNA for testing of laboratory samples is an attractive alternative to labour-intensive manual methods when higher throughput is required. However, it is important to maintain the maximum detection sensitivity possible to reduce the occurrence of type II errors (false negatives; failure to detect the target when it is present), especially in the biomedical field, where PCR is used for diagnosis. We used blood infected with known concentrations of *Trypanosoma copemani* to test the impact of analysis techniques on trypanosome detection sensitivity by PCR. We compared combinations of a manual and an automated DNA extraction method and two different PCR primer sets to investigate the impact of each on detection levels. Both extraction techniques and specificity of primer sets had a significant impact on detection sensitivity. Samples extracted using the same DNA extraction technique performed substantially differently for each of the separate primer sets. Type I errors (false positives; detection of the target when it is not present), produced by contaminants, were avoided with both extraction methods. This study highlights the importance of testing laboratory techniques with known samples to optimise accuracy of test results.

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1. Introduction

Accurate diagnosis of the haemoprotozoan parasite *Trypanosoma* is of great concern for physicians and veterinarians worldwide, especially in tropical areas of developing countries where particularly pathogenic species exist. Human infection with *Trypanosoma brucei* gives rise to the chronic and acute forms of human African trypanosomiasis (sleeping sickness) via infection with *Trypanosoma brucei gambiense* or *Trypanosoma brucei rhodesiense*, respectively, in sub-Saharan Africa and is fatal if left untreated (Kennedy, 2013). American trypanosomiasis (Chagas disease) is caused by *Trypanosoma cruzi*, and can be fatal in both

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its chronic and acute forms (Kirchhoff, 1993). There is increasing evidence that trypanosomes are not only problematic for humans and livestock, but may also have impacts on wildlife, especially in cases where other threatening processes are present (e.g. black rhino, *Diceros bicornis*, Clausen, 1981; dromedary camels, Mihok et al., 1994; Australian marsupials, Thompson et al., 2009, 2014b).

In Australia, exotic trypanosome species have been inadvertently introduced with feral species such as the European rabbit *Oryctolagus cuniculus* (Hamilton et al., 2005) and black rats *Rattus rattus* (Pickering and Norris, 1996). These introductions can have potentially devastating results to immunologically naïve native species; for example, the arrival of black rats carrying fleas infected with *Trypanosoma lewisi* is likely to have caused the extinction of the native Maclear's rat *Rattus macleari* and probably Bulldog rat *Rattus nativatis* on Christmas Island (Wyatt et al., 2008; see also Thompson et al., 2014a). More recently, new endemic trypanosome species have been identified and linked to declines in populations of the endangered woylie, *Bettongia penicillata* (Wayne et al., 2013; Thompson et al., 2014b). Accurate assessment of the impacts of trypanosomes on humans, livestock and wildlife relies on sensitive and reliable testing techniques.

Detection of pathogens within biological samples using molecular techniques (most commonly by polymerase chain reaction; PCR) is an efficient and often more sensitive alternative to traditional means, such as blood smear microscopy. It is possible to remove some level of observer error by using molecular detection techniques, and studies have shown situations where molecular techniques detect a pathogen that are missed by more traditional means (Smith et al., 2008). It is estimated that 20–30% of *T. brucei* infections are undetected by traditional microscopy screening methods (Robays et al., 2004). However, molecular techniques are not perfect and require quantifiable sensitivity testing to detect the margin of errors present when analysing biological samples.

PCR is a relatively simple and effective method for detecting trypanosome infections in whole blood samples (Desquesnes and Davila, 2002), with techniques continually being refined. The process of detecting trypanosomes by molecular methods involves extracting DNA in a blood sample (including both host and parasite DNA), then using PCR to exponentially replicate a specific sequence of DNA that is unique to the target (Kirchhoff et al., 1996).

When assessing host response to parasitic infections, it is crucial that detection techniques are reliable and have the best sensitivity possible. Much effort is concentrated on modification of PCR primers and reaction conditions to optimise specific detection, however there are few studies dealing with the sensitivity and reliability of different extraction methods, particularly using large numbers of replicates of the same samples.

Since the number of trypanosomes can be particularly low in biological specimens (Eastern barred bandicoot, *Perameles gunnii*, Bettiol et al., 1998), the DNA extraction step is particularly important in harvesting the maximum amount of high quality DNA for optimal detection by PCR. Although this study is specific to detecting trypanosomes within whole blood samples, the concept of extracting pathogenic DNA from host samples is common in medical research; for example, detection of *Chlamydia* within tissue samples (Apfalter et al., 2001), bacterial DNA from human faecal samples (McOrist et al., 2002) and fungal pathogens from blood (Loffler et al., 1997).

The DNA extraction process has traditionally involved manual pipetting methodology, with each sample being processed independently. Conventional manual extractions are cheap and often require inexpensive consumables, but can be more time-consuming and may become impractical when processing large numbers of samples. An alternative is small batch robotic DNA extraction systems such as the Maxwell® 16 Instrument (Promega); able to process up to 16 samples simultaneously (Krnajski et al., 2007). Small batch robotic systems may also assist in maintaining consistency in results when compared to variations of manual extraction batches due to human error and experience involved with individual processing.

The goal of our study was to compare the performance and sensitivity of two different nested PCR assays with trypanosome DNA extracted from blood using manual and automated methods. The analysis included diagnostic sensitivity for biological specimens of known concentrations (presence of type II errors), a contamination study (presence of type I errors) and a comparison of extraction times and costs.

2. Methods

2.1. Trypanosome sample preparation

Trypanosoma copemani were collected from infected woylies (*B. penicillata*) via whole blood (Thompson et al., 2013) and cultured in medium. *T. copemani* cultures were maintained as per (Botero et al., 2013) until there were adequate numbers of trypanosomes for the current experiment. The concentration of trypanosomes per mL was calculated by counting individual amastigote and trypomastigote forms in 10 μL of media using a haemocytometer chamber. From this known concentration, we diluted the solution with clean media and carefully pipetted appropriate quantities into clean vials. Trypanosome-free horse blood (250 μL) was added to each vial of parasites and mixed thoroughly with a pipette. Samples were stored at −20 °C until thawed for extraction. This work was carried out under the Department of Environment and Conservation animal ethics permit DEC AEC 2010/01, and Murdoch University animal ethics permit W2337/10.

2.2. Experimental design

The following known concentrations used were; 256, 128, 32, 16, 4 and 0 trypanosomes per 0.3 mL sample. Each of these individual concentrations was replicated 15 times for each of the two different DNA extraction techniques. These values represent a spectrum of very low to moderate levels of parasitaemia in blood (ILRAD, 1983). It was necessary to include 15 replicates of zero parasites for both techniques to ensure that no false positives occurred due to contamination during the experimental protocol.

2.3. DNA extraction

We tested two extraction methods; a manual method (Wizard® Genomic DNA Purification Kit, Promega Corporation) and an automated extraction (Maxwell® 16 LEV Blood DNA Kit, Promega Corporation). Both of these kits were designed specifically for DNA extraction from whole blood. Samples were run according to the manufacturer's instructions for frozen blood samples; the Maxwell® 16 Forensic Instrument (Cat. # AS1000) (which is required for the Maxwell® 16 LEV Blood DNA kits) was run using version 4.71 firmware for forensic casework. These two extraction methods differ substantially in price per sample and time required for the extraction (see Table 1). DNA was eluted in 50 μL of DNA rehydration solution for both protocols and stored at −20 °C prior to use.

2.4. PCR primers and methods

All samples underwent one of two nested PCR protocols; one using *Trypanosoma* genus-specific primers and one using *T. copemani* species-specific primers. Details of the primer sets can be found in Table 2. PCR reaction conditions were as per McInnes

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Table 1
Cost and time attributes of the two extraction techniques.

Type	Automated	Manual
Kit name	Maxwell® 16 LEV blood DNA kit	Wizard® genomic DNA purification kit
Cost per sample	$7.50	$1.50
Additional consumables per sample	1 × 1000 μL Pipette tip	2 × 1.5 mL Eppendorf tubes, 10 × 1000 μL and 3 × 200 μL pipette tips
Samples processed simultaneously	16	Limited by centrifuge size
Total time for extraction	1 h	2.5 h
Handling time included	20 min	2.5 h

et al. (2009), except for increasing the annealing temperature in the cycling phase to 56 °C (rather than 52 °C). We used 2 μL of DNA template solution for each reaction.

There were 15 replicates of each trypanosome concentration, for each extraction technique, and for each primer pair (15 replicates × 7 concentrations × 2 extraction techniques × 2 primer sets = 420 samples in total). The DNA extractions were distributed among 96 well plates for PCR, with each plate containing extractions from both techniques and of varying trypanosome concentrations; plates were prepared this way to eliminate experimental variations of PCR reagents and protocols. Following PCR, each sample was confirmed as positive or negative according to presence of a fluorescent band following agarose gel electrophoresis. We also ran negative controls on each plate for the primary and secondary step of the nested PCR to check for contamination.

2.5. Data analysis

The experimental design was a nested three factorial for primer type (species or genus-specific primer) and extraction method (automated or manual) across different trypanosome concentrations. Data were determined to be quasibinomial (residual deviance/degrees of freedom > 1), so a general linear model was constructed of all three factors, and analysed by ANOVA in statistical program R, using package "stats" (Crawley, 2012).

3. Results

There was a significant relationship between detectability and trypanosome concentration ($F_{1,26} = 30.13$, $P < 0.001$), where detection success increased with increasing trypanosome concentration across all treatments. This caused significant positive interactions between concentration and primer ($F_{1,23} = 7.203$, $P = 0.014$), as well as concentration and DNA extraction method ($F_{1,22} = 14.60$, $P < 0.001$). These relationships differed significantly between the different primers used ($F_{1,25} = 4.50$, $P = 0.046$) (where species-specific primers had greater success), and different DNA extraction method used ($F_{1,24} = 8.45$, $P = 0.008$) (where manual extraction proved more successful than automated extraction). There was no interaction of extraction method and primer used ($F_{1,21} = 56.84$, $P = 0.240$).

By correlating our percentage of successful detection with increasing trypanosome concentration for each of the four combinations of extraction method and primers (Figs. 1 and 2), we were able to interpolate the levels of infection required to produce positive samples with different levels of confidence, as described in Table 3.

From these data we conclude that the order of sensitivity for our protocols (listed here for 95% detection success; see Table 3) are;

(1) Manual extraction with genus-specific primers; 221.7 trypanosomes per 0.3 mL sample (i.e. a concentration of 739 parasites per mL of blood).
(2) Manual extraction with species-specific primers; 320.7 trypanosomes per 0.3 mL sample (i.e. a concentration of 1069 parasites per mL of blood).
(3) Automated extraction with species-specific primers; 1324 trypanosomes per 0.3 mL sample (i.e. a concentration of 4413 parasites per mL of blood).

Our manual extraction method proved to be substantially better at detecting low concentrations of parasites, with a 50% chance of detecting ~20 parasites/mL. Neither method produced Type I errors (false positives). From this we can conclude that there was no DNA contamination present for either method.

4. Discussion

Our data demonstrate that the analytical sensitivity of trypanosome detection by PCR is significantly affected by both primer type and extraction method used. Many researchers focus on optimising molecular techniques used post extraction, but this study demonstrates the need to check and verify the efficacy of the DNA extraction technique as well. Furthermore, we highlight the importance of quantifying detection sensitivity with multiple replicates of known parasitic concentrations to accurately interpret results.

As expected, there was a significant relationship between trypanosome concentration and detection. Concentrations of 426 parasites per mL or higher were consistently detected. An exception to this was the combination of the manual DNA extraction protocol and the species-specific primers; which had 100% detection for parasite concentrations of 128 per sample or higher.

Table 2
PCR primers used to replicate sections of the *Trypanosoma* 18S rRNA gene, using nested protocols.

Reaction	Step	Name	Primer sequence	Source	Product size (bp)
T. copemani species-specific reaction	Primary reaction	S825F	5′-ACC GTT TCG GCT TTT GTT GG-3′	Maslov et al. (1996)	959
		SLIR	5′ACA TTG TAG TGC GCG TGT C-3′	McInnes et al. (2011)	
	Secondary reaction	WoF	5′- GTG TTG CTT TTT TGG TCT TCA CG-3′	McInnes et al. (2011)	457
		WoR	5′-CAC AAA GGA GGA AAA AAG GGC-3′	McInnes et al. (2011)	
Trypanosoma genus specific reaction	Primary reaction	SLF	5′-GCT TGT TTC AAG GAC TTA GC-3′	McInnes et al. (2009)	1500
		S762R	5′-GAC TTT TGC TTC CTC TAA TG-3′	Maslov et al. (1996)	
	Secondary reaction	S823F	5′-CGA ACA ACT GCC CTA TCA GC-3′	Maslov et al. (1996)	904
		S662R	5′-GAC TAC AAT GGT CTC TAA TC-3′	Maslov et al. (1996)	

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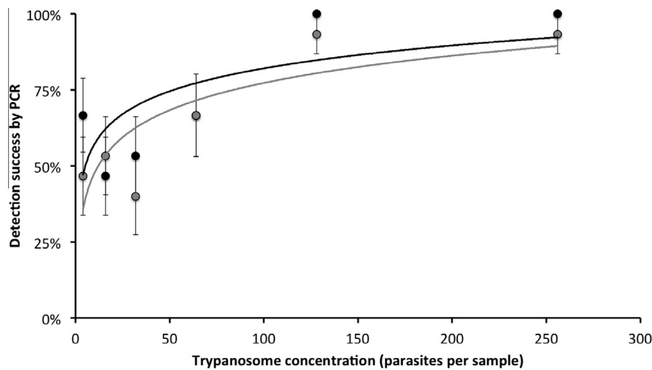

Fig. 1. Species-specific primers: Sensitivity levels of *T. copemani* detection from whole blood, using two different DNA extraction techniques and species specific primers. Manual extraction: solid black circles, automated extraction: grey circles. Error bars indicate 95% confidence intervals.

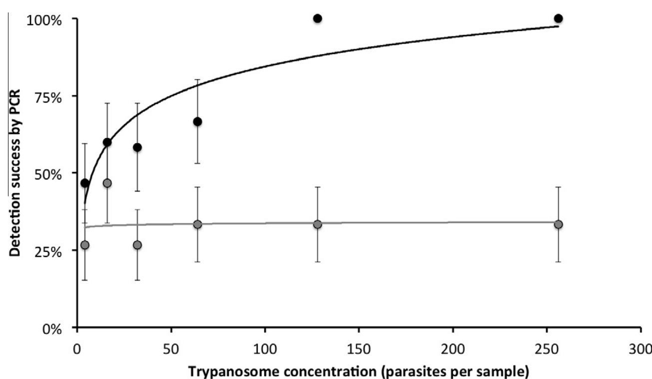

Fig. 2. Genus specific primers: Sensitivity levels of *T. copemani* detection from whole blood, using two different DNA extraction techniques and genus specific primers. Manual extraction: solid black circles, automated extraction: grey circles. Error bars indicate 95% confidence intervals.

Table 3
Number of trypanosomes required in a 0.3 mL blood sample for each method, for differing detection confidences.

Detection sensitivity (%)	Species-specific primers		Genus-specific primers	
	Automated	Manual	Automated	Manual
95	397.2	320.7	4.15E+68	221.7
80	124.2	81.0	2.20E+52	74.2
75	84.3	51.2	8.00E+46	51.5
60	26.3	12.9	4.15E+30	17.2
50	12.1	5.2	5.75E+19	8.3

Overall, the detection sensitivity for our samples was improved by using manual DNA extraction. Regardless of the trypanosome concentration, only 30% of the samples containing trypanosomes presented as positive when extracted using the automated Maxwell® 16 system and screened with the genus-specific primers (Fig. 1). Although it is likely that higher concentrations of cultured trypanosomes would eventually produce a consistently positive result, for the purposes of biological specimens with potentially low parasitaemia, low concentrations were more meaningful.

Although more time consuming, the manual method tested appears to outperform automated extraction method in trypanosome detection and cost-effectiveness. Other research has shown the Maxwell® 16 automated system to be an effective DNA extraction method for situations of sampling from an environment that is rich in target DNA (Foley et al., 2011; Davis et al., 2012). In the case of pathological studies where the majority of DNA present is non-target (e.g. host) DNA, manual extraction methods were found to be more successful (Durnez et al., 2009; Affolabi et al., 2012). Perhaps, as appears to be the case with our samples, successful automated protocols are targeting shorter DNA segments.

We identified significantly different detection sensitivities between primer sets for the automated extraction process. Both primer sets targeted the same gene region of trypanosome DNA, with each extraction replicate of a given trypanosome concentration containing a similar numbers of target gene regions. A possible cause for the varying sensitivity of the different primer sets could be the difference in length of the target region of DNA. The target region for the genus-specific primer was 1.5 times the length of the species primer set. Longer amplicons are useful for target specificity – in this case, the 18S gene is common to a variety of taxa (Meyer et al., 2010) and a longer amplicon is useful to differentiate between them. However, as DNA within a sample deteriorates, long DNA fragments become less common and therefore less likely to amplify (McCarty and Atlas, 1993; Wiegand and Kleiber, 2001).

The addition of "host" blood to the replicated samples is important to the process, both for realism and for the creation of a physical DNA pellet required during the manual DNA extraction technique used here. If we used only trypanosomes in media, there would not be sufficient DNA in the solution to create this pellet and the target DNA would have been lost during the DNA extraction protocol. It also adds a more practical aspect to the experiment, as trypanosome detection is usually required from either whole blood or tissue samples, which contain host DNA in excess.

Several other studies have compared DNA yield from automated and manual extraction methods. Although less time consuming, automated methods do not tend to outperform manual extraction methods (Durnez et al., 2009; Lindner et al., 2011; Affolabi et al., 2012). However, Maxwell® appears to be one the better low throughput robotic systems (Davis et al., 2012), especially when also considering material costs (see Table 1).

The varying DNA yield of the two DNA extraction techniques used here is possibly attributable to key differences in the purification process. Generally, manual extraction processes involves chemical and physical removal of cellular proteins and salts, leaving behind purified DNA, whereas automated systems use physical mean, such as paramagnetic particles to attract DNA and move it from well to well during the extraction process (Krnajski et al., 2007). We suspect that this difference may mean the automated system is either less efficient at picking up long, intact chains of DNA, or can become flooded by "host" DNA and therefore, is less likely to pick up relatively low concentrations of target parasitic DNA. Another possible cause could be the presence of some inhibitors from the automated DNA extraction kit that is less favourable for long chain replication.

The final aspect of interpreting detection sensitivity of biological samples is placing some context on whether our detection limits are relevant in a virulence setting. There are limited data, especially in a wildlife context, of what levels of parasitaemia correlate with the occurrence of overt symptoms and virulence of parasitic infections. Wild and domestic animals experimentally infected with *Trypanosoma congolense* were observed to have parasite loads of up to 10,000 (oryx and waterbuck) and 1,000,000 (eland and cattle) parasites per mL, along with symptoms of anaemia (ILRAD, 1983). In human African trypanosomaisis (caused by *T. b. gambiense*), parasitaemia can occur between 100 and 10,000 parasites/mL of blood, with the former concentration being below the detection limits of most molecular techniques (Chappuis et al., 2005). Parasitaemia in the realm of thousands of parasites per mL is likely to be the case for wildlife trypanosomaisis as well; the host animal (*B. penicillata*) that the parasites were collected from was apparently healthy with a blood parasite concentration of up to 2000 trypanosomes per mL

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(unpublished data), and lived for a further two years after blood sampling. From this information it seems that our best method (manual extraction followed by genus-specific PCR), which is capable of detecting 663 p/mL with 95% confidence, is a reasonable sensitivity for this biological context.

This study highlights the importance of testing laboratory techniques with multiple replicates of known samples to assess detection limits of pathological samples. Furthermore, each step of the laboratory process should be optimised for maximised sensitivity and reduction of type II errors.

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