

Parasitology

# Comparison of short-term and long-term protocols for stabilization and preservation of RNA and DNA of *Leishmania*, *Trypanosoma*, and *Plasmodium*

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## Abstract

Molecular tools continue to be important in the prevention and control of parasitic diseases. However, using these techniques directly in the field remains a major challenge. Therefore, the preservation of clinical samples collected from endemic field areas for later analysis remains an important preanalytical process. This study aimed at identifying a suitable protocol for stabilization and preservation of RNA and DNA in bioclinical specimens for *Trypanosoma*, *Leishmania*, and *Plasmodium* research. Both spiked and unspiked blood samples were preserved in 7 protocols (different media; storage temperatures). Samples were evaluated for possible degradation of DNA and RNA along the storage duration up to the 10th week. Nucleic acid targets were assessed as follows: (i) *Trypanosoma* and *Plasmodium* RNA analysis was done using real-time nucleic acid sequence-based amplification (RT-NASBA) for 18S rRNA and for stage-specific Pfs25 mRNA, respectively; (ii) *Trypanosoma* DNA assessment analysis was conducted by using a conventional PCR for 18S rDNA; (iii) *Leishmania* RNA analysis was performed with a quantitative NASBA for 18S rRNA and *Leishmania* DNA assessment with an RT-PCR for 18S rDNA. Findings suggested that a newly developed L3<sup>TM</sup> buffer proved to be reliable and suitable for both short- and long-term preservation of parasite nucleic acid material. This buffer is envisaged to be suitable for utilization in field situations where resources are limited.

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## 1. Introduction

Until the beginning of the 1990s, the diagnosis of parasitic diseases like human African trypanosomiasis (HAT), visceral leishmaniasis (VL), and malaria relied on classic microbiological methods (Giemsa-stained smears). Since then, molecular biology has increasingly become relevant to the diagnosis and control of infectious diseases. Various molecular tools have been developed to replace

microscopy, with polymerase chain reaction (PCR; targeting the amplification of DNA) evolving as one of the most specific and sensitive methods for diagnosis of infectious diseases like HAT, VL, and malaria (Anderson et al., 2004; Bromidge et al., 1993; Graig et al., 2003). Different PCR-based assays like restriction fragment length polymorphism, random amplification of polymorphic DNA, reverse transcriptase (RT), amplified fragment length polymorphism, and real-time PCR are tools that have been used in the identification of *Leishmania* and *Trypanosoma brucei* species (Compton, 1991). Other molecular tools targeting RNA include nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification (Notomi et al., 2000). The growing number of molecular tools requires biological material with good long-term

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preservation of nucleic acids (Wiedon et al., 2002). The preparation of high-quality DNA and RNA from various fresh and frozen clinical specimens (e.g., whole blood and cell pellets) is the most important step in downstream molecular applications. It ensures good quality and sufficient quantity of RNA and DNA necessary for molecular assays.

Of the nucleic acids, DNA is relatively stable while RNA is fairly labile and will degrade easily in routine conditions. Therefore, the molecular analysis of RNA in particular is severely hampered as a result of loss from abundant and stable tissue RNases and the high temperatures commonly used in specimen processing (Finkelstein et al., 1999). To circumvent this problem, the RNA in pathological specimens is preserved through removal or inactivation of any RNases present in a biological specimen by addition of commercial products or chemicals (Fregeau et al., 2001). In addition to that, many different protocols for sample storage and subsequent extraction of both RNA and DNA from blood are currently in use. The available storage protocols are based on different storage media from EDTA salt, filter paper to lysis buffers, all of which are maintained at different temperatures ranging from 37 °C to –70 °C. These protocols also vary in their storage capabilities from a few hours, days to months (Avila et al., 1991).

The preservation of parasite DNA has previously been accomplished. For example, the *Trypanosoma cruzi* DNA has been preserved by collecting blood in guanidine/EDTA solution and stored at 37 °C for up to 1 month (Tsui et al., 2002). RNA can also remain stable in EDTA blood stored at 4 °C for up to 24 h. DNA can be stored for long term in high salts (>1 mol) like in the presence of high EDTA (>10 mmol/L) of pH 8.5 (Chen et al., 1990). Other storage methods that involve freezing to preserve DNA and RNA in blood are extensively in use and include plasma storage at –70°C to preserve RNA (Kephart and Shenoi, 1998; Kline et al., 2002; Wang et al., 1992). Blood samples have also been stored on filter paper. The commercial FTA classic card (filter paper) technology is ideal for long-term preservation of blood at room temperature (Chaorattanakawee et al., 2003; Snowden et al., 2002). The filter paper methods of storage in general provide a convenient, simple, and economical way of preservation and distribution of different types of nucleus acid samples for epidemiologic investigations. Samples from patients with *Plasmodium falciparum* for PCR have been stored for long durations of more than 4 years on filter paper (Lahiri et al., 1992).

Other combined protocols have successfully been used, including the process of mixing the guanidine isothiocyanate buffer (L6 lysis buffer) with blood and storing in liquid nitrogen for RNA preservation (Ambion, 2006).

The present study was aimed at identifying and developing a standard protocol that can be used for stabilization and conservation of both RNA and DNA (of *Leishmania*, *Trypanosoma*, and *Plasmodium* species) from bioclinical samples. We focused on the use of a recently

developed storage buffer, the so-called L3™ buffer (soon available under the brand name Angero NA™ from Mallinckrodt Baker, Phillipsburg, NJ. A manuscript detailing the development of this buffer is in preparation by Beld et al.). Furthermore, we sought to identify a protocol that is easily compatible with a simple extraction method. This research is necessitated by the inevitable time delay between blood (sample) draw and processing that can greatly influence the outcome of a result. It is well documented that for each class of microorganisms, the sample preparation and nucleic acid extraction method could greatly influence the reliability of the test results (Behzadbahani et al., 1997; Grob et al., 1992). This therefore necessitated the need to compare selected methods for their efficacy and applicability in the preservation of nucleic acid materials from the 3 parasites species for use in endemic countries.

## 2. Materials and methods

The study focused on assessing storage and preservation of RNA because it is easily degradable compared to DNA. We therefore examined 18S ribosomal RNA, which is relatively better protected against degradation due to ribosome protection and mRNA (from *P. falciparum*) which degrades comparatively faster. We selectively carried out preservation, extraction, and detection of nucleic acid as per availability of resources.

### 2.1. Parasite strains

*Leishmania donovani* strain MHOM/SD/68/IS, *Trypanosoma brucei gambiense* isolate, and *P. falciparum* strain NF54 gametocytes obtained from fresh cultures were used to spike the samples.

### 2.2. Preparation of seeded and unseeded blood samples

From the stock parasite concentrations of 10<sup>6</sup> parasites per milliliter, final parasite concentrations for *Leishmania* and *Trypanosoma* were made to 10<sup>3</sup> parasites per milliliter of blood, while for *P. falciparum*, parasite concentration was made to 10<sup>5</sup> parasites per milliliter of blood. Twenty-five milliliters of EDTA blood obtained from a healthy donor was used as a negative control (unspiked).

### 2.3. Control samples

From the stock parasite concentrations, a single batch of serial dilutions in blood was made to obtain a control series of 10<sup>6</sup>, 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup> (for *P. falciparum*) and 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup>, 10<sup>1</sup> (*Trypanosoma* and *Leishmania*) and a negative sample for all the 3 parasite species. These samples were stored at –70 °C and eventually a set removed at each extraction time point and used for making the standard curve for quantification in the real-time assays (see also Table 1).

Table 1

A summary of the 7 storage protocols that were evaluated in the current study

	Protocol	Blood volume	Storage medium and volumes	Target for preservation	Storage temperature
1	Reference	50 $\mu$ L	L <sub>6</sub> buffer: 950 $\mu$ L	DNA and RNA	–70 °C
2	Silica	200 $\mu$ L	L <sub>6</sub> buffer + silica: 1.2 mL + 40 $\mu$ L silica	DNA and RNA	–20 °C
3	L3 <sup>TM</sup> buffer	200 $\mu$ L	L <sub>3</sub> buffer: 200 $\mu$ L	DNA and RNA	26 °C
4	L3 <sup>TM</sup> buffer	200 $\mu$ L	L <sub>3</sub> buffer: 200 $\mu$ L	DNA and RNA	–20 °C
5	L3 <sup>TM</sup> buffer	200 $\mu$ L	L <sub>3</sub> buffer: 200 $\mu$ L	DNA and RNA	4 °C
6	F. Paper	50 $\mu$ L	Filter paper: 50 $\mu$ L spot	DNA and RNA	26 °C
7	QIAmp	200 $\mu$ L	As-1 buffer: 200 $\mu$ L	DNA only	26 °C

#### 2.4. Storage protocols

Each storage protocol (Table 1) comprised 2 sets of samples: a spiked sample (with  $10^3$  parasites per milliliter of *Trypanosoma*,  $10^3$  parasites per milliliter *Leishmania*, and  $10^5$  parasites per milliliter of *P. falciparum* gametocytes all mixed in the same blood sample) and non spiked sample. Earlier experiments had shown that there was no cross-reaction between the 3 parasite species when spiked in single sample and subjected to the molecular tests used here (van der Meide et al., 2008).

Short description of storage protocols (see also Table 1) are as follows:

1. Reference protocol: this protocol is derived from the original Boom extraction protocol (Boom et al., 1990); here, we refer to it as the “reference protocol” because it has proven over time to be very reliable and acts as the gold standard in our laboratory: 50  $\mu$ L blood was mixed with 950  $\mu$ L L<sub>6</sub> buffer [5 mol/L guanidine isothiocyanate, 0.1 mol/L Tris (pH 6.4), 20 mmol/L EDTA, 1.2% (wt./vol.) Triton X100] and stored at –70 °C.
2. Silica protocol: this protocol has proved to be a reliable “in-field” method with a disadvantage of requiring a centrifuge and –20 °C facilities on site but can be adapted to most field setups: 200  $\mu$ L blood was mixed with 1.2 mL L<sub>6</sub> buffer. Then, 40  $\mu$ L silica (obtained from Sigma, St. Louis, MO) was added followed by 5 min of mixing. The tube was centrifuged for 1 min at 12 000 rpm and the supernatant was discarded. The sediment containing the silica-bound DNA/RNA was stored at –20 °C.
3. L3<sup>TM</sup> buffer protocol: the L3<sup>TM</sup> buffer (soon available under the brand name Angero NA<sup>TM</sup> from Mallinckrodt Baker USA) is an invention by the University of Amsterdam and had shown promising preliminary results; we therefore chose to subject it to 3 storage temperatures: 200  $\mu$ L blood was mixed with 200  $\mu$ L L3<sup>TM</sup> buffer and stored, respectively, at –20 °C, 4 °C, and 26 °C.
4. QIAmp protocol: a commonly used commercial method but for DNA preservation and extraction only: 200  $\mu$ L blood was mixed with As 1 buffer (Qiagen, Hombrechtikon, Switzerland) and stored at 26 °C.

5. Filter paper protocol: an approximately 50  $\mu$ L of blood per drop was spotted 3 times on filter paper, allowed to dry, and stored at 26 °C.

All protocols, except protocol 3, were tested at one temperature, which is in principle the optimal operational temperature of the protocol. Protocol 3 was tested at 3 different temperatures because this was the experimental protocol. Each sample in the 7 protocols was made in triplicates (for both spiked and unspiked samples). The extraction and detection of DNA and RNA from the triplicate spiked and unspiked samples in the 7 protocols was done on day 1, day 3, week 1, week 2, week 4, week 6, and week 10 of storage.

#### 2.5. Extraction of nucleic acids (DNA and RNA)

All storage protocols with the exception of the QIAmp (AS-1 buffer) protocol from Qiagen were compatible with the Boom extraction method (Boom et al., 1990). The filter paper required an elution step done by punching out 3 disks (6 mm each) from the dried blood spots, placing them into an Eppendorf tube, and adding 900  $\mu$ L of L<sub>6</sub> buffer. The tube was placed onto a rotation mixer for 10 min, after which the filter paper disks were discarded. The L<sub>6</sub> buffer mixture was then subjected to the Boom method by the addition of silica and further processed through the following steps of the Boom method of extraction described below.

In the Boom method of extraction, the RNA/DNA in the blood samples was first isolated by lysis in L<sub>6</sub> buffer. The isolated RNA/DNA was then bound to silica (filter paper protocol proceeded from this step) and washed twice with L<sub>2</sub> wash buffer (5 mol/L guanidine thiocyanate, 50 mmol/L Tris, pH 6.4), then washed twice with 70% ethanol and once with acetone. The silica was dried at 56 °C for 10 min and RNA was eluted in RNase-free water for 10 min at 56 °C. The purified RNA/DNA was stored at –70 °C until analysis.

The QIAmp mini kit was used for samples stored in AS-1 buffer (Qiagen protocol). Proteinase K solution was added to samples initially stored in AS-1 buffer to lyse the cells. This was followed by addition of AS-2 buffer that acts as a stabilizing buffer. The mixture was then washed once in 70% absolute ethanol, followed by second wash in AW-1 buffer and finally a third wash with AW-2 buffer. AE buffer was then used for elution of DNA material at room temperature.

for 5 min. (QIAGEN, 2003). The purified DNA was then stored at  $-70^{\circ}\text{C}$  until analysis.

### 2.6. Assay conditions for the molecular tests used in parasite target detection

Established molecular DNA/RNA detection assays were used in the present study. The *Trypanosoma* and *Plasmodium* RNA analyses were done using the real-time NASBA for 18S rRNA and for Pf 25S mRNA, respectively (Mugasa et al., 2008; Schneider et al., 2004; Schoone et al., 2000). *Trypanosoma* DNA analysis was performed using the conventional PCR for 18S rDNA, respectively (Salotra et al., 2001, Schneider et al., 2004; Schoone et al., 2000). The *Leishmania* RNA analysis was done using the qualitative (QT)-NASBA for 18S rRNA (van der Meide et al., 2005) and the *Leishmania* DNA analysis was done using the real-time PCR for 18S rDNA (van der Meide et al., 2008).

### 2.7. Data analysis

The quantitative results were obtained in triplicate for each time point and the outliers in each triplicate identified using the Dixon Q test (Dixon, 1950). Repeated-measures analysis of variance was done using the SPSS program with a 95% confidence interval with a significance level of  $P \leq 0.05$ .

## 3. Results

### 3.1. *Leishmania* RNA quantities

There was no significant difference in the mean RNA levels along the storage duration for the reference protocol ( $P = 0.221$ ), L3<sup>TM</sup> buffer at  $4^{\circ}\text{C}$  ( $P = 0.127$ ), L3<sup>TM</sup> buffer at  $-20^{\circ}\text{C}$  ( $P = 0.070$ ), and silica protocol ( $P = 0.072$ ). The mean RNA levels in the L3<sup>TM</sup> buffer at  $26^{\circ}\text{C}$  remained stable up to the second week of storage; the levels started dropping toward the fourth week where there were low levels

of RNA detectable and by the sixth week RNA was undetectable in the samples. For the filter paper protocol, the mean RNA levels remained stable up to the sixth week of storage and by the 10th week of storage the RNA was undetectable. The decrease in RNA levels along the storage duration was significant in the L3<sup>TM</sup> buffer at  $26^{\circ}\text{C}$  ( $P = 0.008$ ) and filter paper ( $P = 0.002$ ) protocols (see Fig. 1).

### 3.2. *Trypanosoma* RNA quantities

There was no significant difference in the mean RNA levels along the storage duration for the reference protocol ( $P = 0.328$ ), L3<sup>TM</sup> buffer at  $4^{\circ}\text{C}$  ( $P = 0.381$ ), L3<sup>TM</sup> buffer at  $-20^{\circ}\text{C}$  ( $P = 0.247$ ), and silica protocol ( $P = 0.077$ ). The mean RNA levels in the L3<sup>TM</sup> buffer at  $26^{\circ}\text{C}$  remained stable up to the second week of storage, there was a decrease in levels showed by the fourth week, and by the sixth week RNA was undetectable in the samples. The mean RNA levels remained stable in the filter paper protocol up to the sixth week of storage and by the 10th week of storage the RNA was undetectable. These decreases in RNA levels along the storage duration were significant in the L3<sup>TM</sup> buffer at  $26^{\circ}\text{C}$  ( $P = 0.020$ ) and filter paper ( $P = 0.002$ ) protocols (see Fig. 2).

### 3.3. *P. falciparum* RNA quantities

There was no significant difference in the mean RNA levels along the storage duration for the reference protocol ( $P = 0.129$ ), L3<sup>TM</sup> buffer at  $4^{\circ}\text{C}$  ( $P = 0.137$ ), L3<sup>TM</sup> buffer at  $-20^{\circ}\text{C}$  ( $P = 0.351$ ), and silica protocol ( $P = 0.199$ ). The mean RNA levels in the L3<sup>TM</sup> buffer at  $26^{\circ}\text{C}$  remained stable up to the second week of storage; by the fourth week of storage RNA levels were undetectable in the samples. For the filter paper protocol, the mean RNA levels remained stable up to the sixth week of storage and by the 10th week of storage RNA was undetectable. These decreases in RNA levels along the storage duration were

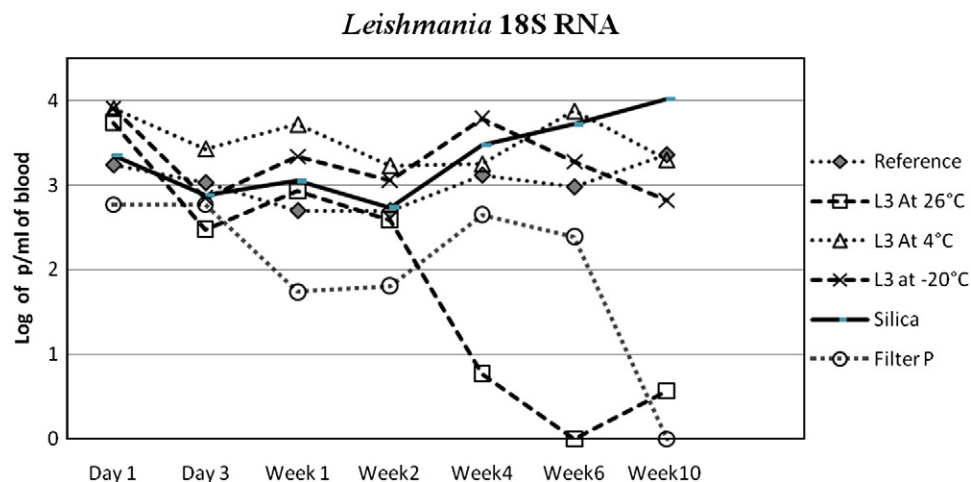


Fig. 1. Detection of *Leishmania* 18S rRNA (mean log p/mL) using the QT-NASBA assay on spiked blood samples after different storage durations.



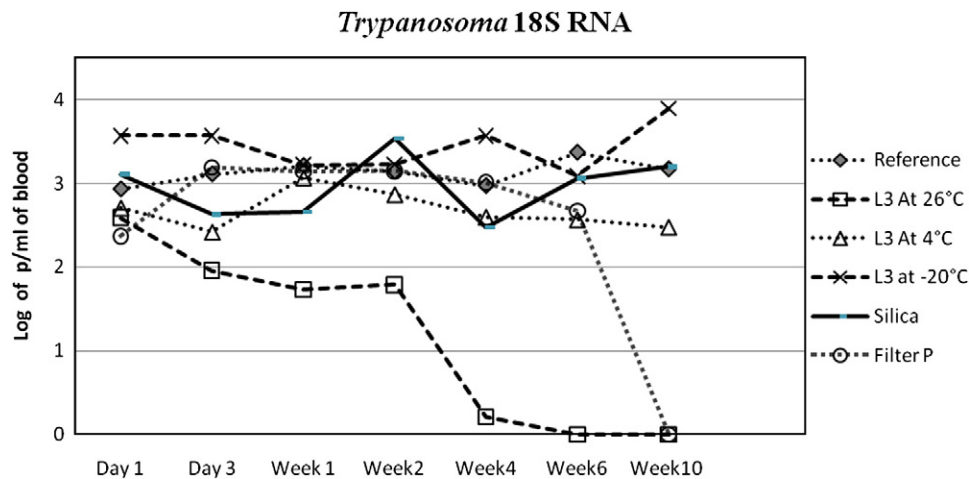


Fig. 2. Detection of *Trypanosoma* 18S rRNA (mean log p/mL) using the real-time NASBA assay on spiked blood samples after different storage durations.

significant in the L3<sup>TM</sup> buffer at 26 °C ( $P = 0.002$ ) and filter paper ( $P = 0.011$ ) protocols (see Fig. 3).

### 3.4. *Leishmania* DNA quantities

There was no significant difference in the mean DNA levels along the storage duration for all the protocols: reference protocol ( $P = 0.249$ ), L3<sup>TM</sup> buffer at 4 °C ( $P = 0.053$ ), L3<sup>TM</sup> buffer at -20 °C ( $P = 0.596$ ), silica protocol ( $P = 0.056$ ), L3<sup>TM</sup> buffer at 26 °C ( $P = 0.222$ ), filter paper ( $P = 0.874$ ), and the QIAmp method ( $P = 516$ ). However, DNA levels remained constantly low in the filter paper protocol from day 1 of storage and remained constant at these low levels along the whole storage period (see Fig. 4).

### 3.5. *Trypanosoma* DNA signals

The DNA gel electrophoresis results showed that all the storage protocols were positive for DNA signal on all days of storage apart from the filter paper protocol in which the

DNA was present only up to the sixth week of storage and negative on the 10th week.

## 4. Discussion

Ensuring optimized field collection, preservation, storage, and purification of specimens will greatly improve the results from molecular tests. Therefore, additional information toward addressing this need will be handy for research and diagnosis of parasitic diseases in general. Findings from this study are expected to contribute toward such efforts.

Our findings reveal that DNA from both *Leishmania* and *Trypanosoma* parasites was well preserved by all the protocols under study up to the end of the storage duration with the exception of preservation on the filter paper. From the filter paper, very low amounts of DNA extracts were obtained for *Leishmania*. This could be attributed to a possible challenge in extraction and recovery of nucleic acid

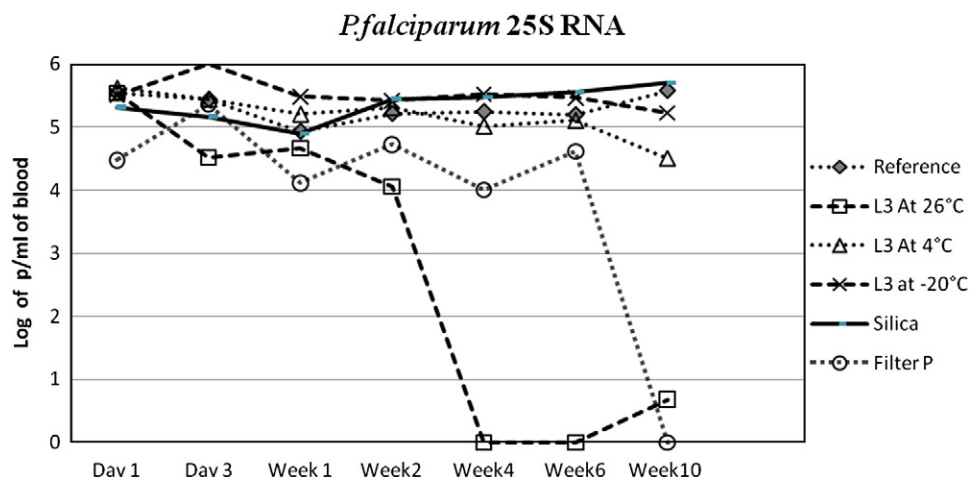


Fig. 3. Detection of *P. falciparum* 25 mRNA (mean log p/mL) using the RT-PCR assay on spiked blood samples after different storage durations.

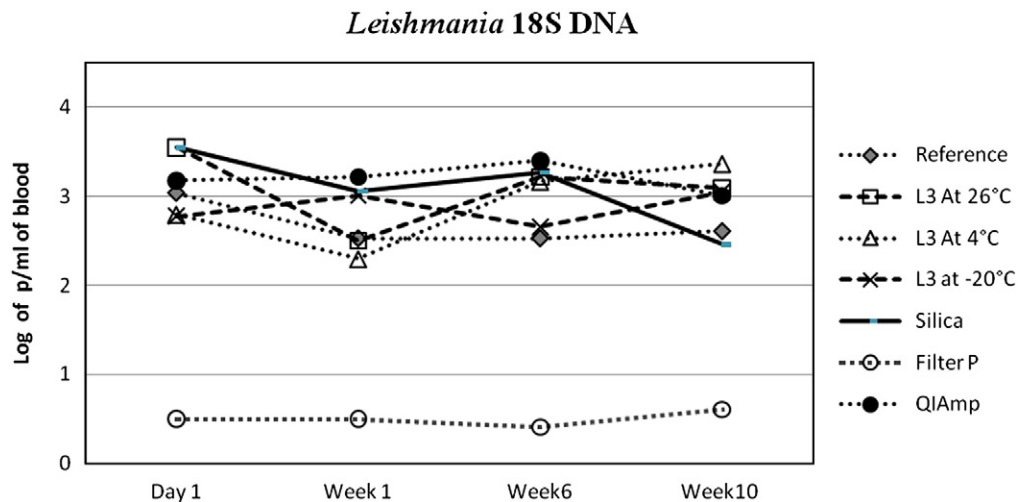


Fig. 4. Detection of *Leishmania* 18S rDNA (mean log p/mL) using the real-time PCR assay on spiked blood samples after different storage durations.

materials from the filter paper leading to loss of the already lower parasite copies. Preservation on filter paper may require higher parasite loads subsequently analyzed in highly sensitive assays. DNA preserved in the AS-1 buffer (QIAmp protocol) was equally robust as shown by preservation in the L3™ buffer protocol at temperatures of 4 °C and –20 °C, respectively. DNA stored in AS-1 buffer at ambient temperature in the dark is known to remain stable for up to 3 months (Deborggraeve et al., 2006). Apart from the filter paper protocol, all the DNA storage protocols in the current study were comparable to one of the most widely used commercially available method of preservation (QIAGEN, 2003).

The preservation of RNA has always been a major challenge to scientists, therefore making it a less explored target as compared to DNA. In the current study, RNA preservation from *Leishmania*, *Trypanosoma*, and *Plasmodium* was well achieved in all the protocols apart from the filter paper and the L3™ buffer at ambient temperature. The

silica protocol with storage at –20 °C has proved to be a valuable method because of its capabilities to preserve blood for a long time before actual extraction.

Storage and recovery of nucleic acid from samples stored on filter paper may have been less efficient than other methods undertaken in the current study. For *Leishmania* and especially *Trypanosoma*, the diagnosis can be missed when samples have been preserved on filter paper followed by analysis in molecular assays with poor sensitivity (Table 2). The drop in *Plasmodium* RNA in the filter paper at week 10 could either be due to the fragility of mRNA or to poor recover of nucleic acid material from the filter paper. We could not obtain information on any studies conducted on preservation of *Plasmodium* mRNA for comparison. In general, the filter paper method might be good in preservation of nucleic acid (both DNA and RNA) from high copy number targets (like *Plasmodium* due to higher parasitemia) and not as good enough for low copy targets (like *Trypanosoma* and *Leishmania* with lower parasitemia).

Table 2

Primers used in the molecular assays

*Trypanosoma* real-time NASBA for 18S rRNA

Forward primer: Tryps Nas F2 generic: 5'GATGCAAGGTCGCATATGAGGATTCTTGCTTTTCG CG 3'

Reverse primer: Tryps Nas Rev T7: 5'AATTCTAATACGACTCACTATAGGGCTCGGACTCTTGTTCTC 3'

*Trypanosoma* PCR for 18S rDNA

Forward primer TrypF1 : 5'GGATTCCTTGCTTTTCGC 3'

Reverse primer Tryp 6 rev: 5'CTCGGACTCTTGTTCTCA C 3'

*Leishmania* NASBA for 18S rRNA

Forward primer Leish gen forw.: 5'GATGCAAGGTCGCATATGAGCCAAAGTGTGGAGATCGAAG 3'

Reverse primer Leish Rev. T7: 5'AATTCTAATACGACTCACTATAGGGAGAAGGGCCGGTAA AGGCCG AAT AG 3'

*Leishmania* PCR for 18S rDNA

Forward primer Leish18Snas 6 for: 5' GGATTCCTTTGTAATTGC 3'

Reverse primer leish18S 6 rev.: 5' GATCAAGTAGGTTTCGAC 3'

*Plasmodium* Real time NASBA for Pf s25 mRNA

Reverse primer ECL NASBA: 5'-AATTCTAATACGACTCACTATAGGGAGAAGGCATTTACCGTTACCACAAGTTA-3'

Forward primer ECL NASBA: 5'-GACTGTAAATAAACCATGTGGAGA-3'

Pfs25 molecular beacon: 5'-Texas\_Red-CGATCG-CCCGTTTCATACGCTTGTAACGATCG-DABSYL-3'

This method is easy to accomplish, affordable, and can be conveniently applied in any setup for sample collection. It offers a convenient source for storing specimens as there is no need for pretreatment and no need to maintain a cold chain during shipment of samples.

The L3<sup>TM</sup> buffer recently developed by the University of Amsterdam had shown promising preliminary results in preservation of naked DNA and RNA elsewhere (Beld et al, manuscript in preparation). Of the L3 buffer protocols, storage at 4 °C and –20 °C were efficient in both DNA and RNA stabilization, while the storage protocol in this buffer at ambient temperature was found to be less efficient. This observation was confirmed by Beld and Minnaar (manuscript in preparation). The reasons for the difficulty in RNA preservation at ambient temperatures can be attributed to the combined actions of the abundant and stable tissue RNases and in some cases the prevailing temperature conditions when handling specimens (Finkelstein et al., 1999). The findings of the current study support the documented fact that RNases remain highly active at ambient temperatures when compared to storage at temperatures about and below 4 °C.

Storage at lower temperatures is however cumbersome for field establishment because freezing facilities are minimal and constant power supply is not guaranteed. However, in a field situation where resources are minimal, attaining a 4 °C temperature can be achieved by the use of cool boxes with constant change of ice packs. In many established laboratories, ice packs have been proven to maintain this temperature for up to 30 h.

The current study suggests that the L3<sup>TM</sup> buffer is a good option for transport, or for short-term or long-term preservation of parasite RNA and DNA. Furthermore, this storage method can provide the benefit of flexibility in storage temperatures for preservation of parasite nucleic acids in human blood samples. The practical application of the L3<sup>TM</sup> buffer is recently confirmed in 3 studies in which clinical specimens for leishmaniasis (Sudan; Kenya) and trypanosomiasis (Uganda; DR Congo) were successfully collected using this buffer under harsh field conditions (Basiye et al., 2010; Matovu et al., 2010; Saad et al., 2010).

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