DEVELOPMENT OF STANDARD PROTOCOLS FOR SAMPLING, PREPARATION AND STORAGE OF BIO-CLINICAL MATERIALS FOR *TRYPANOSOMA, LEISHMANIA* AND *PLASMODIUM* RESEARCH.

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DECLARATION

I Frank Lusasi Basiye, duly declare that this thesis is my original work and has not been presented for a degree in any other university or any other award.

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DEDICATION

I dedicate this work to my loving wife, Rachel Bonyo for her moral support at all times, her endurance and patience in my long stay away during the course of my project, to my late mum Mrs. E. M. Basiye who would have been very proud of this achievement.

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AGPC	Acid-Guanidine-Phenol-Chloroform
dNTP	Deoxyribonucleotide Triphosphate
dUTP	Deoxyuridine Triphosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethelynediaminetetraacetic Acid
ECL	Electrochemiluminescent
FTA	Flinders Technology Associates
GE	Guanidium-EDTA
GuSCN	Guanidine thiocyanate
HAT	Human African Trypanosomiasis
HCL	Hydrochloric Acid
KOH	Potassium Hydroxide
Log p/ml	Log of parasites per milliliter
$MgCL_2$	Magnesium Chloride
NA	Nucleic Acid
NASBA	Nucleic Acid Sequence Based Amplification
NaOH	Sodium hydroxide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription PCR

RT-PCR	Also means - Real time PCR
SiO_2	Silica (Silicon Dioxide)
SOP	Standard Operating Procedures
TE	Tris-EDTA
TDR	Tropical Diseases Research
Tris-buffer	Trisamine (Hydroxymethylaminoethane) buffer.
VL	Visceral Leishmaniasis
WHO	World Health Organization
w/v	Weight of solute per volume of solvent

ABSTRACT

According to WHO, Human African Trypanosomiasis (HAT) or sleeping sickness, Human Visceral Leishmaniasis (VL) and, Malaria all caused by protozoan parasites, are severely neglected diseases. The prevention and control of these diseases remain a major challenge to scientists. Molecular techniques continue to be exploited as a major tool in the prevention and control of parasitic diseases. However, the feasibility and dynamics of using these techniques directly in the field remain a major challenge. This is mainly due to the technicalities involved in molecular methods and the logistical constraints that prevail in the field situations. Therefore, the preservation of samples drawn from the endemic areas (field) to be sent for analysis several hours, days or weeks later to established laboratory setups remains an important pre-analytical task. The aim of this study was to develop a standard protocol for sample preparation, extraction and storage of bio-clinical materials for *Leishmania*, *Trypanosoma* and *Plasmodium* research. Seven different protocols were evaluated. Each protocol had two sets of blood samples; one set spiked with parasites and another set of plain blood samples (non-spiked). These samples were preserved in different media (either buffer or filter paper) and stored under different temperatures. DNA and RNA extraction was carried out at seven time points (days 0 to week 10) and analysis done by the use of quantitative and qualitative PCR and NASBA assays. The results obtained at each time point for the seven protocols were compared along the storage duration. A newly developed buffer, the L3 buffer proved to be very reliable in short term and long term preservation of parasite RNA and DNA in the spiked samples. It is envisaged to be ideal for utilization in field situations where bio-clinical samples for molecular work require preservation.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Human African Trypanosomiasis (HAT) or sleeping sickness, Human Visceral Leishmaniasis (VL) and malaria are caused by protozoan parasites. HAT and VL causative parasites belong to the phylum; Mastigophora, order; Kinetoplastida. While malaria is caused by parasites from the *Plasmodium species.* These three diseases are categorized as severely neglected diseases according to WHO criteria (WHO, 2001).

HAT or sleeping sickness is caused by *Trypanosoma brucei (T. b) gambiense* in West and Central Africa or *Trypanosoma brucei (T. b) rhodesiense* in East and Southern Africa. Over 60 million people living in 36 sub-Saharan countries are threatened with the disease, while between 300, 000 and 500,000 are actually infected (WHO, 2001).

VL is caused by parasites from *Leishmania donovani* complex; In the Old World (Africa, Asia, and Europe) VL is caused by the subspecies *L. d. donovani, L .d. infantum*, East African *L. donovani*, and Sudanese *L. donovani.* In the New World (South and Central America) VL is caused by *Leishmania donovani chagasi* (Hyams *et al.*, 1995). According to WHO 350 million people are at risk with 12 million infected persons. An estimated 500,000 new cases of VL occur annually with 90% of these numbers existing in five countries: Bangladesh, Brazil, India, Nepal and Sudan (WHO, 2000).

Malaria one of the leading parasitic diseases in the word, can be caused by four species in humans; *Plasmodium falciparum, Plasmodium ovale, Plasmodium vivax*

and *Plasmodium malaria. Plasmodium falciparum* is the most pathogenic species and is highly prevalent in the African continent. Malaria causes 200-300 million clinical cases and over 1 million deaths each year (WHO, 2005; Guerra *et al.*, 2006).

HAT also known as sleeping sickness entered what is now Kenya from Uganda in the form of Gambian sleeping sickness in about 1901 and quickly spread along the Kenyan shores and islands of Lake Victoria and by 1910 the disease had spread 25 miles inland along the Kuja and Migori rivers and their tributaries. It was not until 1950, when the use of insecticides (DDT) proved successful subsequently the Kuja-Migori endemic area was cleared of flies and disease, as well as the South and Central Nyanza lake shores and islands. By 1965 Gambian sleeping sickness had virtually disappeared from Kenya. A more virulent form of the disease, Rhodesian sleeping sickness, may have also spread to Kenya from Uganda, it appeared in the Lambwe Valley, South Nyanza, in about 1959, other outbreaks were eventually reported to occur in Alego, in Central Nyanza, in 1964, in Samia in 1976 and also along the lakeshore in South Nyanza in 1981. Sleeping sickness has been restricted primarily to the Western and Nyanza Provinces of Kenya with Lambwe Valley being the only remaining endemic area in Kenya (Wellde *et al.*, 1989).

In Kenya VL is endemic in Machakos, Kitui, West Pokot, Meru, Baringo and Turkana districts. In 2000, VL outbreaks were reported in three districts of Kenya's North Eastern Province; Mandera, Wajir and Garissa. In Garissa district the cases seemed to be concentrated within the Dadaab refugee camp (WHO, 2000). In Baringo district where the highest number of cases exists, the disease has a focal distribution in the dry, hot areas below 1500 meters above sea level (Schaefer *et al.*, 1994).

Until the beginning of the 1990s, the biological diagnosis of these parasitic diseases relied on classical microbiological methods (Giemsa stained smears). Since then, however molecular biology has increasingly become relevant to the diagnosis and control of infectious diseases. Various molecular tools have been developed to replace microscopy, with PCR evolving as one of the most specific and sensitive method for the diagnosis of infectious diseases (Bromidge *et al.*, 1993; Graig *et al.*, 2003). Information on *Trypanosoma*, *Leishmania* and *Plasmodium* DNA sequences has been exploited for the development of PCR diagnostic assays for diagnosis (Schallig and Oskam, 2002). Different PCR based assays like PCR-RFLP, RAPD, RT-PCR, Real Time -PCR and AFLP are tools used in the identification of *Leishmania* and *T. brucei* species (Compton, 1991). Other molecular tools include NASBA and Northern Blotting techniques. These growing numbers of molecular diagnostic tools that are currently available require material with good long-term preservation of morphology of nucleic acid and antigenic structures (Wiedon *et al.*, 2002). The preparation of high quality DNA and RNA from various samples sources for example, whole blood and cell pellets, both fresh and frozen, is the most important first step in molecular work. It ensures good quantity and quality of RNA and DNA necessary for molecular assays. The advantages of using the molecular techniques mentioned above over the traditional methods (serology, direct microscopic examination, hemoculture and xenodiagnosis) is that most of them like PCR can distinguish between infection with any of the hemoflagellates, do not depend on the immunocompetence of the patient, they are sensitive enough for use with chronic patients, and can distinguish a current infection from a previous one or a newborn infection from a maternal one (Avila *et al.*, 1993).

Many different protocols for sample storage and eventual extraction of 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 at different temperatures ranging from 37 ° C to -70 ° C. These protocols are also based on varied storage durations from a few hours, days to months of storage after sample collection (Halfon *et al.*, 1996). Most DNA extraction protocols are based on phenol – chloroform extraction, salting out, digestion-free extraction, boiling and microwaving. While most RNA extraction protocols are based on protein K extraction, Silica, TRIzol, and guanidine isothiocyanate. For both DNA and RNA extraction, different commercial kits are also available and laboratories have developed in-house protocols based on the above methods to suit the nature of work they conduct (Armaleo and Cleric, 1995; Crespo *et al.*, 1997; Lee *et al.,* 1998). There has therefore arisen a need to standardize the storage and processing protocols in order to obtain uniform or at least comparable results.

1.2 Literature Review

1.2.1 Global distribution of African Trypanosomiasis, Leishmaniasis and Malaria

As illustrated in Fig 1, African Trypanosomiasis occurs in the tsetse fly areas of sub-Saharan African of between 15° N and 20 ° S. The vectors (tsetse flies) of the disease are only found in Africa. *T. b. gambiense* is found in West Africa and western Central Africa extending from Senegal across to Sudan and down to Angola while T. *b rhodesiense* is found in East Africa, Central Africa and Southern Africa extending from Ethiopia down to Botswana. The distribution of both parasite species overlaps in

the region of the great lakes of East Africa. There are also extensive areas within the geographical boundaries where sleeping sickness is not found (WHO, 1986).

Visceral Leishmaniasis also known as kala-azar in the New World (South America and Central America) is caused by *Leishmania donovani chagasi* while in the Old Word (Africa, Asia and Europe) it is caused by *Leishmania donovani donovani*, *Leishmania donovani infantum*, East African *Leishmania donovani* and Sudanese *Leishmania donovani. L .d. donovani* is found in the Indian subcontinent and southwest Asia. *L. d. infantum* is endemic in North Africa, Middle East, northern China and central Asia and southern parts of Europe. *L. d. chagasi* is found in parts of Central and South America (WHO, 1984).

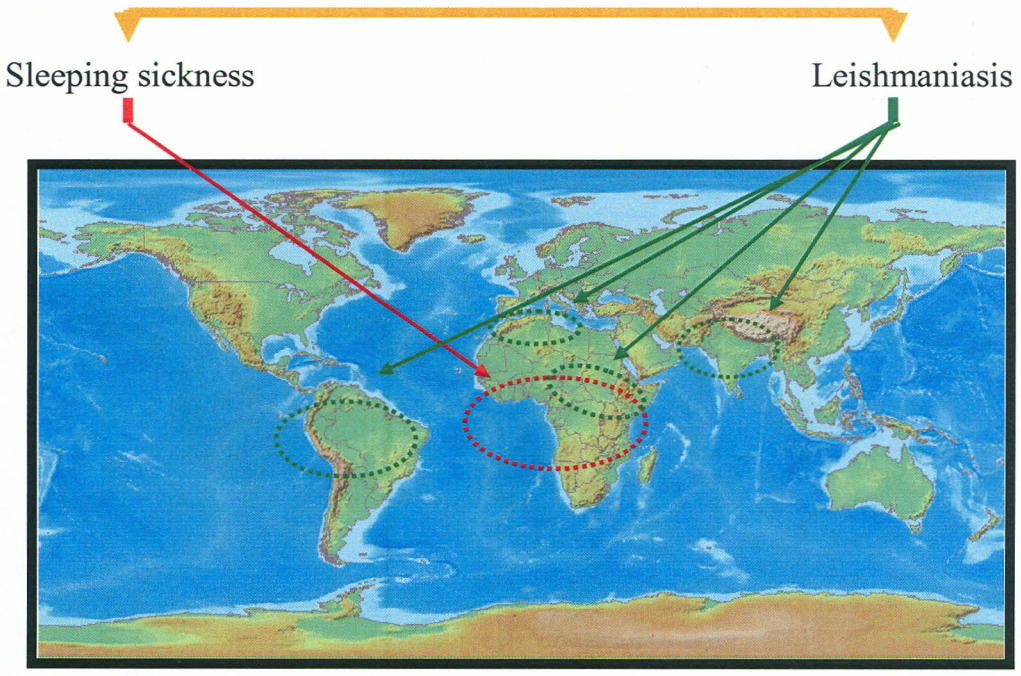


Fig 1a: Global distribution: Leishmaniasis and African Trypanosomiasis

As illustrated in Fig 1b, malaria primarily occurs in the tropical and sub tropical areas, especially Asia, Africa and Central South America as shown in figure 1. It is estimated that 40 % of the world's population lives in malaria endemic area. It causes 300 million episodes of acute illness every year. The mortality levels are greatest in the sub-Saharan Africa where children below five years of age are most vulnerable resulting in 90 % of the deaths due to malaria (Breman, 2001).

The four species of human Plasmodia have different geographical distributions. *P. falciparum* is predominant in Africa. *P. vivax* is found mostly in Asia, Latin America and parts of Africa. *P. ovale* is found mostly in West Africa and the islands of the western Pacific and *P. malarie* is found scattered worldwide (http:// www CDC .gov/malaria).

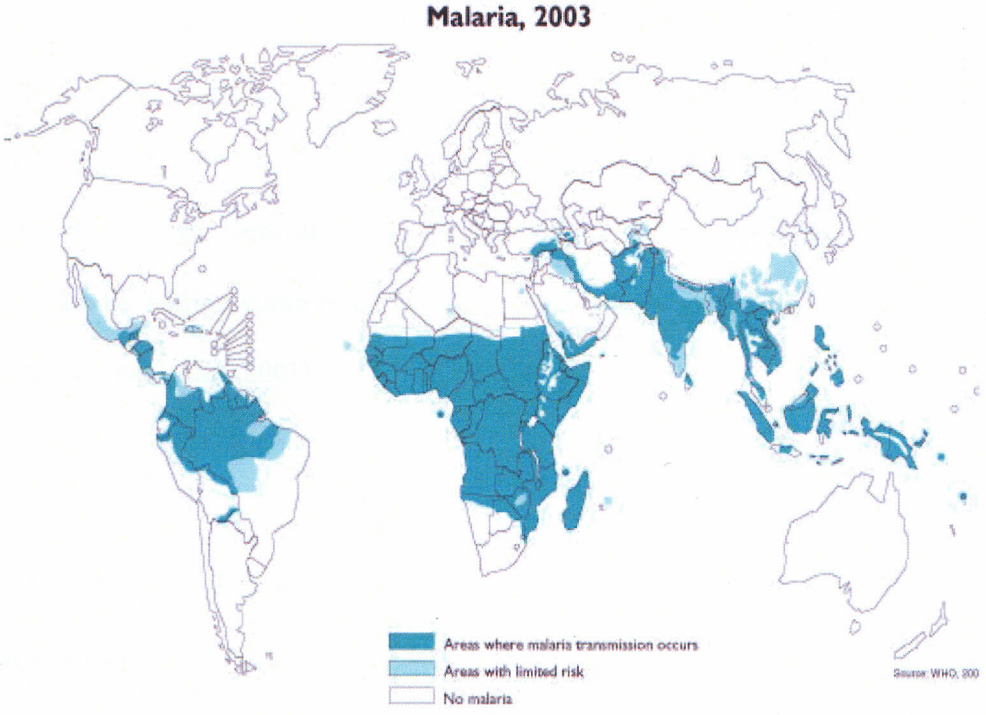


Fig 1b: Global distribution: Malaria

1.2.2 Degradation of nucleic acid material.

Most of the time nucleases are the enemy of the molecular biologist who is trying to preserve the integrity of RNA or DNA samples. A variety of non-specific nucleases have been discovered, isolated and characterized. They differ among other things in substrate specificity, cofactor requirements, and whether they cleave nucleic acids internally (endonucleases), chew in from the ends (exonucleases) or attack in both of these modes. In many cases, the substrate specificity of a nuclease depends upon the concentration of enzyme used up in the reaction process, with high concentrations promoting less specific cleavages.

Of the nucleic acids, DNA is relatively stable while RNA is fairly labile with its degradation occurring frequently in routine conditions. Therefore, the molecular analysis of RNA in particular, is severely hampered as a result of RNA loss secondary to the combined actions of the abundant and stable tissue RNAses and the high temperatures commonly used in specimen processing (Finkelstein *et al.*, 1999). To circumvent this problem, the targeted sequences in pathological specimens are preserved through removal or inactivation of any RNAses present in a biological specimen by adding commercial products or chemicals to the specimen to stabilize RNA (Fregeau *et al.*, 2001).

1.2.3 Storage and preservation of bio-clinical material

Since the logistics of using molecular techniques directly in the field are at present not very feasible, several methods have been developed to enable blood samples and other bio-clinical material to be processed several days later. The molecular procedure may

not be infallible in amplified materials that have been subjected to rough handling in the pre-analytic phase. Blood is the most important material for molecular diagnosis in parasitic disease like leishmaniasis and trypanosomiasis, and when drawn at collection centers, delays of up to 24 hours from collection time to processing time have been experienced. Preservation of blood remains the most important challenge due to its complex nature and its components. Therefore, most methods that are able to preserve DNA and RNA in blood will most likely apply to other bio-clinical martial.

The preservation of T. *cruzi* DNA has previously been accomplished by collecting blood in guanidine/EDTA solution which preserves the parasite DNA at 37° C for at least one month (Avila *et al.*, 1991). Elsewhere, RNA concentration has been shown to remain stable in uncentrifuged EDTA blood stored at 4 ° C for up to 24 hours. For long term preservation of DNA, it is best stored in high salt (>1M) in the presence of high EDTA (> 10mM) at pH 8.5 (Tsui *et al.*, 2002). A method of freezing of blood has also been used for preservation of DNA and RNA, but according to guidelines published by the American Association for Clinical Chemistry, whole blood for RNA isolation should not be frozen directly (Chen *et al.*, 1990). Instead, a simple pretreatment of whole blood samples to remove erythrocytes and plasma prior to freezing of leukocytes has been known to greatly increase the quality of RNA compared to quality of RNA stored under sub-optimal conditions (Kephart & Shenoi, 1998). Some studies have shown that plasma samples stored frozen at -70 ° C gave a good yield of RNA for molecular analysis (Wang *et al.*, 1992). Blood samples have also been stored on filter paper. This method of storage provides a simple and economical method of storage, preservation, and distribution of nucleic acid samples

for molecular analysis. It has been used for the preservation and storage of blood samples in general (Kline *et al.*, 2002). Commercial extraction-free filter paper-based methods like the FTA classic card technology have been developed for a variety of applications for long term preservation of blood at room temperature (Snowden *et al.*, 2002).

Other storage protocols combining the different storage conditions are in existence and have successfully been used, an example is the guanidine isothiocyanate (GuSCN) L6 lysis buffer and blood mixture followed by storage in liquid nitrogen for RNA isolation at a later date (Lahiri *et al.*, 1992). Other kits are commercially available for use; such as one based on RNAse-free water (with 0.1mM EDTA) or TE buffer (10mM Tris, 1mM EDTA) which indicates that RNA can be stable at -80 ° C for up to one year without degradation (Ambion, 2006).

1.2.4 Extraction and purification of bio-clinical materials for molecular analysis

Many DNA and RNA purification protocols have been developed, most of them being commercially available. These protocols are based on organic solvents like phenol or chloroform, enzymes like *proteinase* K, silica beads, and non-organic salts like EDTA, buffers or different combinations of these reagents (Lahiri *et al.*, 1992).

A rapid method that avoids the use of organic solvents (phenol/chloroform) and eliminates completely the use of *proteinase* K has been described. This method is accomplished by salting out and precipitation of the cellular proteins in saturated sodium chloride. The whole procedure takes less than an hour to completion, without

compromising the yield and quality of DNA (Lahiri *et al.*, 1992). Guanidium EDTA (GE) method based on the use of chaotropic agents to disrupt cells and inhibit nucleases was used successfully in PCR detection of *Trypanosoma cruzi* in blood. Blood samples collected in GE were boiled to disperse the template throughout the lysate, thus increasing the probability that samples contained at least one template of DNA (Avila *et al.*, 1991).

Different methods have been employed in processing samples for VL molecular analysis. *Proteinase* K based methods were proved to be superior to GE based methods for buffy coat and whole blood samples in a comparative study (Lachaud *et al.*, 2001). An acid guanidium phenol/chloroform (AGPC) method from a modified protocol was described and used for tissue RNA extraction (Chomzynski & Sacchi, 1987). A DNA extraction method, QIAmp, was designed to provide a fast and easy procedure for purification of total DNA for reliable PCR and Southern blotting. Total DNA can be purified from whole blood, plasma, serum, buffy coat, bone marrow, other body fluids, lymphocytes, cultured cells tissue and forensic specimens by use of this method (QIAGEN, 2003).

Among the most popular extraction methods is the Boom method, an ideal method for rapid purification of nucleic acid (covalently closed circular, relaxed circular and linear double stranded DNA; single-stranded DNA; and rRNA) directly from human serum and urine. The method is based on the observation that in the presence of high concentrations of the chaotropic agent GuSCN, nucleic acid will bind to diatoms or silica or glass particles. The method is simple, rapid and a reliable protocol for small

scale purification of DNA and RNA from human serum and Urine (Boom *et al.*, 1990).

1.2.5 Molecular tools for post storage and preservation (assay methods).

Molecular tools are widely in use in the diagnosis of infectious diseases; this has applied to both *Leishmania*, *Trypanosoma* and *Plasmodium* research and diagnosis. PCR is the most used molecular tool in infectious disease diagnosis; it can be performed on any biological sample including skin, tissue, blood and bone marrow. Many centers have evaluated the use of PCR for diagnosis of VL and have considered it extremely efficient and reliable. When PCR is performed on blood, the sensitivity for the detection of *Leishmania* DNA ranges from 80.8% to 92.5 % (Anderson *et al.*, 1997; Lachaud *et al.*, 2001; Salotra *et al.*, 2001). A quantitative real time PCR is becoming more available, mainly for research purposes. It is an established methodology that uses fluorescent labels to enable the continuous monitoring of amplicon throughout the reaction. A real-time PCR assay for detection of *Trypanosoma brucei* DNA in human blood samples was developed and was recommended for use in routine clinical laboratory practice (Becker *et al.*, 2004).

Nucleic Acid Sequence Based Amplification (NASBA) another molecular technology, is based on the concurrent activity of reverse transcriptase (RT), RNAse H, and T7 RNA polymerase, together with two primers to produce amplification (Sooknanan *et al.*, 1995; Kievits *et al.*, 1991). This is a technology with potential for broad application in RNA amplification and detection. With some modifications, NASBA can be adjusted to quantify RNA levels; this is called QT-NASBA. This makes it possible to quantify the actual numbers of the infectious agents in the clinical samples (Schoone *et al.*, 2000; Deiman *et al.*, 2002). The NASBA technique can be

perfomrd in the background of DNA in a sample, and, in addition, allows easy detection of stage specific expressed genes (e.g Pf 25S for specific detection of the gametocyte stage of *P. falciparum* (Shneider *et al.*, 2004)). Other techniques like Western blotting and Northern blotting have also been extensively used in the diagnosis and identification of antigens in VL cases (Burns *et al.*, 1993).

1.2.6 In-house storage protocols

Several protocols for preservation and extraction of DNA and RNA from bio-clinical materials are in use in different institutions. Some protocols are developed from commercially available kits and reagents while others are based on existing established in-house methods. The selected protocols are those that have been used at the study site at various times for preservation of nucleic acid material.

The L6 buffer method of preservation of bio-clinical material is one of the methods that have been widely used in preservation of DNA and RNA. It is a modification developed from the Boom method of extraction. L6 buffer is made up of GuSCN, Tris HCL, EDTA and Triton X-100. The L6 buffer has more often been referred to as the lysis/binding buffer because of the capability to perform both actions. Guanidine on its own is a very strong protein denaturant, therefore a GuSCN combination provides the perfect lysing and nuclease-inactivating properties of the chaotropic agent GuSCN that are bound in lysis/binding buffer L6 (Rashid *et al.*, 2006). GuSCN is a chaotropic salt which rapidly solubilizes cells and tissues while inactivating cellular ribonucleases. Additionally GuSCN provides an efficient environment for nucleic acid hybridization. All of the RNA and DNA in the sample are eventually available for hybridization. After lysing of cells and inactivation of nucleases, the samples are

then stored at -70°C for up to several months as they await extraction and purification (Boom *et al.*,1990).

A protocol based on Silica is also a modification from the Boom method of extraction. This protocol is based on the binding capacity of nucleic material from samples when subjected to silica or glass particles. The protocol involves the use of L6 buffer and silica particles to make a preservation step. Silica is also known to provide one of the most reliable means of purifying genomic DNA, plasmid DNA and RNA for routine molecular biology applications. In the presence of high salt concentrations a high affinity of the negatively charged DNA and RNA to the positively charged silica particles is created. The DNA and RNA hence become tightly bound to the silica particles. These molecules of DNA and RNA can later be eluted under low ionic strength. In this protocol, L6 buffer is used to lyse the cells and then silica added to the sample/L6 buffer mixture. The nucleic acid material that is set free in the sample after the lysis bind to the silica particles in the presence of the high salts (GuSCN). The nucleic acid material once bound to silica can then be stored at -20°C in form of dry silica particles which can be recovered at a later date (Boom *et al.*, 1990).

A protocol based on the L3 buffer is an invention of the University of Amsterdam, and is at the moment yet to obtain patenting rights and remains a trade secret. However, pilot experiments have shown that naked DNA and RNA material preserved in L3 buffer have remained stable for several months of experiment. The DNA and RNA material preserved in the L3 buffer have been stored under various temperatures ranging from 26°C, 4 °C, -20 °C to -80 °C successfully (unpublished data by Marcel *et al*).

The Filter paper method of preservation of DNA and RNA material has been shown to work very well in preservation of various samples for various types of molecular work. In some instances the filter papers have undergone some improvements by incorporation or impregnation with specialized binding chemicals to enhance their performance. A protocol based on plain filter paper without any enhancing substances can prove to be very easy to use, affordable and very convenient for field studies (Snowden *et al.*, 2002).

A popular commercial method for DNA storage and extraction from blood samples is the QIAmp method from Qiagen Company. The protocol based on this method has an advantage that it can be used with all common anticoagulants, such as heparin, EDTA, or citrate. The protocol is based on a Kit that has a DNA preservation (AS 1 buffer) which is a special stabilizing reagent. When whole blood is mixed with an equal volume of this buffer, it can be stored at room temperature for up to 12 weeks before proceeding with the QIAmp purification procedure. The AS1 buffers has been known to be enhanced in efficiency by mixing it with a second buffer (AS 2 buffer) also available in the Kit, DNA is then stable in the blood lysate for up to 6 months at almost any temperature, even in tropical regions. Samples stored in these buffers are then processed in the QIAmp Blood protocol for DNA purification (QIAGEN, 2003).

1.3 Statement of the problem

Although molecular tools are becoming more and more essential in the diagnosis of infectious disease, standardization in their use is far from being achieved. It is now well documented that for each class of microorganisms, the sample preparation and

nucleic acid extraction method could greatly influence the outcome and reliability of the test (Behzadbehbahani *et al.*, 1997; Grob *et al.*, 1992).

Time delay between blood draw and processing is one of the major pre-analytical variables in RNA and DNA studies; which has led to the development of several sample preservation and preparation methods. This necessitates the need to compare some of these methods for their efficacy, cost and applicability in developing countries.

The study sought to identify an ideal method that would be very rapid and economical and at the same time produce good yield of high quality nucleic acid material for use among research partners in *Trypanosomiasis*, *Leishmaniasis* and *Plasmodium* endemic areas.

1.4 Hypothesis

An ideal standard protocol that is efficient, cost effective and applicable for sampling, preparation and storage of bio-clinical materials is achievable.

1.5 Objectives

1.5.1 Main objective

To develop standard protocols for sampling, preparation and storage of bio-clinical materials for *Leishmania, Trypanosoma and Plasmodium* molecular research.

1.5.2 Specific objectives

(I). To develop a standard protocol for stabilization and conservation of bio-clinical samples for *Leishmania, Trypanosoma and Plasmodium* molecular analysis.

(II). To develop a standard protocol for extraction and conservation of RNA from bio-clinical samples for *Leishmania, Trypanosoma and Plasmodium* molecular analysis.

(III). To develop standard protocols for extraction and conservation of DNA from bio-clinical samples for *Leishmania* and *Trypanosoma* molecular analysis.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study site

The study was conducted at the KIT Biomedical Research, Royal Tropical Institute, The Netherlands.

2.2 Study design

This was a comparative experimental study.

2.3 Study samples

2.3.1 Parasite types

The parasites strains used in the experiment to spike blood samples were; *Leishmania donovani* strain MHOM/SD/68/IS, *Trypanosoma b. gambiense* isolate and *Plasmodium falciparum* strain NF54 gametocytes obtained from fresh cultures and spiked into human blood samples to simulate infected blood.

2.3.2 Sample size

The sample size was determined based on the experimental design. The study comprised of seven protocols with a 10^3 parasite per ml concentration sample in triplicate for each of the seven time points making **21** samples and a zero parasite concentration sample in triplicate for each of the seven time points and making a second set of **21** samples. Therefore each storage protocol had a total of **42** samples. Five reference control samples (for the standard curve) were processed alongside the other samples at each time point of extraction. The were **35** control samples in total, making the overall sample size for all the seven protocols in triplicate at all time points (control samples included) to be **329** in number.

2.4 Experimental design

The study entailed seven storage protocols that differed in media (either buffers or filter paper) and/or temperature. Samples in the seven protocols were spiked with the counted and known amounts of parasites. Each protocol was made up of two sets of samples; a spiked sample (with 10^3 parasites per ml of *Trypanosoma*, 10^3 parasites per ml *Leishmania* and 10^5 parasites per ml of *Plasmodium falciparum* gametocytes all mixed in the a single sample), and a second set; a plain sample (zero parasites concentration). Each sample in the seven protocols for each time point was made into triplicates. The triplicate samples of the spiked and the zero parasite concentrations samples from each of the seven protocols were removed from storage at each stipulated time points of extraction ranging from day 0 to week 10 of storage and extraction followed by molecular analysis done. The *Leishmania*, *Trypanosoma* and *Plasmodium* RNA and *Leishmania* DNA assays were done quantitatively while the *Trypanosoma* DNA was qualitatively done by gel electrophoresis to establish whether there was any deterioration in each of the protocols along the storage duration. The mean value of the triplicate quantitative results obtained for the all samples were used to plot graphs to show the change over time.

2.4.1 Laboratory procedures.

2.4.1.1 Counting of parasites

The parasite mentioned above were freshly obtained from culture were counted using a standard Burker counting chamber to obtain the number of parasites per ml. The parasite/buffer concentration was either diluted or concentrated to make a final stock concentration of 10^6 parasites/ml. The 10^6 parasites/ml was spiked into blood to make a final parasite concentration of 10^3 parasites/ml for both *Trypanosoma* and

Leishmania while a 10^6 p/ml was spiked to attain a final concentration 10^5 p/ml for the *Plasmodium falciparum* gametocytes.

2.4.1.2 Preparation of seeded blood samples (Blood/Parasite dilution)

The experiment required approximately 65 ml of blood obtained from an individual who had no remote possibility of having exposure to any of the two diseases. The samples were obtained through the existing ethical codes. The samples were as follows:

(a) Spiked samples

To 25ml of freshly obtained EDTA blood, 25 µl of 10^6 parasites per ml of *Trypanosoma brucei gambiense,* 25 µl of 10^6 of *Leishmania donovani* parasites/ml and 2.5ml of 10^6 of *P. falciparum* gametocytes were added to the same blood and mixed thoroughly. The final parasite concentration for Leishmania and Trypanosoma was 10^3 parasites per ml of blood while for *Plasmodium falciparum* were 10^5 parasites per ml of blood.

(b) Plain samples (negative controls)

25 ml of freshly obtained plain EDTA blood had nothing added to it.

2.4.1.3. Preparation of positive control samples

Positive control blood samples were made from the stock 10^6 parasites/ml, different serial dilutions were made to obtain a control series of 10^5 (E5) (for plasmodium only) and 10^4 (E4), 10^3 (E3), 10^2 (E2), 10^1 (E1) and a 10^0 (E0) sample for all the three parasite species.

2.4.1.4 Storage protocols

The sample preparation scheme for the seven storage protocols and the control series was as follows.

	Protocol	Sample Vol	Storage Medium	Temperature	Target
1	**Reference**	50 µl	L_6 buffer	-70° C	DNA and RNA
2	**Silica**	200 µl	L_6 buffer & silica	-20° C	DNA and RNA
3	**L3 at 26°C**	200 µl	L_3 buffer	26° C	DNA and RNA
4	**L3 at -20°C**	200 µl	L_3 buffer at	-20°C	DNA and RNA
5	**L3 at 4°C**	200 µl	L_3 buffer	4 ° C	DNA and RNA
6	**F. paper**	50 µl	filter paper	26° C	DNA and RNA
7	**QIAmp**	200 µl	As-1 buffer	26° C	DNA only
Control	**Control**	50 µl	L_6 buffer	-70° C	DNA and RNA

2.4.1.5 Extraction time points

The extraction and purification of DNA and RNA from samples in the seven protocols was done on day1, day3, week 1, week 2, week 4, week6 and week 10 of storage.

2.4.1.6 Methods for DNA and RNA extraction.

For the control, reference, L3 buffer at -4°C, L3 buffer at 26°C, L3 buffer at -20 °C, Silica and filter paper samples, the Boom extraction method was used for both DNA and RNA purification/extraction (Boom *et al.,* 1990). In this method the RNA/DNA was isolated by lysis in L6 buffer (5 M guanidine isothiocyanate, 0.1 M Tris pH 6.4, 20 mM EDTA, 1.2% [wt. /vol.] Triton X100). RNA/DNA was bound to silica and the silica was washed twice with an L2 wash buffer (5 M guanidine thiocyanate, 50mM Tris pH 6.4), 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 QIAmp mini kit from Qiagen was used for samples stored in AS-1 buffer. The entire process of the QIAmp procedure was done in specially designed spin columns

that reduce the possibilities of contamination. Proteinase K solution was added to samples initially stored in AS-1 buffer to lyse the cells followed by AS -2 buffer that also acts as a stabilizing buffer, the mixture was washed once in 70 % absolute ethanol. Another wash in AW-1 buffer followed by one wash with AW- 2 buffer was done. AE buffer was used for elution of DNA material at room temperature for 5 min. (QIAGEN, 2003). The purified samples from both extraction methods were then stored at -70°C as they awaited analysis.

2.4.1.7 Molecular Analytical methods

The following molecular assays were used for the different parasite molecular targets in the spiked, preserved and extracted samples at the various points of extraction (storage duration).

Trypanosoma: Real time NASBA for 18S rRNA
Conventional PCR for 18S rDNA

Leishmania: QT-NASBA for 18S rRNA
Real time PCR for 18S rDNA

Plasmodium: Real time NASBA for Pf 25S mRNA

a) ***PCR*** – The principle of the conventional PCR for *Trypanosoma* 18S rDNA was based on the enzymatic amplification of a fragment of DNA that is usually flanked by two 'primers', short oligonucleotides that hybridized to the opposite strands of the target sequence and then prime synthesis of the complementary DNA sequence by DNA polymerase (an enzyme). The chain reaction was a three-step process, denaturation, annealing, and extension, which repeated itself in several cycles. At each stage of the process, the number of copies doubled from two to four, to eight, and so on. The reactions were controlled by changing the temperature using a special heat-stable Taq polymerase (reagents). After 20 cycles, roughly 1 million copies were

in existence, or enough material of the desired DNA for detection by the conventional means such as gel electrophoresis in this case. (Anderson *et al.*, 1997; Lachaud *et al.*, 2001; Salotra, 2001).

The RT-PCR for the *Leishmania* 18S rDNA was based on the key feature that the amplified DNA was quantified as it accumulated in the reaction in *real time* after each amplification cycle. This method of quantification made use of a fluorescent dye that intercalated with the double-stranded DNA. An increase in DNA product during the PCR therefore lead to an increase in fluorescence intensity and was measured at each cycle, thus allowing the DNA concentrations to be quantified (Schulz *et al.*, 2003).

b) *NASBA*- The NASBA principle used in the quantification of *Trypanosoma* 18S rRNA and the *P. falciparum* 25S mRNA was based on the concurrent activity of the enzyme reverse transcriptase (RT), RNAseH and T7 RNA polymerase, together with two primers to produce amplification. This process occurred at a temperature of 41° C without the need of adding intermediate reagents and resulted in the exponential amplification of RNA and DNA product within 90 minutes, producing as the major product antisense, single-stranded RNA. A molecular beacon used in the reaction was employed to detect the amplified product in a real time instrument that gave a quantitative output (Schoone *et al.*, 2000). For the quantification of the *Leishmania* 18S rRNA, the ECL-NASBA technique was used, where the amplified products were detected by electrochemiluminescence. In this procedure capture probes coupled to magnetic beads, a wild-type and a quantitative (Q-RNA) specific ruthenium labeled oligonucleotide detection probe are employed (Greijer *et al.*, 2001).

2.4.2 Data Analysis

The quantitative results were obtained in triplicate for each analytical point and the outliers in each triplicate results identified using the Dixon's Q-test (Dixon, 1950). The data was entered into a computer using the SPSS program outliers replaced within the SPSS functions and the data subjected to General Linear Model statistics to obtain the Repeated Measures ANOVA. The sphericity correction was done using the method advocated by Greenhouse and Geisser in 1959. The p values obtained at 95% confidence limits showed whether there was any significant difference in the data in each of the storage methods from the values obtained at baseline (day1) along the storage duration to the last point of analysis (week 10). The data was stored in a computer memory, flash disk and in form of hard copy.

CHAPTER THREE

RESULTS

To address the objective of this study, both *Leishmania*, *Trypanosoma* RNA/DNA were stored/stabilized under six protocols for RNA/DNA preservation (Reference, L3 buffer at 26 °C, L3 buffer at 4°C , L3 buffer at -20°C , Filter paper and Silica methods) and one protocol for DNA stabilization (AS-1 buffer for QIAmp). As an additional RNA experiment, *Plasmodium* gametocyte RNA in blood was preserved in the six protocols for RNA/DNA preservation. The *Leishmania* RNA and DNA, *Trypanosoma* RNA and *Plasmodium* RNA levels in these samples were analyzed using quantitative methods while *Trypanosoma* DNA was analyzed by a qualitative method.

The quantitative and qualitative results obtained from the assays used in this study were meant to evaluate and compare the efficacy of each of the methods in the preservation and stabilization of both DNA and RNA in the spiked samples.

For all the protocols, the results for the spiked and non-spiked blood samples were obtained. For the quantitative methods, the non spiked samples remained negative and gave values equivalent to zero. Therefore the non-spiked samples are not discussed here in the results section. For the qualitative methods, the gel showed bands for the spiked samples and no bands for the non spiked samples these are all illustrated in the gel plates.

3.1 RNA recovery after different preservation protocol

3.1.1 *Leishmania* RNA quantities

The *Leishmania* 18S rRNA result output from the ECL-NASBA assay was obtained in triplicate for each time point. The results output was quantified by a regression based on the control samples and the log of parasites per milliliter of blood obtained (log p/ml). The means of the triplicate results at each time point as were used to plot graphs (Figs 2a and 2b).

The average log of parasites per ml of *Leishmania* RNA for all the methods was expected to remain at log 3.0 p/ml during the storage duration for an ideal method. The Reference method had an average of log 3.02 p/ml. The L3 buffer at 26°C method had the lowest average value of log 1.87 p/ml while the L3 buffer at 4°C had the highest average value of log 3.53 p/ml. The L3 buffer at -20°C had an average of log 3.29 p/ ml; this was a higher score than that of the Reference method while the Filter paper method had an average value of log 2.05 p/ml (Table 1).

Table 1: Mean *Leishmania* 18S rRNA p/ml by storage period

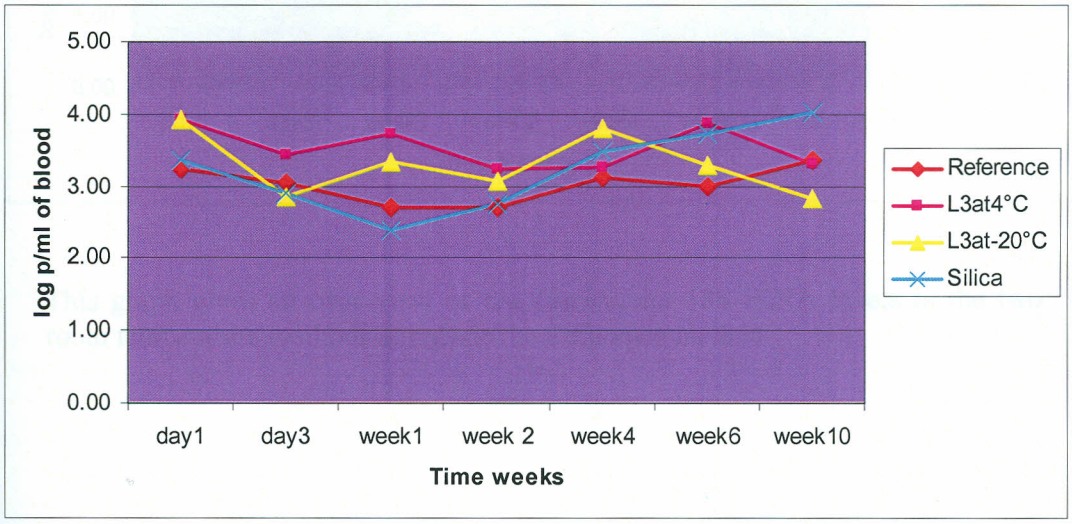
Method	day1	day3	week1	week 2	week 4	week 6	week 10	Avg
Reference	3.24	3.03	2.70	2.70	3.12	2.98	3.36	**3.02**
L3 at 26°C	3.74	2.48	2.93	2.59	0.77	0.00	0.57	**1.87**
L3 at 4°C	3.91	3.43	3.72	3.23	3.25	3.88	3.30	**3.53**
L3 at -20°C	3.91	2.85	3.34	3.06	3.79	3.28	2.82	**3.29**
Silica	4.35	2.88	2.05	2.74	3.48	3.72	4.02	**3.22**
F. paper	2.77	2.77	1.74	1.81	2.65	2.39	0.00	**2.05**

The values reported are means of logs of *Leishmania* 18S rRNA p/ml in blood for the assays run in triplicate at different time points.

The data was subjected to the repeated measures ANOVA at 95% confidence intervals and the following observations made: There were significant differences between the average log p/ml of the Reference method and the following methods; L3 buffer at 26°C ($p \leq 0.001$) and Filter paper ($p \leq 0.001$). There were no significant differences between the Reference method and the following methods; L3 buffer at 4°C ($p=0.250$), L3 buffer at -20°C ($p=0.054$) and the Silica method $p=0.202$.

The mean RNA levels in the Reference, L3 buffer at -20°C, L3 buffer at 4°C and Silica methods did not show any major changes along the storage duration. The raw data for these refrigeration methods was subjected to the repeated measures analysis of variance to establish whether there was any significant difference in the mean log p/ml for each storage method along the storage duration. The findings showed that there was no significant difference in the mean RNA levels along the storage duration for the Reference method ($p=0.221$), L3 buffer at 4°C ($p= 0.127$), L3 buffer at -20°C ($p=0.070$), and Silica methods $p=0.072$ (Fig 2a).

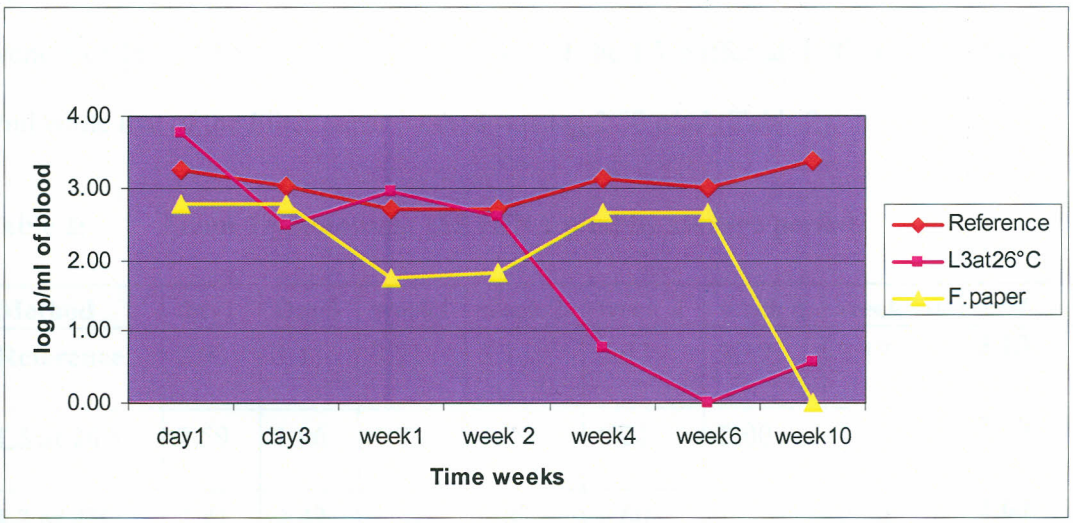
Fig 2a: Mean log p/ml of *Leishmania* 18S rRNA from refrigerated samples



This graph gives an overview of *Leishmania* 18S rRNA levels in the four refrigeration methods.

The mean RNA levels in the L3 buffer at 26°C remained stable up to the second week of storage, the levels started dropping and at the fourth week there were low levels of RNA detectable and after six weeks no RNA was detectable in the samples. For the Filter paper method, the mean RNA levels remained stable up to the sixth week of storage and by the tenth week of storage the RNA was undetectable. The raw data for these room temperature methods was subjected to the repeated measures analysis of variance to establish whether there was any significant difference in the mean log p/ml for each storage method along the storage duration. Further analysis indicated that there were statistically significant differences in the RNA levels along the storage duration in the L3 buffer at 26 °C p= 0.008 and the Filter paper p= 0.002 (Fig 2b).

Fig 2b: Mean log p/ml of *Leishmania* 18S rRNA from room temperature samples



This graph gives an over view of the *Leishmania* 18S rRNA levels in the two room temperature methods alongside the Reference method.

3.1.2 *Trypanosoma* RNA quantities

The *Trypanosoma* 18S rRNA results output from the Real-Time NASBA assay were obtained in triplicate for each time point. The quantitative results output was obtained in threshold cycle values which were converted to log of parasites per milliliter of blood obtained (log p/ml). The mean of the triplicate results at each time points were used to plot the graphs (Figs 3a and 3b).

The average log of parasites per ml of *Trypanosoma RNA* for all the methods was expected to remain at log 3.0 p/ml during the storage duration. The Reference method had an average value of log 3.13 p/ml. The average of the L3 buffer at 26°C method had the lowest average value of log 1.18 p/ml, while the L3 buffer at -20 °C had the highest average value of log 3.45 p/ml. The Silica method on the other hand had an average of log 2.95 p/ml. The average value of the L3 buffer at 4 °C was log 2.68 p/ml while that of the Filter paper method was log 2.50 p/ml (Table 2).

Table 2: Mean *Trypanosoma* 18S rRNA p/ml by storage period.

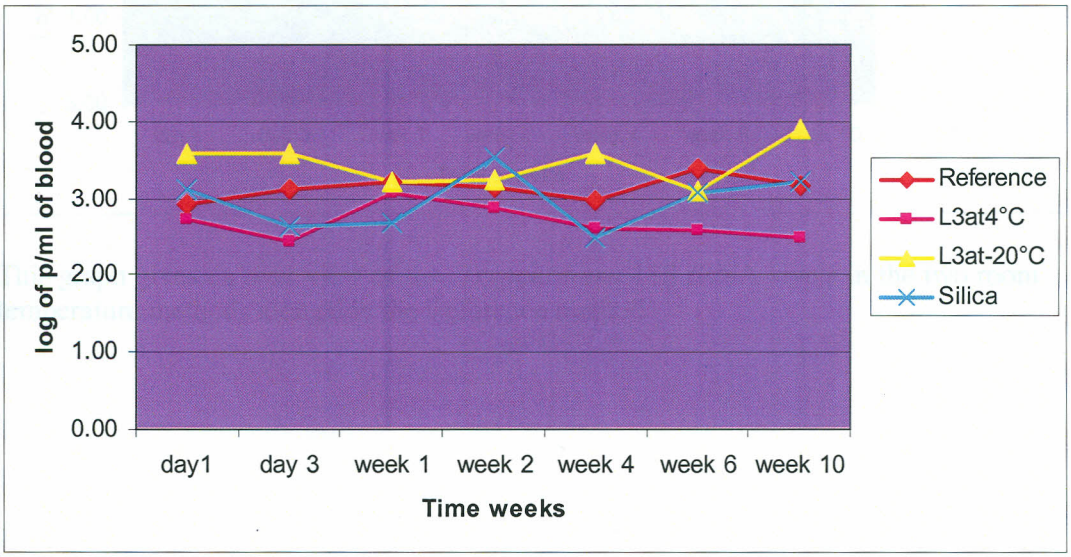
Method	day1	Day3	week1	week 2	week 4	week 6	week 10	Avg
Reference	2.93	3.11	3.21	3.14	2.97	3.37	3.17	**3.13**
L3 at 26°C	2.59	1.96	1.73	1.79	0.21	0.00	0.00	**1.18**
L3 at 4°C	2.71	2.42	3.07	2.87	2.60	2.57	2.48	**2.68**
L3 at-20°C	3.57	3.57	3.22	3.23	3.57	3.09	3.90	**3.45**
Silica	3.11	2.63	2.66	3.54	2.47	3.06	3.20	**2.95**
F. paper	2.37	3.19	3.14	3.15	3.01	2.67	0.00	**2.50**

The values reported are means of logs of *Trypanosoma* 18S rRNA p/ml in blood for the assays run in triplicate at different time points.

Upon subjecting the data to the repeated measures ANOVA at 95% confidence intervals, the outcome showed that there were significant differences between the mean log p/ml of the Reference method and all the other methods with the following p values; L3 buffer at 26°C ($p \leq 0.001$), L3 buffer at -20°C ($p = 0.002$) and Filter paper ($p \leq 0.001$) L3 buffer at 4°C ($p \leq 0.001$) and the Silica method ($p = 0.021$).

The mean RNA levels in the Reference, L3 buffer at -20°C, L3 buffer at 4°C and Silica methods did not show any major changes along the storage duration. The raw data for these refrigeration methods was subjected to the repeated measures analysis of variance to establish whether there was any significant difference in the mean log p/ml for each storage method along the storage duration. The findings showed that there was no significant difference in the mean RNA levels along the storage duration for the Reference method ($p=0.328$), L3 buffer at 4°C ($p = 0.381$), L3 buffer at -20°C ($p=0.247$), and Silica methods $p=0.077$ (Fig 3a).

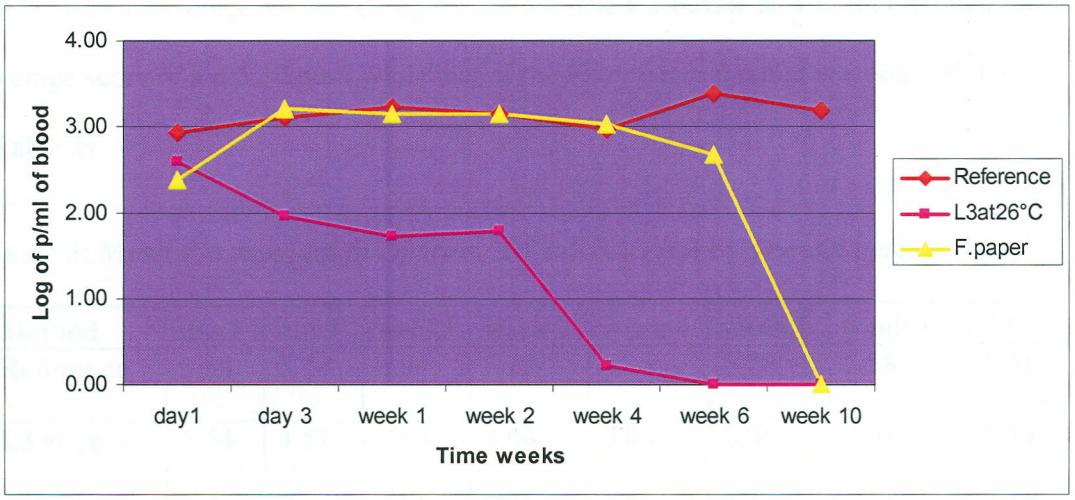
Fig 3a: Mean log p/ml *Trypanosoma* 18S rRNA from refrigerated samples.



This graph gives an overview of *Trypanosoma* 18S rRNA levels in the four refrigeration methods.

The mean RNA levels in the L3 buffer at 26°C remained stable up to the second week of storage, the levels showed a decreased by the fourth week and by the sixth week of storage RNA was not detectable in the samples. As for the Filter paper method, the mean RNA levels remained stable up to the sixth week of storage. However, by the tenth week of storage there was no RNA detected in the samples. The raw data for these room temperature methods was subjected to the repeated measures analysis of variance to establish whether there was any significant difference in the mean log p/ml for each storage method along the storage duration. Further analysis indicated that there were statistically significant differences in the RNA levels along the storage duration in L3 buffer at 26 °C (p=0.020) and filter paper p=0.002 (Fig 3b).

Fig 3b: Mean log p/ml *Trypanosoma* 18S rRNA from room temperature samples.



This graph gives an over view of the *Trypanosoma* 18S rRNA levels in the two room temperature methods alongside the Reference method

3.1.3 *Plasmodium falciparum* RNA quantities

The *Plasmodium falciparum* 25S mRNA analysis was done by use of the Real-Time NASBA assay and the results obtained in triplicate for each time point. The quantitative results output was obtained in threshold cycle values which were converted to log of parasites per ml by a regression based on the control samples. The mean of the triplicate results at each time point were used to plot the graphs (Figs 4a and 4b) .

The average log of parasites per ml of Plasmodium *falciparum* 25S mRNA was expected to be about log 5.0 p/ml. The Reference method had an average value of log 5.31 p/m. The L3 buffer at 26°C method had the lowest average value of log 2.78 p/ml, while the Silica method had the highest average of log 5.37 p/ml. L3 buffer at -20 °C had an average value of log 5.52 p/ml. The L3 buffer at 4°C method had an average score of log 5.17 p/ml while that of the Filter paper method was log 3.90 p/ml (Table 3).

Table 3: Mean *Plasmodium falciparum* 25S mRNA p/ml by storage period.

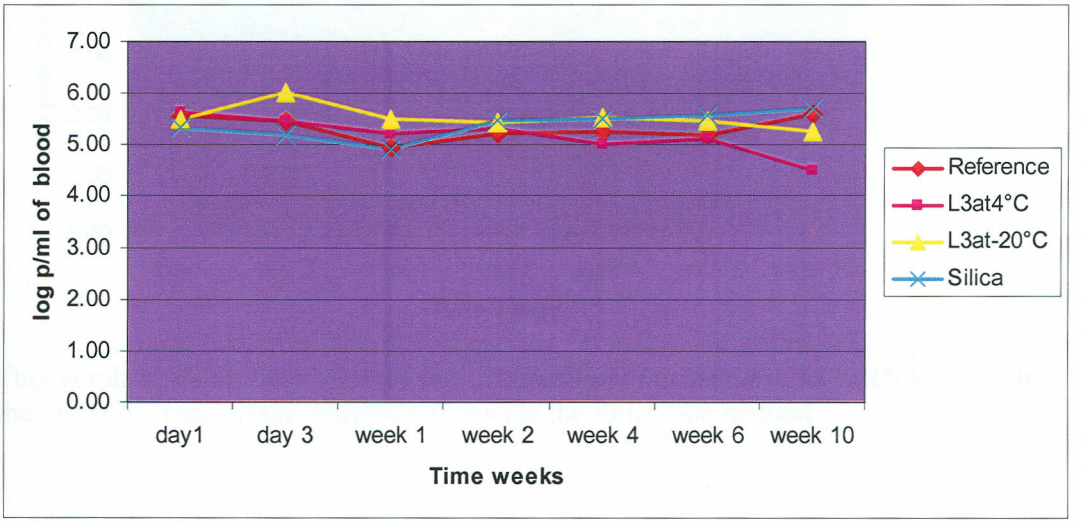
Method	day1	day3	week1	week 2	week 4	week 6	week 10	Avg
Reference	5.54	5.44	4.94	5.22	5.25	5.20	5.58	**5.31**
L3 at 26°C	5.54	4.52	4.67	4.06	0.00	0.00	0.68	**2.78**
L3 at 4°C	5.62	5.45	5.21	5.31	5.02	5.11	4.51	**5.17**
L3 at-20°C	5.51	6.01	5.49	5.42	5.53	5.47	5.23	**5.52**
Silica	5.32	5.17	4.90	5.45	5.48	5.56	5.71	**5.37**
F. paper	4.48	5.37	4.12	4.73	4.01	4.62	0.00	**3.90**

The values reported are means of logs of *Plasmodium falciparum* 25S mRNA p/ml in blood and standard deviations for the assays run in triplicate at different time points.

The data was subjected to the repeated measures ANOVA at 95% confidence intervals and the following observations made: There were significant differences between the average log p/ml of the Reference method and the following methods; L3 buffer at 26°C ($p \leq 0.001$) and Filter paper ($p \leq 0.001$). And there were no significant differences between the Reference method and the following methods; L3 buffer at 4°C (p=0.404), L3 buffer at -20°C (p=0.209) and the Silica method (p= 0.665).

The mean RNA levels in the Reference, L3 buffer at -20°C, L3 buffer at 4°C and Silica methods did not show any major changes along the storage duration. The raw data for these refrigeration methods was subjected to the repeated measures analysis of variance to establish whether there was any significant difference in the mean log p/ml for each storage method along the storage duration. The repeated measures analysis of variance showed that there was no significant difference in the mean RNA levels along the storage duration for the Reference method (p=0.129), L3 buffer at 4°C (p= 0.137), L3 buffer at -20°C (p=0.351), and Silica methods p=0.199 (Fig 4a).

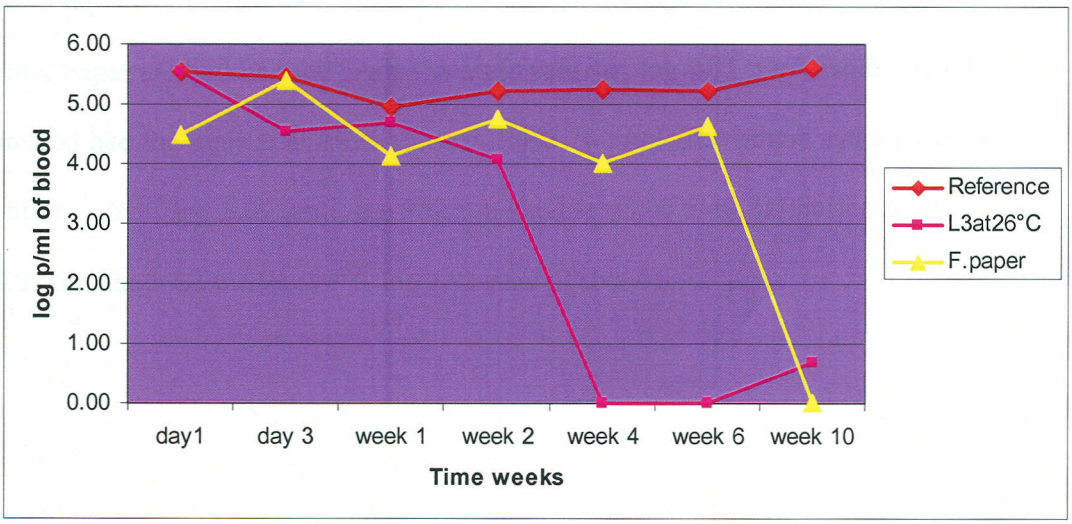
Fig 4a: Mean log p/ml *Plasmodium falciparum* 25S mRNA from refrigerated samples



This graph gives an overview of *Plasmodium falciparum* 25S mRNA levels in the four refrigeration methods.

The mean RNA levels in the L3 buffer at 26°C remained stable up to the second week of storage, by the fourth week of storage there was no RNA that could be detected in the samples. For the Filter paper method the mean RNA levels remained stable up to the sixth week of storage and by the tenth week of storage there was no RNA that could be detected in the samples at that point. The raw data for these room temperature methods was subjected to the repeated measures analysis of variance to establish whether there was any significant difference in the mean log p/ml for each storage method along the storage duration. Further analysis indicated that there were statistically significant differences in the RNA levels along the storage duration in L3 buffer at 26 °C (p=0.002) and filter paper (p=0.011), there were statistically significant differences in the RNA levels along the storage duration (Fig 4b).

Fig 4b: Mean log p/ml *Plasmodium falciparum* 25S mRNA from room temperature samples.



This graph gives an over view of the *Plasmodium falciparum* 25S mRNA levels in the two room temperature methods alongside the Reference method

3.2 DNA recovery after different preservation protocols

For the DNA assays, an extra storage method was evaluated along the initial six RNA/DNA storage methods. The seven methods that were compared for DNA preservation are the reference, L3 buffer at 4°C, L3 buffer at -20°C, L3 buffer at 26°C, filter paper, silica methods and the QIAmp method. The *Leishmania* 18S rDNA results were quantitative while the *Trypanosoma* 18S rDNA results were qualitative.

3.2.1 *Leishmania* DNA quantities.

The *Leishmania* 18S rDNA analysis was done by use of the Real-Time PCR (RT-PCR) assay and the results obtained in triplicate for each time point. The quantitative results output was obtained in threshold cycle values which were converted to log of parasites per ml by a regression based on the control samples.

The average log of parasites per ml of Leishmania 18S rDNA was expected to be about log 3.0 p/ml. The Reference method had an average value of log 2.68 p/ml. The Filter paper method had the lowest average value of log 0.05 p/ml, while the QIAmp method had the highest average value of log 3.20 p/ml. The others averages were L3 buffer at 26°C log 3.09 p/ml, the Silica method log 3.00 p/ml, L3 buffer at -20 °C log 2.87 p/ml and L3 buffer at 4°C log 2.91 p/ml (Table 4).

Table 4: Mean *Leishmania* 18S rDNA p/ml by storage period.

Method	Day 1	Week 1	Week 6	Week 10	Average
Reference	3.04	2.53	2.53	2.61	**2.68**
L3 at 26°C	3.55	2.50	3.22	3.09	**3.09**
L3 at 4°C	2.80	2.30	3.17	3.37	**2.91**
L3 at-20°C	2.78	3.01	2.66	3.05	**2.87**
Silica	3.55	3.06	3.27	2.46	**3.09**
Filter paper	0.50	0.50	0.41	0.61	**0.50**
QIAmp	3.18	3.22	3.40	3.01	**3.20**

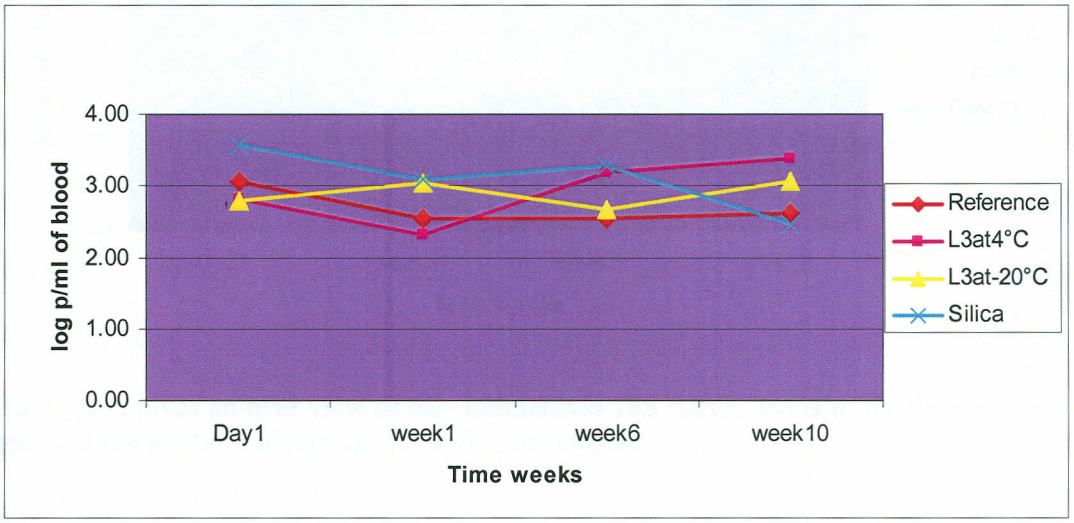
The values reported are means of logs of *Leishmania* 18S rDNA parasites/pm in blood and standard deviations for the assays run in triplicate at different time points

The data was subjected to the repeated measures ANOVA at 95% confidence intervals and the following observations made: There were significant differences between the average log p/ml of the Reference method and the QIAmp ($p= 0.007$), L3 buffer at 26°C ($p= 0.018$), Silica method ($p= 0.033$), and Filter paper ($p=0.003$). There were no significant differences between the Reference method and the L3 buffer at 4°C ($p= 0.088$) and the L3 buffer at -20°C ($p=0.426$).

The mean DNA levels in the Reference, L3 buffer at -20°C, L3 buffer at 4°C, and the Silica method did not show any major changes along the storage duration. The raw data for these refrigeration methods was subjected to the repeated measures analysis of variance to establish whether there was any significant difference in the mean log p/ml for each storage method along the storage duration. The statistical analysis

showed that there was no significant difference in the mean DNA levels along the storage duration for the Reference method (p=0.249), L3 buffer at 4°C (p= 0.0.53), L3 buffer at -20°C (p=0.596), and Silica methods p=0.056 (Fig a).

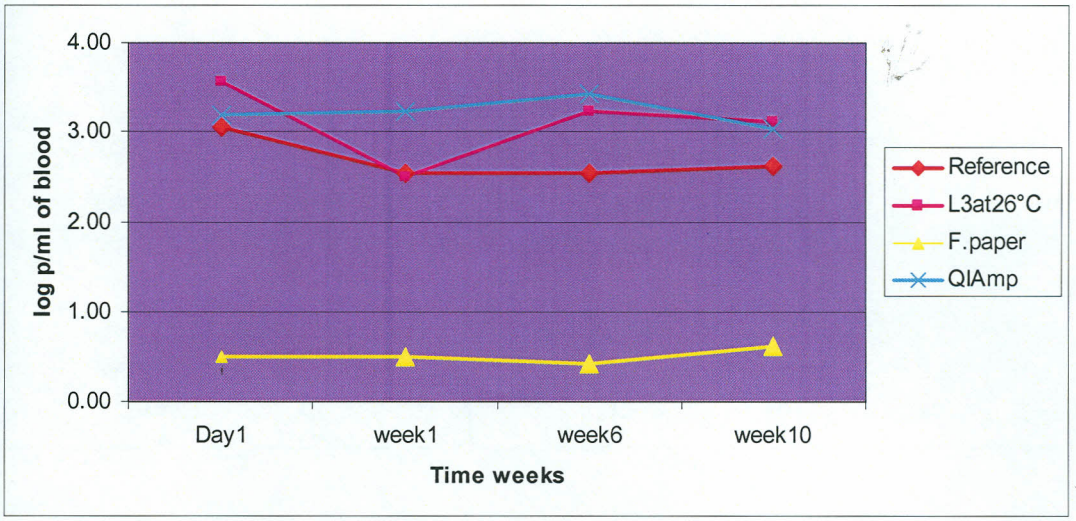
Fig 5a:Mean log p/ml *Leishmania* 18S rDNA from refrigerated samples



This graph gives an overview of *Leishmania* 18S rDNA levels in the four refrigeration methods.

In the Filter paper method, low mean DNA levels were recovered from the day 1 of storage and this managed to remain stable at these low levels along the whole storage period. The raw data for these room temperature methods was subjected to the repeated measures analysis of variance to establish whether there was any significant difference in the mean log p/ml for each storage method along the storage duration. The analysis indicated that there were no statistically significant differences in the DNA levels along the storage duration in the L3 buffer at 26 °C (p=0.222), Filter paper (p=0.874) and the QIAmp (p=516) methods (Fig 5b).

Fig 5b: Mean log p/ml *Leishmania* 18S rDNA from room temperature samples



This graph gives an over view of the *Leishmania* 18S rDNA levels in the three room temperature methods alongside the Reference method

3.2.2 *Trypanosoma* DNA signals

The *Trypasoma* 18S rDNA analysis was done by use of a programmed conventional PCR assay in a thermocycler followed by detection of the amplified product by way of gel electrophoresis with ethidium bromide staining.

Plates 1 and 2 show the PCR amplicons of 18S rDNA for all the seven methods at the start of the study (day 1) and at the end of the study (week 10). Each sample was done in triplicate; spiked samples were expected to show bands which signified the presence of DNA while the non-spiked were not expected to show any bands to signify the absence of DNA.

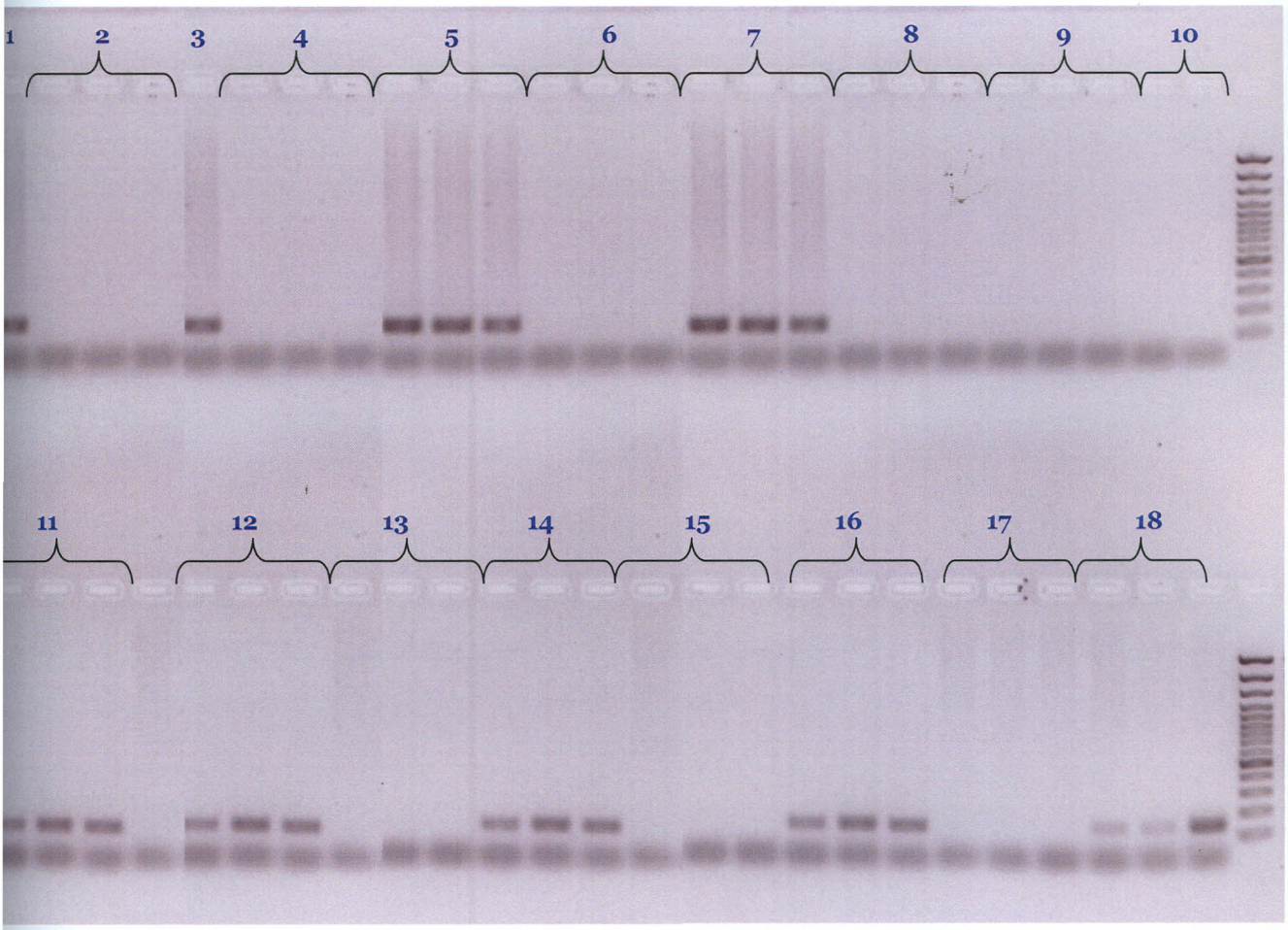


Plate 1: Gel electrophoresis day 1 Trypanosoma 18S rDNA PCR amplicons

Positive control samples.....1 & 3
Reference method plain samples.....4
L3 buffer at 26°C plain samples.....6
L3 buffer at -20 °C plain samples.....13
L3 buffer at 4°C plain sample.....9
Silica method plain sample.....8
Filter paper method plain sample.....17
Qiagen method plain sample.....15
Nuclease free water.....10

Negative control samples.....2
Reference method spiked sample...5
L3 buffer at 26°C spiked sample....7
L3 buffer at -20°C spiked sample..14
L3 buffer at 4°C spiked samples...12
Silica method spiked samples.....11
Filter paper spiked sample.....18
Qiagen method spiked sample.....16

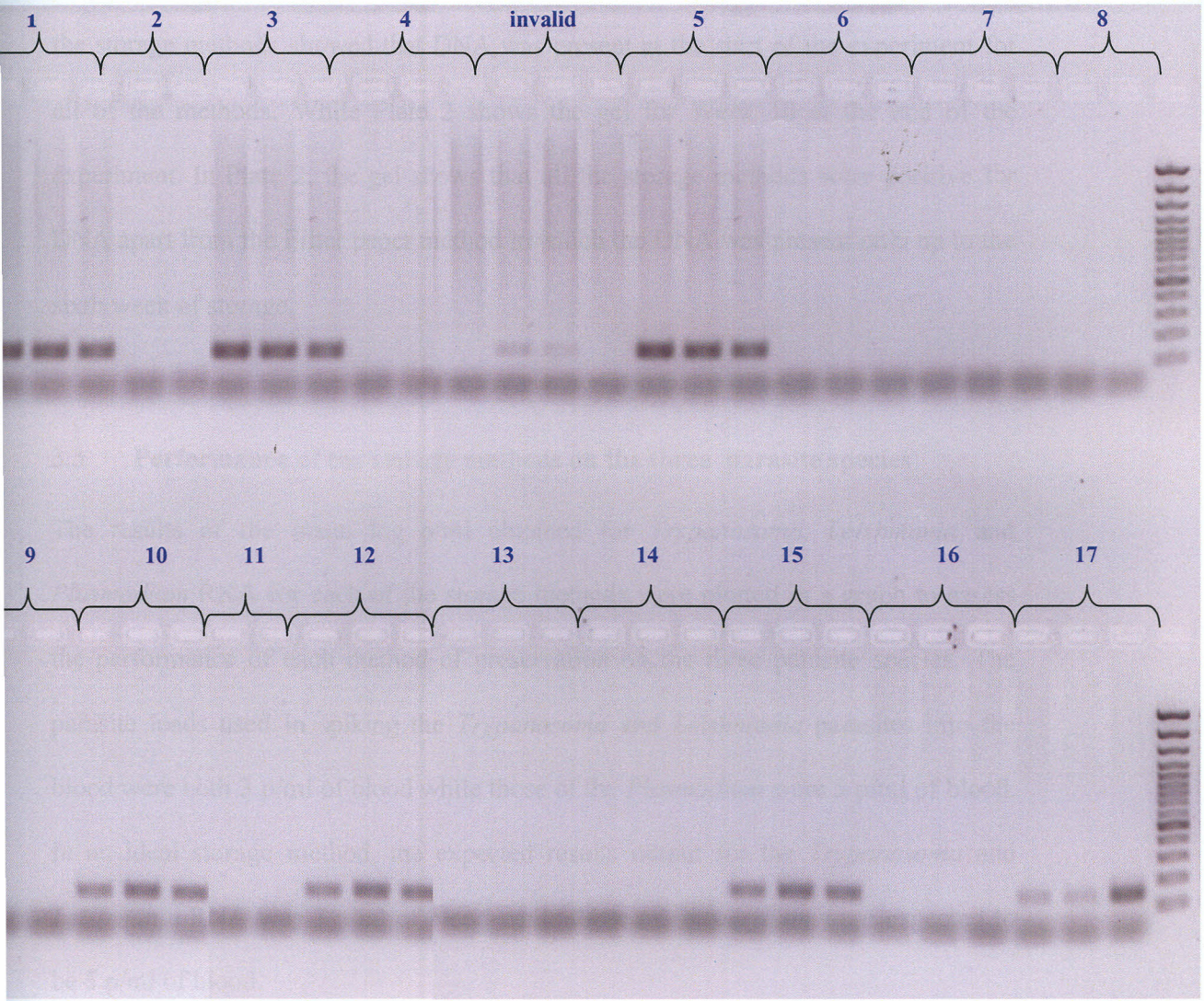


Plate 2: Gel electrophoresis week 10 Trypanosoma 18S rDNA PCR amplicons

Positive control samples……..1
Reference method plain samples……...2
L3 buffer at 26°C plain samples..……4
L3 buffer at -20 °C plain samples……....6
L3 buffer at 4°C plain sample……...7
Silica method plain sample……..…….9
Filter paper method plain sample……13
Qiagen method plain sample……16
Nuclease free water……...11

Negative control samples……8
Reference method spiked sample…3
L3 buffer at 26°C spiked sample….5
L3 buffer at -20°C spiked sample..10
L3 buffer at 4°C spiked samples...12
Silica method spiked samples.....15
Filter paper spiked sample……14
Qiagen method spiked sample…...17

Plate 1 shows the DNA results for Day 1 of analysis; in this gel all the samples of all the storage methods showed that DNA was present at the start of the experiment for all of the methods. While Plate 2 shows the gel for Week 10 at the end of the experiment. In Plate 2, the gel shows that all the storage methods were positive for DNA apart from the Filter paper method in which the DNA was present only up to the sixth week of storage.

3.3 Performance of the storage methods on the three parasite species

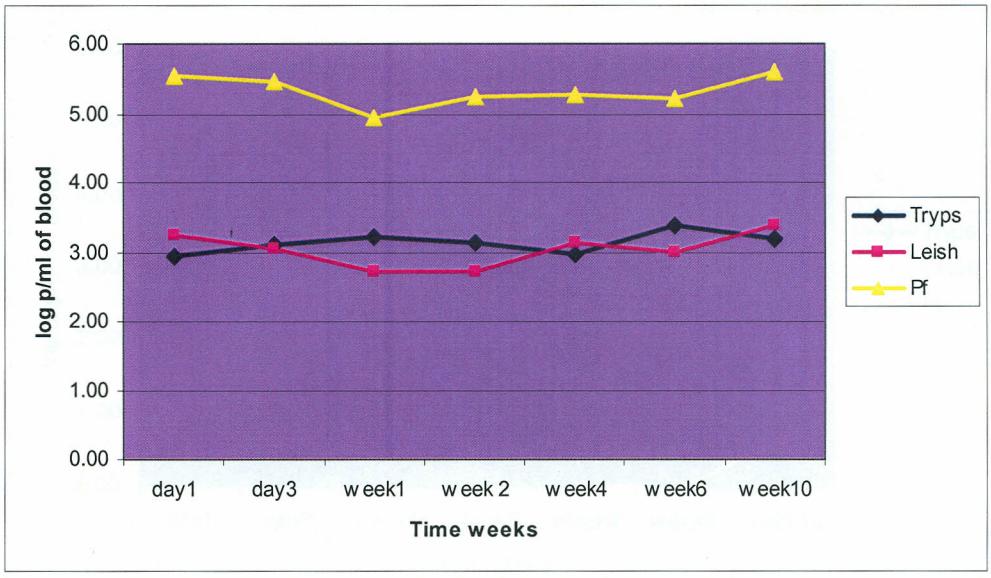
The results of the mean log p/ml obtained for *Trypanosoma*, *Leishmania* and *Plasmodium* RNA for each of the storage methods were plotted in a graph to assess the performance of each method of preservation on the three parasite species. The parasite loads used in spiking the *Trypanosoma* and *Leishmania* parasites into the blood were both 3 p/ml of blood while those of the *Plasmodium* were 5 p/ml of blood. In an ideal storage method, the expected results output for the *Trypanosoma* and *Leishmania* RNA was 3 p/ml of blood while those of *Plasmodium* were expected to be 5 p/ml of blood.

3.3.1 The Reference method

The Reference method of preservation was able to maintain the RNA from all the three parasites in a stable state up to the last day of the study (week 10). The RNA levels that were detected from all the three parasites remained constant from the first to the last day of storage. There were no significant changes seen in the mean RNA log p/ml along the storage duration for all these parasite species. All the p values were greater than 0.05, of the three parasite species the *Plasmodium* RNA underwent a

biggest change (p=0.129) with *Trypanosoma* showing the smallest change (p= 0.328) while *Leishmania* RNA parasites had a change whose p value was 0.221 (Fig 6).

Fig 6: Reference method mean RNA values in log p/ml



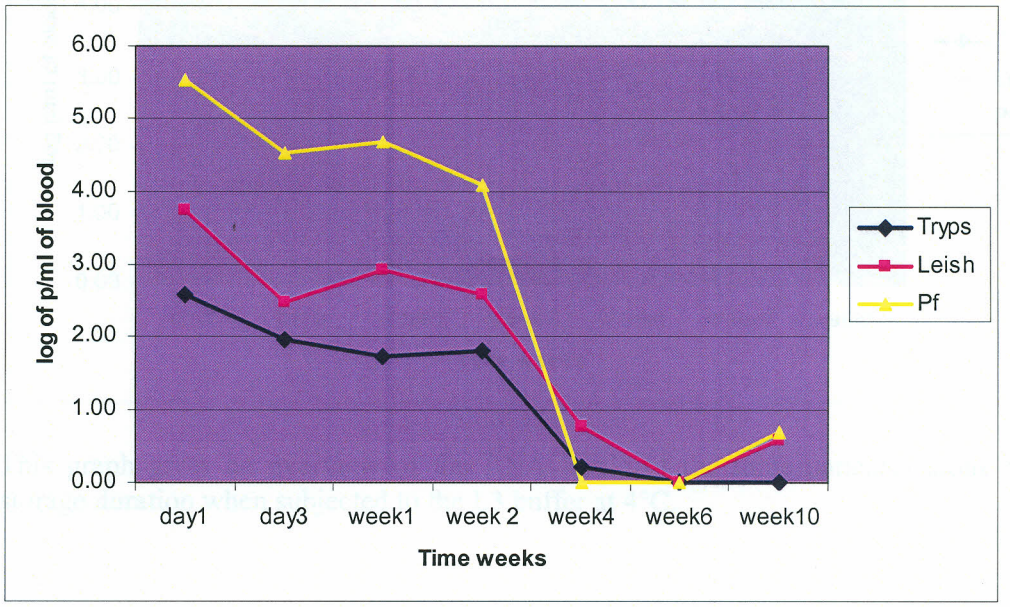
This graph gives an overview of the RNA levels of the three parasites along the storage duration when subjected to the Reference method

3.3.2 The L3 buffer 26°C method

For the L3 buffer at 26°C method, the *Leishmania* 18S rRNA, *Plasmodium falciparum* 25S mRNA *Trypanosoma* 18S rRNA mean RNA values in all the parasites species did not remain stable to the end of study. The RNA from all the three parasites could only be detected up to the second week of storage. The RNA levels in the *Plasmodium* deteriorated very fast to the point that by the fourth week of storage there was no RNA that could be detected in the sample. The *Trypanosoma* and *Leishmania* RNA remained stable up to the second week after which there was a slow deterioration towards the sixth week to a point that RNA could not be detected anymore. For all the parasites, there was a statistically significant change in the RNA

levels along the duration of storage with the following p values: *Leishmania* (p=0.008), *Trypanosoma* (p=0.020) and *Plasmodium* p=0.002 (Fig 7).

Fig 7: L3 buffer at 26°C method mean RNA values in log p/ml

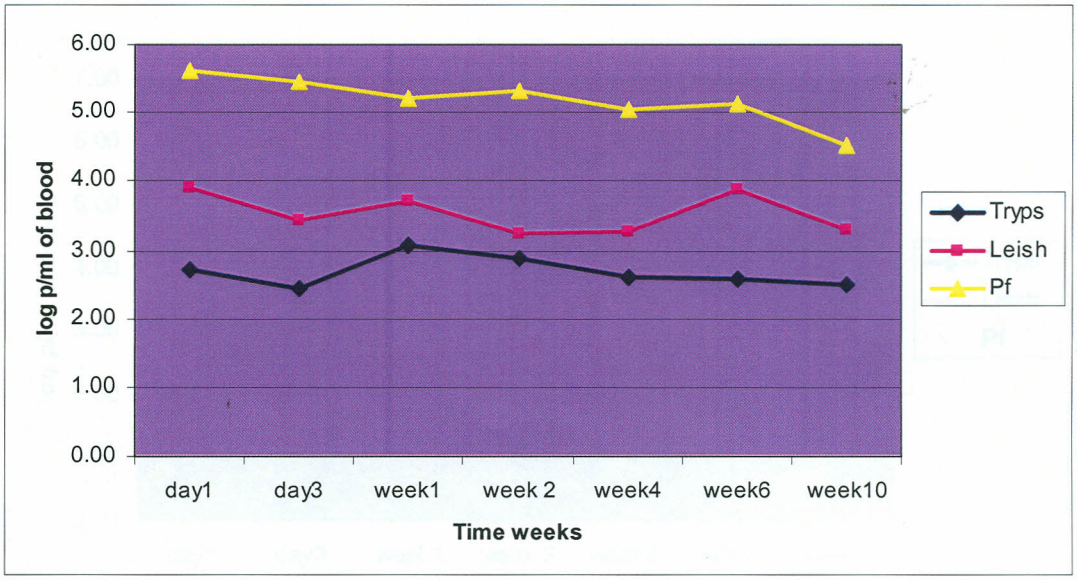


This graph gives an overview of the RNA levels of the three parasites along the storage duration when subjected to the L3 buffer at 26°C.

3.3.3 The L3 buffer 4°C method

The L3 buffer 4°C method of preservation was able to preserve the RNA from the three parasites species stable up to the last day of the study (week 10). For all the three parasites there was no statistically significant difference in the RNA levels during the duration of storage. The p values for the three parasites species were greater than 0.05, of the three the *Leishmania* RNA underwent a bigger change (p=0.127) with *Trypanosoma* showing the smallest change (p= 0.381) while *Plasmodium* RNA parasites value had a change p=0.137 (Fig 8).

Fig 8: L3 buffer at 4°C method mean RNA values in log p/ml

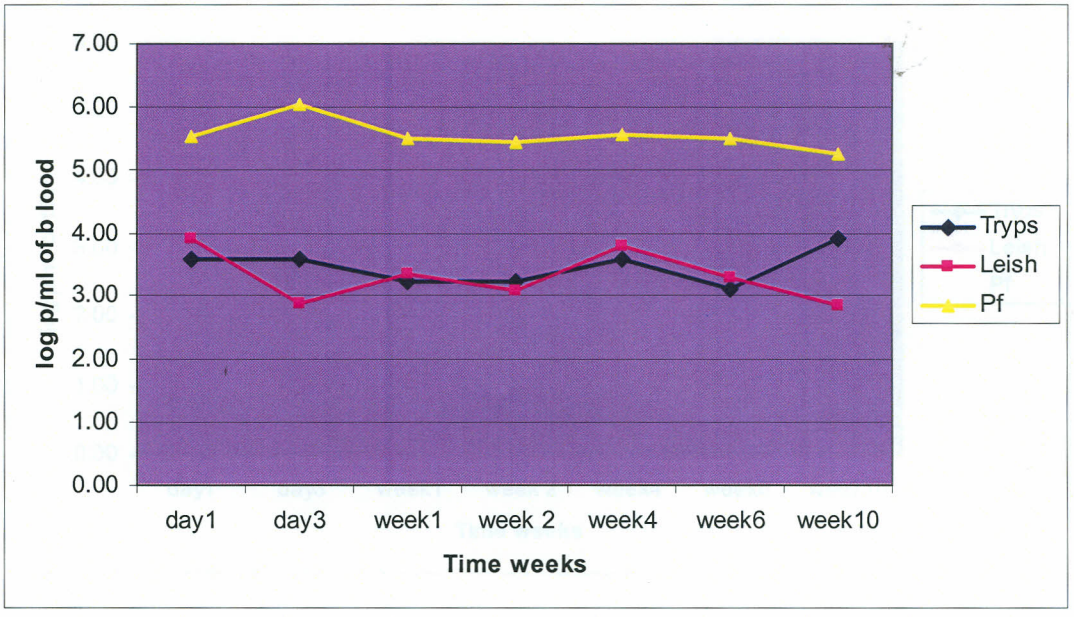


This graph gives an overview of the RNA levels of the three parasites along the storage duration when subjected to the L3 buffer at 4°C.

3.3.4 The L3 buffer -20°C method

The L3 buffer -20°C method was able to preserve the RNA from all the three parasites species up to the last day of the study (week 10). The RNA levels from all the parasites remained fairly constant from the first day of storage to the last day of storage. For all the three parasites there was no statistically significant difference in the mean RNA levels during the duration of storage. With all the P values being greater than 0.05, of the three the *Leishmania* RNA underwent a bigger change (p=0.070) with *Plasmodium* showing the smallest change (p= 0.351) while *Trypanosoma* RNA had a p value = 0.247 (Fig 9).

Fig 9: L3 buffer at -20°C method mean RNA values in log p/ml

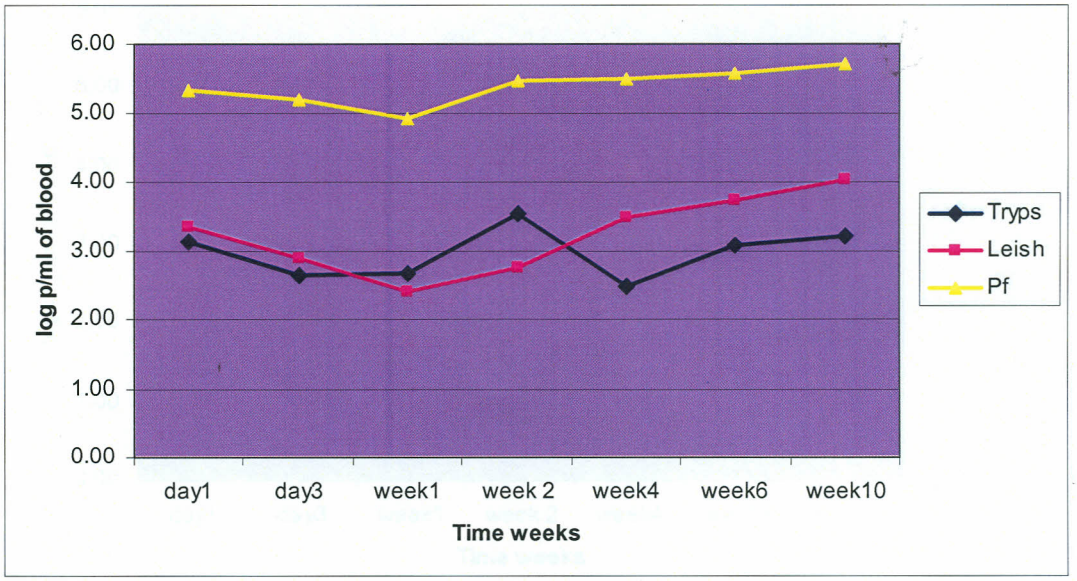


This graph gives an overview of the RNA levels of the three parasites along the storage duration when subjected to the L3 buffer -20°C.

3.3.5 The Silica method

The Silica at -20°C method achieved an effective preservation in all the parasites up to the end of the study duration. The mean RNA from all the three parasites remained fairly stable up to the last day of the study (week 10). For all the three parasites there was no statistically significant difference in the mean RNA values during the duration of storage. All the p values were greater than 0.05. Of the three the *Leishmania* RNA underwent a bigger change (p=0.072) with *Plasmodium* showing the smallest change (p= 0.119) while *Trypanosoma* RNA had a p value of =0.077 (Fig 10).

Fig 10: Silica method mean RNA values in log p/ml

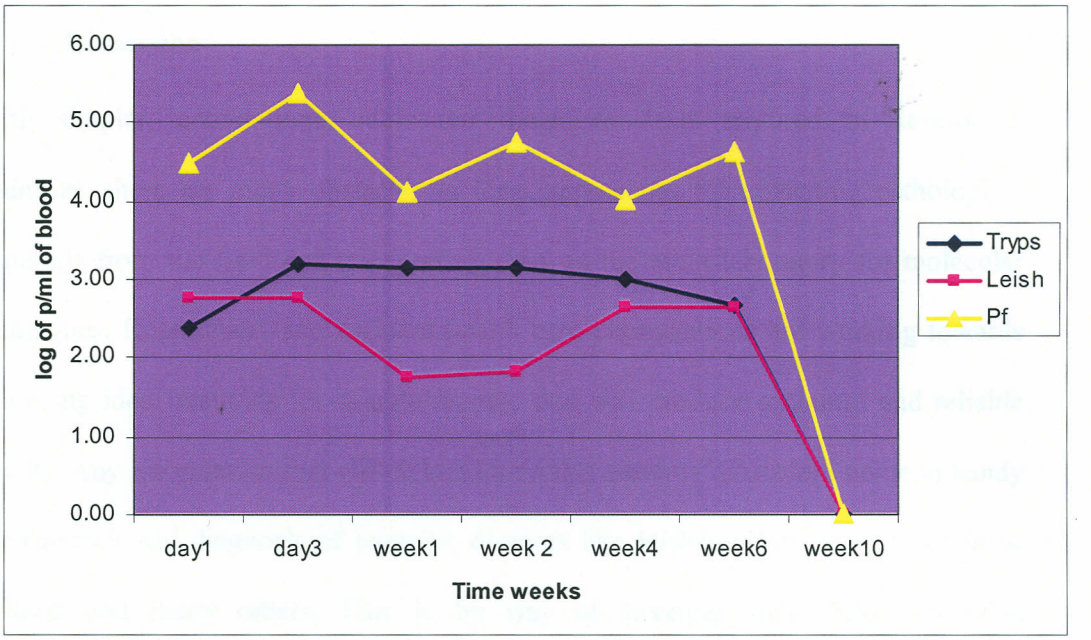


This graph gives an overview of the RNA levels of the three parasites along the storage duration when subjected to the Silica method

3.3.6 The Filter Paper method

For the Filter paper method, the preservation in all the parasites was not very efficient. Storage in this method preserved and kept stable the RNA from all the three parasites up to the sixth week. The results were very inconsistent along the storage duration with the mean RNA levels in the *Trypanosoma* and *Plasmodium* showing fluctuations and between the sixth week and tenth week the RNA were at undetectable levels in the samples. *Leishmania* RNA remained stable up to the sixth week and deteriorated till the tenth week where the RNA was undetectable in the samples. For all the parasites, there was a statistically significant change in the mean RNA levels along the duration of storage with the following p values: *Leishmania* ($p=0.002$), *Trypanosoma* ($p=0.002$) and *Plasmodium* $p=0.011$ (Fig 11).

Fig 11:Filter paper method mean RNA values in log p/ml



This graph gives an overview of the RNA levels of the three parasites along the storage duration when subjected to the Filter paper method.

CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

With simpler, conventional molecular diagnostic tests required in developing countries, there are many obstacles to ideal approaches for collecting pathological materials from the field and transporting them to the main laboratory for molecular techniques. Researchers and national health care organizations are working towards achieving ideal methods for sample storage that will produce optimum and reliable results. Any information that will help address this need will therefore come in handy for research and diagnosis of parasitic diseases like leishmaniasis, trypanosomiasis, malaria and many others. This is by way of ensuring ideal field collection, preservation, storage and purification of specimens before molecular studies are performed. Therefore findings from this study are expected to contribute a great deal towards such efforts.

The major findings from this study indicate that the L3 buffer methods where 200µl of blood was put in 200 µl of L3 buffer then subjected to three different storage temperatures (26°C, 4°C and -20°C), are ideal for transport, short term and long term preservation of parasite RNA and DNA in human blood samples. The L3 buffer is a new discovery whose details including mode of preservation and its constituent reagents still remain a trade secret at the moment as it is currently undergoing the patenting process by the discoverers (Marcel *et al.*, of the University of Amsterdam). Of the three storage temperatures subjected to the L3 buffer, the 26°C storage temperature proved to be the least stable method in RNA preservation for all the three parasite species with stability in the RNA guaranteed up to the second week of

storage. The L3 buffer at 26 °C was efficient in the preservation of both *Leishmania* and *Trypanosoma* DNA to the end of the storage duration. This temperature requires minimal facility hence making storage to be very ideal in a field situation where equipment could be lacking. The L3 buffer at this temperature can be utilized as an ideal transport medium where preservation is required over a short duration (during transit) no need of an expensive cold storage transportation method like on dry ice. Two weeks stability of RNA and DNA in L3 buffer at 26°C can allow transportation to any part of the world with no additional expenditures. This temperature can also provide ideal short term storage while awaiting purification prior to analysis.

Storage at the -20°C temperature in L3 buffer was fairly reliable, and efficient in the preservation of both the DNA and RNA of all the parasites tested, but there were few cases where samples formed a clot during storage and could not be used in the study. The reasons for the clot formation still remain unexplained, though the percentage of samples that formed the clot at -20 °C was approximately 2%. The clot formation at this temperature had a strong correlating with the duration of storage. Around 98% of the samples remained in good condition up to the end of the study. In the meantime, attaining -20°C in the field can prove to be an expensive venture and not easy to achieve in most cases as a freezer of that capability is required.

The storage in L3 buffer at 4 °C was very reliable and efficient in the preservation of both DNA and RNA of all the parasites tested. This method is ideal and attaining 4 °C may not require very sophisticated cold storage refrigerators as this temperature can be achieved by the use of cool boxes with constant changes of ice packs that are pre-frozen. In many established laboratories, the ice packs have proved to maintain this

temperature for up to 30 hours while special storage packs like the wizards storage packs can go for up to 96 hours. This method of storage has very minimal limitations and is ideal in the field with good management of the cool box. The major challenge is therefore to make the L3 buffer easily and readily available and also affordable once it has gone through all the legal requirements of patenting and production for use. The L3 buffer in general has done very well in pilot studies where naked DNA and RNA were preserved at various temperatures with highly positive outcomes (unpublished data and personal communication from the discovers of this buffer). At the moment, no records are available to indicate any other studies done on this buffer and part of the data generated here will be used in justifying the relevance of this buffer.

In this study, the refrigeration temperature methods performed better than the ambient temperature methods in the preservation of parasite RNA. In general the cold storage methods L3 buffer at 4 °C, L3 buffer at -20°C, Silica at -20°C and the Reference method at -70°C were more efficient and reliable in the preservation of both RNA and DNA when compared to the ambient temperature methods (the Filter paper at 26°C and L3 buffer at 26°C). The reason to this difference could be attributed to the fact that most of the RNAses and DNAses or other proteins that would break down the parasite RNA and DNA in the samples are rendered inactive by the low temperatures while they remain active at ambient temperatures. The cold storage methods are however more expensive for field establishment because they require refrigeration or freezing facility which will eventually need to run on electricity. In the field situation where resources are minimal and in some cases power supply is not guaranteed, a simple but efficient method would still be the method of choice.

Refrigeration storage has previously been found superior to the ambient temperature storage where a sample of DNA in biopsy tissue on Filter paper impregnated with GenoFix was preserved at room temperature for one year and seven months while a similar sample of the same tissue similarly treated was stored for three and a half years at –20°C with the DNA remaining stable (Fregeau *et al*, 2001. These findings strongly suggest that refrigeration methods are more likely to perform much better than ambient temperature methods even when all the other factors like reagents, volumes and parasite loads are kept constant.

It was observed that the preservation of RNA was fairly difficult to attain as compared to the preservation of DNA. Whereas DNA was successfully stored for ten weeks , RNA could only be stored for two and six weeks in the L3 buffer at 26°C and the Filter paper methods respectively. The reasons for the difficulty in RNA preservation can be attributed to among many other things, the combined actions of the abundant and stable tissue RNAses and in some cases the high temperatures commonly used when handling specimens (Finkelstein *et al.*, 1999). To circumvent this problem that is associated with the presence of the many types of RNAses that are found in pathological specimens, their removal or inactivation from the specimens is achieved by the addition of commercial products or chemicals (that eventually stabilize the RNA) to the biological specimen (Fregeau *et al.*, 2001).

Recent discoveries have also attributed the fragility of RNA to auto-degradation, whereby individual RNA is able to degrade itself or other RNA molecules in the surrounding . These RNA molecules that act as enzymes are called ribozymes. A ribozyme (from ribonucleic acid enzyme, also called RNA enzyme or catalytic RNA)

is an RNA molecule that catalyzes a chemical reaction. The reactions catalyzed by natural ribozymes often involve the cleavage or exchange of phosphodiester bonds in RNA molecules. For example, ribonuclease P trims the 5' ends of transfer RNA molecules. *In vivo* ribonuclease P operates as an RNA-protein complex, but it is the RNA component that catalyses the RNA cleavage reaction. Before this discovery, enzymes, which are defined as catalytic proteins, were the only known biological catalysts. Biologists had traditionally thought that all enzymes are proteins This view was overturned in the 1980s, when Thomas Cech of Yale University discovered that some RNA molecules can also act as enzymes. The term *ribozyme* was first introduced by Kelly Kruger *et al.* in 1982 in a paper published in Cell (Cech and Bass 1986; Breaker and Joyce 1994).

Elsewhere for the Filter paper preservation, the problem of RNAses has been tackled by the addition of an alcohol-based tissue fixative, GenoFix as mentioned by Fregeau *et al* in 2001. Also a Filter paper impregnated with GuSCN has been shown to preserve HIV RNA for 28 days at room temperature (Fiscus *et al.*, 1998). However, a set of results of storage on Filter paper in this particular study gave a result that could not be interpreted. DNA due to its structural nature and the lack of many stable DNAses existing in tissues or in the environment make it more stable and easy to handle as compared to the more labile RNA. Since not much is known about the L3 buffer at 26°C, the temperature of (26°C) may be the major factor that affects its performance. At ambient temperature, RNA preservation may be difficult to attain even with the addition of chemical substances that render the RNAses inactive.

In this study, the Reference method preserved *Trypanosoma* and *Leishmania* DNA and RNA and *Plasmodium* RNA for the ten weeks efficiently. This method is a

GuSCN based method (the main active substance in L6 buffer is GuSCN). Nucleic acid samples preserved in this method have been shown to contain a good yield of both RNA and DNA in a single sample. Preservation of *Leishmania tropica* RNA and DNA in plain GuSCN has been accomplished from skin tissue. The tissue was obtained from the lesions, put on a slide and preserved in a tube with GuSCN and stored at 4°C for one month with success (Noyes *et al.*, 1998). In another study EDTA blood samples from malaria patients were stored efficiently in this buffer for several months (Schoone *et al.*, 2000).

The advantages attributed to the Reference method are that a small sample volume is required (50 µl), and can be made locally in any laboratory after obtaining its constituent reagents. The Reference method still remains crucial for use in the well equipped major molecular biology laboratories and remains a major quality control method for preservation. The disadvantages on the other hand are that this method of preservation is expensive as it is not easy to have the -70°C storage in a field set up.

With the Filter paper method storage at 26°C, the RNA remained stable for up to six weeks of storage while DNA was preserved but in low amounts for the whole duration of storage. The poor sensitivity and low nucleic acid recovery observed in the filter paper samples make it to be unreliable and unsatisfactory in the preservation of nucleic acids in clinical specimens for molecular investigations. This can be attributed to the RNAses that break down the RNA. The other factor that may lead to the poor performance of the filter paper is the poor recovery of the nucleic acid material from the filter paper. The extraction and purification if not well carried out, may not recover the actual amount of RNA and DNA. To improve on the recovery of

earlier preserved nucleic material from malaria parasites in dried blood spots on filter paper, it was soaked overnight in a tube containing 100 µL of phosphate-buffered saline (PBS) at 4°C. This treatment enhanced greatly the yield of the targeted DNA (Chaorattanakawee *et al.*, 2003). Low sensitivity has also been observed in PCR assays due to low amounts of DNA in filter paper preserved samples. In one study, storage on filter paper in the first 30 days showed a decreased sensitivity in the PCR for different types of samples. While finding from another study suggested that the longer the samples were stored, the drier the blood spots became and the stronger the fixation of hemoglobin and other blood proteins on the filter paper (Farnert *et al.*, 1999; Chaorattanakawee *et al.*, 2003). In the first study mentioned above, for the first purification of samples stored for less than four years the heme and proteins on the filter paper were suspected to have inhibited the PCR resulting in poor sensitivity of the assay. In some cases such inhibition was too much to the extent that it led to false negatives. These may explain partly the results obtained in this study where very low DNA levels were detected in the *Leishmania* PCR assay. However, collecting blood spots on filter paper is a convenient way to keep parasite DNA for epidemiologic investigations (Farnert *et al.*, 1999).

The Silica method was very sensitive, stable and reliable in the preservation of both *Trypanosoma* and *Leishmania* DNA and RNA, and also the Plasmodium RNA for the entire study duration. The mode of preservation where the silica provides an ideal binding surface for both DNA and RNA once lysis of cells and inactivation of RNAses has been achieved (by the L6 buffer) in the blood enabled efficient preservation of both RNA and DNA when stored at -20°C. This method of preservation is robust and convenient because the final recovery of the nucleic acid

from the Boom purification process guarantees a high yield of both the RNA and DNA from the preserved sample. A similar silica protocol was used in a field study conducted in Western Kenya by Mens *et al* (unpublished data).

Results obtained from this study showed that both malaria parasite mRNA and rRNA remained stable for at least two months on dry silica at -20 °C. The qualities of both mRNA and rRNA in this study were confirmed by way of quantitative assays that proved the effectiveness of preservation. Further claims indicate that samples preserved by this method could remain stable at -20 °C for up to six months or even longer(unpublished data). Advantages that can be attributed to this method are; the reagents that are required in silica preservation can be made locally in any laboratory once the constituent chemicals are available and molecular assays conducted on samples preserved under silica method remain highly sensitive. However, attaining preservation at a temperature of -20°C may not be easy as one must have a refrigerator and in some cases lack of power remains a problem. The fact that a refrigerator and a centrifuge are required to carry out this method, renders it expensive and inappropriate for the field as these equipment may not be guaranteed. The Silica method has many steps and also requires other additional reagents like the L6 buffer making it very cumbersome to use in the field particularly when dealing with many samples.

On the other hand, the Qiagen method which involves the storage of 200 µl of blood in an equal volume AS-1 buffer at 26°C was a reliable and efficient method but only for DNA storage. Findings from this study show that this method preserved both the *Leishmania* and *Trypanosoma* DNA efficiently for the entire storage duration. In a

study conducted by Deboggraeve *et al.* (2006). *Trypanosoma* DNA was preserved successfully in the AS-1 buffer (Qiagen protocol) at ambient temperature until the time of extraction. It was observed in the same study that *Trypanosoma* DNA could remain stable for up to three months in the dark without loss of DNA quality (Deborggraeve *et al*, 2006). In another study blood samples initially confirmed ether positive or negative by serological and parasitological tests for trypanosomes and *P. falciparum* were stored for one month in the dark at -20°C without deterioration of the DNA. The yield of DNA obtained later from these samples was of good quality and quantity (Radwanska *et al.*, 2002). An advantage of this method is that sample preservation can be attained in the field and later transported to the main lab for later purification by the QIAmp blood mini kit. The AS-1 buffer itself is easy to handle in the field because of its stability at ambient temperature (26°C). This makes it less expensive because it requires minimal facility and equipment. The specially designed spin columns that are used during the purification process reduce the possibilities of contamination of samples during the continuous washing and elution steps. The main disadvantage of this method as compared to the other methods under study was its inability to be used for RNA preservation. All the other methods under study were able to preserve both DNA and RNA concurrently in the same sample without the need for a separate reagent. This method therefore is only ideal for the preservation of DNA alone when RNA is not required from the specimen.

In this study blood was the specimen of choice since it is the most accessible human tissue from which appreciable amounts of RNA and DNA can be recovered. It is also the most expendable, since a substantial amount can be collected with no ill effects and it is rapidly regenerated. Blood also has a unique ability to report events that are

widely disseminated, since the design of the circulatory system ensures that blood cells are in intimate contact with tissues in essentially all parts of the body. This means that circulating blood has the potential to act as sentinel for cells for the surveillance of distant tissues affected by infection, cancer, inflammation, and genetic and metabolic diseases (Whitney et al., 2003). On the other hand due to its complex nature, it is believed that it is much more difficult to preserve RNA and DNA in blood than other tissues mainly due to its biochemical nature and its composition. Blood may be having too many enzymes and substances that are able to easily breakdown both DNA and RNA. Therefore results obtained in this study may be easily adapted to be utilized in collection of other bio-clinical material from other tissues and organs of the body for parasitic molecular studies.

4.2 Conclusions

In general, this study is anticipated to make a major contribution to molecular work by the information it has generated on the various storage and preservation methods. It is easy for one to pick out whichever method that suits their scientific needs as most of the methods above would work perfectly and would be ideal. The determining factor for which method one chooses will mainly be based on the capacity or availability of facility and affordability of an interested individual or institution.

The main conclusion from the findings in this study is that L3 buffer is the most ideal method for transport and storage (both short-term and long-term preservation) of nucleic acid material from field areas to main laboratories prior to DNA and RNA analysis.

For long term preservation of nucleic acid, a protocol based on the L3 buffer at 4 °C is the specific protocol of choice because it is efficient enough in the preservation of both DNA/RNA from *Trypanosoma*, *Leishmania* and RNA from *Plasmodium* parasites. It was reliable, stable and can be handled easily in the field where a cool box can be used to attain the 4°C. Specimens stored in L3 buffer at 4°C will have both DNA and RNA well preserved within the same sample. A protocol based on this method can be recommended for storage for up to two and a half months based on the findings for the duration in which the study was conducted.

For transportation and/or short term preservation of nucleic acid material, a protocol based on L3 buffer at 26 °C will be the method of choice. At this temperature, this buffer will be able to store *Trypanosoma, Leishmania* and *Plasmodium* RNA for an optimal one week and not longer than two weeks. The method at this temperature is equally ideal for long term preservation of DNA.

From the findings of this study, standard protocols based on the L3 buffer are now being developed (see appendix for initial draft). The final Standard Operating Procedures (SOPs) that will be generated will be used among the Tryleidiag (Trypanosomiasis and Leishmaniasis Diagnosis) research partners for sample collection from the field. The samples collected by the partners will be used in one of the major phases of a multi-centre study whose main aim is to develop a simplified and rapid molecular assay for diagnosis and (sub-) species identification of VL and HAT. These partnership (Tryleidiag) comprises of four African countries Kenya, Uganda Sudan and Congo and four European partners Belgium, Netherlands, France and Denmark who are mainly involved in the *Trypanosoma* and *Leishmania* research.

4.3 Recommendations

- Further studies should be carried out to evaluate the viability of combining different storage protocols. There is need to try out the possible improvement on some of the better performing methods. An example of the methods that can be combined is the silica method with the L3 buffer method at 26°C. If this is achieved, there is a potential of producing a very reliable and convenient storage method at an ambient temperature. This may however not make the methods cheaper but will be more focused on improving the efficiency in performance of either of the two methods. Another foreseen combination is the possibility of impregnation of the filter paper with the L3 buffer at -20°C in an effort to circumvent the clotting problem that was witnessed in the L3 buffer at -20°C and at the same time improve the weaknesses perceived to be in the Filter paper method.

- Further studies need to be conducted on the L3 buffer at -20°C to establish the causes of clotting that was seen in some of the samples. These studies should be conducted with a focus on different concentrations of the main constituents/reagents that make up the L3 buffer. Focus should also be placed on different sample to buffer volume ratios to establish the best combinations that will not cause clotting.

- Studies for evaluating the filter paper method of storage /preservation of *Trypanosoma* and *Leishmania* parasites needs to be conducted. The filter paper has been used successfully to preserve both DNA and RNA of viruses, bacteria and also other parasites like *Plasmodium* in many studies conducted

by various scientists. Different sample volumes and different concentrations of parasites should be used in these studies mainly focusing on *Trypanosoma* and *Leishmania* parasites. The filter papers in these studies should also be subjected to different storage temperatures.

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http://www CDC. gov / malaria (April 23, 2004)

APPENDICES

APPENDIX 1: Important Lab Procedures

1.1 Counting of parasites

Method: Counting Chamber Burker- (method will be performed as per the manufacturers' instructions).

-Parasite cultures were diluted with of 1% formalin PBS and mixed.

- A cover slide was then fixed on a clean counting chamber

-10 µl of diluted culture was pipetted between the cover slip and the counting chamber, mounted onto the microscope and left for 5 min. This allowed the fluid to flow evenly in all the cells and also allow the parasites to settle.

- 25 squares of the counting chamber were examined using the 10x objective and parasites in those squares counted. The parasites lying on the left and top borders of the squares were not counted.

Since the depth of chamber = 0.1 mm,

One square = 0.004 cubic mm,

25 squares = 0.1 cubic mm,

1 ml is 1000 x 0.1 cubic mm = 10,000 cubic mm,

Therefore number of parasites counted in 25 squares was multiplied by 10,000.

Hence the number of parasites per ml was obtained from the formula below.

Parasite count in 25 squares x dilution x 10,000 = Parasites per ml of fluid.

After obtaining the number of parasites per ml, the parasite/PBS mixture was either diluted or concentrated to make a stock parasite concentration of 10^6 parasites/ml. The stock parasite dilution was used in the subsequent seeding of samples.

1.2 Reagents preparation

Silica preparation: the improved sedimentation method

- 60 grams of SiO_2 was added to 500 ml of double distilled water and left to stand for 24 hours at room temperature.
- 430 ml of the supernatant was aliquoted and disposed off.
- Double distilled water was added to the deposit to make a total volume of 500 ml then mixed and left to stand for 5 hours.
- 440 ml of the supernatant was aliquoted and disposed.

600 µl HCL (32% w/v) was added to the silica suspension ,mixed, aliquoted in fresh eppendorf tubes and sterilized in an autoclave for 15 minutes. The silica was then ready for use.

L_6 - Lysis buffer preparation

- 120 grams of GuSCN was put into a 300ml beaker and 100ml 0.1M Tris HCL, pH 6.4 added.
- The solution was heated to 65° C and 22ml of 0.2 M EDTA (pH 8.0 with NOH) added.
- 2.6 grams Triton X-100 (Packard Instruments) was added and the solution kept in the dark.

L_2 - Wash buffer preparation

- 120 grams of GuSCN was put in a beaker and 100ml of 0.1 M Tris HCL (p.H 6.4) added and mixed. The solution was then stored at room temperature for use.

Other reagents were obtained ready for use these were,

- Acetone
- 70% ethanol

1.3 Procedure for DNA and RNA extraction.

The following DNA and RNA extraction procedures were used on the samples for purification of DNA and RNA at each time point of analysis (Day 0 - week 8)

a) **Boom extraction method**; (for protocol 1-6 samples). (Boom *et al,* 1990)

Principle

The method was based on the lysis and inactivating capabilities of guanidine thiocyanate and the binding features of silica particles. The DNA/RNA binds to the silica so that inhibiting substances can be washed out. After the washing the DNA/RNA is eluted from the silica and stored in pure water or buffer.

Procedure

- 30 µl of silica was added to the sample from each storage protocol and vortexed till the silica is well mixed in the sample.
- This mixture was then shaken in a gyratory shaker for 10 min at 150 r.p.m.
- The mixture was centrifuged for 5 sec at 1200 r.p.m. in an eppendorf centrifuge. (The same speed and timing applied for the centrifuge for each washing procedure).
- The supernatant from each vial was removed by suction using a new tip for each vial and leaving the silica deposits at the bottom of the vials.
- The deposit was washed twice with 1ml L_2 buffer and each time the buffer was removed by suction leaving the deposits.
- The deposit was again washed two times with 1ml 70% ethanol and the deposit left at the bottom of the vial.
- This was then washed once with 1ml of acetone.

- The silica deposit was dried in heat block with the vials open for 10 min at 56°C.
- 50µl of nuclease free water (double distilled water) was added to the deposits, vortexed then left to incubate for 2 min 56°C.
- The vials were then centrifuged for 1 minute at 1400 r.p.m.
- 35 µl of the supernatant was then collected carefully leaving the silica particles in the vial and transferred to a fresh vial.
- The supernatant containing the extracted DAN and RNA was analyzed or stored stable at -70°C when not ready to be analyzed immediately.

b) Qiagen extraction method; (for protocol 7 samples only) (QIamp, 2003)

Principle

Nucleic acids bind specifically to the QIAamp silica-gel membrane while contaminants pass through. PCR inhibitors such as divalent cations and proteins are completely removed in two efficient wash steps, leaving pure nucleic acid to be eluted in either water or a buffer provided with the kit. QIAamp technology yields genomic, mitochondrial, bacterial DNA and parasite DNA in our case.

Procedure

- 20 µl of Proteinase K solution was added to samples initially stored in AS-1 buffer.
- 40 µl of AS -2 buffer was added, vortexed, for 15 seconds then incubated for 10 min at 70 °C.
- 210 µl of absolute ethanol was added and the mixture vortexed for 15 sec.
- The mixture was then applied to the QIamp Spin columns, the caps closed and centrifuged at 8,000 r.p.m for 1 minute.

- The spin columns were placed in new collections tube and the previous collection tubes with the filtrate discarded.
- 500 µl AW-1 buffer was added to the spin column, the cap closed and centrifuged at 8,000 r.p.m for 1 minute.
- The spin columns were placed in new collection tubes again and the previous collection tubes with the filtrate discarded.
- 500 µl AW- 2 buffer was added to the spin column, the cap closed and centrifuged at 14,000 r.p.m. for 1 minute.
- The spin columns were placed in 1.5 ml eppendorf tubes and the previous collection tubes with the filtrate discarded.
- 50 µl of AE buffer was added to the spin column and incubated at room temperature for 5 min.
- The spin column was then centrifuged at 14,000 r.p.m for 1 min, the deposits suspended in the collection column was discarded while the filtrate collected in the eppendorf tubes (containing the DNA material) was stored at -70 °C till the time of processing.

APPENDIX 2: Draft SOP (Clinical sample preservation and nucleic acid extraction)

TITLE Method	Clinical sample preservation and nucleic acid extraction. L3 buffer/silica followed by Boom protocol.
Protocol no.	T-K- 01 (Tryleidiag Kit 01)
Author	Frank L. Basiye
Approved by	Gerard J. Schoone
Authorized by	Dr. Henk D.F.H. Schallig
Institution	KIT Biomedical Research
Target group	All the Tryleidiag study field groups
Authorization Date	21/11/2006

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AIMS

A) **Sample preservation in L3 buffer/silica**

B) **Extraction and purification of DNA and RNA samples in L3 buffer/silica using the Boom method**

List of abbreviations and acronyms

EDTA	Ethelenediaminetetraacetic Acid
SiO_2	Silicon dioxide
CSF	Cerebral spinal fluid
GuSCN	Guanidine thiocyanate
HCl	Hydrochloric Acid
Tris buffer	Trisamine (Hydroxymethylaminoethane) buffer
Tris/HCl	Trisamine (Hydroxymethylaminoethane) buffer and HCl
r.p.m.	Revolutions per minute
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
TE buffer	Tris/HCl /EDTA buffer

1 Purpose

DNA and especially RNA are easily degraded if not preserved well. Storage and preservation of blood and other clinical samples in L3 buffer followed by silica prevents degradation. The DNA and RNA is then purified by extraction using the Boom method.

2 Application

Preservation and storage of clinical material followed by extraction and purification of DNA and RNA from the following samples types:

- -Blood (EDTA)
- -Bone marrow
- -CSF
- -Lymph nodes aspirates
- -Spleenic aspirates

3 Equipment and apparatus

The following equipment and apparatus are required for this protocol.

Vortex mixer	(optional)
Micro centrifuge	(speed up to 14,000 g)
Vacuum pump	
Heat block or water bath	(up to 56°C)
Gyratory shaker	(optional)
Eppendorf tubes	1.5 ml
Pipettes:	1-20 µl, 20-200 µl and 200-1000µl
Pipette tips (plugged)	1-20 µl, 20-200 µl and 200-1000µl
Pipette tips (non-plugged)	0-200 µl,
Sterile gloves	
Lab coats	

4 Reagents

Name	Composition of reagents			
L6 Lysis buffer	GuSCN	Tris	EDTA	Triton X-100
L2 wash buffer	GuSCN	Tris/HCL		
Ethanol 70%				
Acetone				
Silica				
Water (RNAse free)				
L3 buffer	(still a trade secret)			

N/B

All the above reagents should be stored in a dark place at ambient temperature.

PART A	Sample preservation in L3 buffer/silica

1 **Principal:**

When blood and other samples are mixed with L3 buffer and stored at 4°C, the DNA and RNA in the samples are preserved optimally and remain stable for 2 weeks.

After maximum 2 weeks of storage in L3 buffer (or earlier if possible), silica has to be added to the samples. The RNA and DNA in the sample will bind to silica and the samples can then be stored for at least six months at –20°C.

2 **Critical steps**

- ✓ Work in a room dedicated for extraction work
- ✓ Silica should be mixed or vortexed hard for at least 30 seconds before being used.
- ✓ Do not preserve Bone marrow, CSF, lymph nodes aspirates, spleenic aspirates blood/L3 buffer at -20° C, otherwise clotting will occur and this will render the sample invalid.

3 **Preservation procedure: (L3 buffer/silica)**

3a **Preservation in L3 buffer**

- ➢ Aliquot 200 µl of L3 buffer in eppendorf tubes
- ➢ Add 200µl blood, lymph node aspirate or CSF fluid samples to the 200 µl of L3 buffer. For the bone marrow or spleenic aspirate where only small volumes are obtained, a single aspiration will be sufficient for preservation in 200 µl L3 buffer.
- ➢ Mix well by vortexing
- ➢ Label the samples clearly with the
  - o sample I.D
  - o Date of collection
- ➢ Store the sample for a maximum period of 2 weeks at 4 °C. In the absence of 4°C facility, store the sample at ambient temperature (room temperature) for a maximum of 3 days.

3b **Follow up preservation in silica**

- ➢ Add 1 ml L6 buffer to the sample/L3 (now 400µl) mixture and mix well
- ➢ Vortex the silica for 30 seconds until it resuspends uniformly.
- ➢ Add 40µl silica to the sample and buffer mixture.
- ➢ Mix the sample continuously for 5 minutes by vortexing.
- ➢ Centrifuge 15 sec 12000 g

- Remove the supernatant by suction using a vacuum pump. Use a new tip for each sample. To prevent loss of silica particles, leave the last 10 µl drop in the vial.

- Proceed with the Boom extraction as per protocol (part B) or store the sample at -20°C to a later date for the Boom extraction (part B).

- If you decide to store the samples, label them clearly with the
  - Sample I.D.
  - Date of collection
  - Date of preservation at -20°C.

The sample at this point can remain stable for up to six months at -20°C.

The samples preserved and purified under this protocol will only be used for the intended purposes by the Tryleidiag team.

PART B	DNA and RNA extraction (Boom method)

1 Principal:

The method is based on the lysis and inactivating capabilities of guanidine thiocyanate and the binding features of silica particles. The DNA/RNA binds to the silica so that inhibiting substances can be washed out. After the washing the DNA/RNA is eluted from the silica and stored in pure water or TE-buffer.

2 Critical steps

- ✓ Vortexing and shaking of the samples should be done thoroughly ensuring that each time all the silica deposits resuspend uniformly into the mixture.
- ✓ When removing the supernatant by suction, care must be taken not to suck the silica along as nucleic acid material is attached to the silica pellets.
- ✓ A fresh pipette tip should be used for each vial at all the times; tips are a major source of contamination when dealing with many samples.
- ✓ The water in use must be very pure RNAse free water, as any contaminated water will affect eventual outcome of the results during analysis.
- ✓ Traces of silica will remain in the RNA/DNA purified samples and may have an inhibitory effect on amplification assays. Therefore prior to amplification, the sample has to be centrifuged for 1 minute at 12000g in order to precipitate the traces of silica.

3 Extraction Procedure: (Boom extraction)

3.1 Add 1 ml L2 wash buffer to the silica deposit (obtained in part A) and mix (vortex) until a uniform suspension is observed

3.2 Centrifuge for 15 sec. at 12,000 g and discard the supernatant by vacuum pump suction.

3.3 Repeat step 3.1 and 3.2

3.4 Add 1 ml of 70% Ethanol to the silica deposit and mix (vortex) till a uniform suspension is observed.

3.5 Centrifuge 15 sec. at 12,000 g and discard the supernatant using a vacuum pump.

3.6 Repeat step 3. 4 and 3.5

3.7 Add 1 ml of Acetone to the silica deposit and mix (vortex) till you observe a uniform suspension.

3.8 Centrifuge 15 sec. at 12,000 g and discard the acetone using a vacuum pump.

3.9 Dry the open vials with silica deposits in a heat block for 10 min at 56°C

3.10 Add 50 µl RNAse free water or TE buffer to the silica pellets.

3.11 Vortex till the silica pellets are well suspended in the water.

3.12 Incubate the vials in a heat block for 10 min at 56°C.

3.13 Centrifuge 2 min at 12000 g.

3.14 Label new vials corresponding to each sample vial that has been processed in the extraction.

3.15 Transfer 30 µl of the supernatant containing DNA and RNA carefully into each corresponding new vial and discard the silica.

3.16 Label the samples very clearly with the following information.

- -Sample I.D
- -Date of collection
- -Date of extraction and storage at -70°C.

3.17 The samples containing purified RNA and DNA can be analysed immediately or be stored at –20 °C for few weeks to few months–70 °C for months to few years.

N/B

A proper log with the information on the sample (I.D, Date of collection, Date of extraction and location in storage) should be kept in a hard cover book and a copy of the excel sheet in a computer. See the example below.

Sample I.D	Sample Collection Date	Silica Storage Date	RNA/DNA Extraction Date	Storage Location	Comment
K01	23-12-06	1-1-07	5-1-07	-70 °C, shelf A, Raw 3	Sample o.k
K02	23-12-06	Not done	5-1-07	-70 °C, shelf A ,Raw 3	Sample Inadequate

4) **Reference Literature for the Boom extraction method:**

1. Boom et al, 1990, J. Clin. Microbiol. 28: 495-503
2. Boom et al, 1991, J. Clin. Microbiol. 29: 1804-1811

Reagents and Buffers preparation

Note: Use ultra pure (Milli Q) or RNAse free water for the preparation of all solutions

1. **Tris/HCL 1 M solution PH 6.4**
   Dissolve 121 gram TRIS *(Boehringer cat.nr. 708976)* in 800 ml MilliQ water.
   Add 81 ml HCl (36%)*(Merck p.a.)* Check and confirm the pH to be 6.4
   Add MilliQ water up to 1Lt.

   -Dilute Tris/HCl 1:10 for use in preparation of L2 wash buffer.

2. **EDTA 0.2 M pH 8.0**
   Dissolve 37.2 gram EDTA *(Titriplex, Merck, cat.nr. 8418)* and 4.4 gram NaOH (Merck cat.nr. 6498) in a total volume of 500 ml Milli Q water.
3. **L2 wash buffer.**
   Add 833 ml Tris/HCl 0.1 M to 1 kg Guanidiniumthiocyanate (GuSCN *Fluka cat.nr. 50990*) in a flask. Dissolve by heating for 2 hours at 55°C.
   After dissolving the total amount of L2 washing solution is 1650 ml

4. **L6 Lysis buffer.**
   Add to 550 ml L2 washing solution: 61 ml 0.2 M EDTA and
   7.4 grams Triton X100

5. **Ethanol 70%**
   Add 30 ml MQ water to 70 ml Ethanol abs. *(Merck p.a.)*

6. **Acetone**
   Buy ready for use Merck p.a.

7. **TE elution buffer (optional)**
   Tris/HCl 10 mM, EDTA 1 mM pH 8.0

8. **Silica Preparation**

Silica: Silicon dioxide, SiO2, Sigma cat nr. S 5631: Particle size 0.5 to 10 microns; 80% between 1-5 microns.

1. Suspend 60 g Silica in a total volume of 500 ml Milli Q water in a glass cylinder (Height of the column 27.5 cm; width 5 cm)
2. Leave on the table for 24 hours.
3. Dispose supernatant by suction until 60 ml silica solution is left.
4. Add again 500 ml Milli Q water, mix and leave on the table for 5 hours.
5. Dispose supernatant by suction until 60 ml silica solution is left.
6. Add 600 µl HCl (36%).
7. Resuspend by gently shaking.
8. Aliquot 1.2 ml quantities in screw cap tubes (2 ml)
9. Loosen the caps of the tubes and autoclave (20 min. 121C)
10. The resulting silica suspension is usually referred to as Silica SC (Silica Coarse).

N/B

For each reagent and buffer prepared, label clearly with the following information.

- Name of the reagent or buffer.
- Preparation Date.
- Expiry Date.
- Storage temperature.
- This information will vary per reagent.
-

Biosafety and Biohazard information of the reagents and chemicals

General Biosafety and Biohazard statements

- Consider all clinical material potentially infectious as per the biosafety protocols.
- Always wear a clean lab coat and fresh pair of gloves when processing samples
- Consider all chemicals and reagents to be a potential hazard as per the Materials Safety Data Sheets (MSDS).

1) L6 buffer and L2 buffer

L6 buffer contains all the chemicals below (i - v) while L2 buffer contains GuSCN and Tris HCl

i) GuSCN

Acute overexposure may cause irritation. Avoid contact with skin, eyes and clothing. In case of exposure, wash the affected area with soap and water. Remove contaminated clothing. In case of contact with eyes, irrigate the eyes for at least 15 minutes. If ingested, induce vomiting. Wash spill area with copious amounts of water.

ii) HCL

Though the acid used in the preparation of L6 buffer is diluted the following information is for concentrated HCl

HCl is corrosive; swallowing can cause immediate pain and burns of the mouth, throat, esophagus and gastrointestinal tract. It may cause nausea, vomiting, and diarrhea and may be fatal. It can cause redness, pain, and severe skin burns and concentrated solutions cause deep ulcers and discolor skin. Vapors are irritating and may cause damage to the eyes while contact with the eyes may cause severe burns and permanent eye damage. Give large quantities of water or milk if available in case of swallowing and do not induce vomiting when dealing with concentrated acid.

iii) TritonX-100.

Harmful if swallowed, inhaled or in contact with skin, can cause severe eye irritation. Toxicology not fully investigated. The product may contain traces of ethylene oxide or dioxane, which are probable human carcinogens. Wash thoroughly after handling or upon exposure.

iv)Tris buffer

Slightly hazardous, can be an irritant or permeator to the skin, irritant to the eye or when ingested. In case of exposure, rinse affected areas with plenty of water.

v) EDTA -Eye irritant in case of contact, wash off with plenty of water.

2) Ethanol

Ethanol is highly flammable

Ethanol causes skin irritation; repeated contact may cause defatting of the skin and dermatitis. It can also cause eye irritation by direct contact or vapors. Ingestion can cause nausea, vomiting and inebriation; chronic use can cause serious liver damage. Note that "absolute" alcohol, which is close to 100% ethanol, may nevertheless contain traces of 2-propanol, together with methanol or benzene. The latter two are very toxic, while "denatured" alcohol has substances added to it which make it unpleasant and possibly hazardous to consume. Inhalation of high concentrations may cause central nervous system effects characterized by nausea, headache, dizziness, unconsciousness and coma.

Upon exposure, immediately flash the skin or eyes with plenty of water for at least 15 minutes, occasionally lifting the upper and lower eyelids. If swallowed in excess, induce vomiting by giving one teaspoon of Syrup of Ipecac.

3) Acetone (also known as Propanone).

Acetone is highly flammable, and presents a serious fire risk.

Contact with the eyes can cause serious permanent damage. Acetone is harmful if it is swallowed or inhaled. Long-term exposure, for example through breathing in the fumes, can cause liver damage. Repeated skin exposure may lead to defatting and irritation. Upon exposure, immediately flush the skin or eyes with plenty of water, occasionally lifting the upper and lower eyelids. Continue for at least ten minutes and call for medical help. Remove any contaminated clothing (noting that clothing soaked in acetone may present a serious fire risk).If swallowed wash out the mouth with water and call for medical help.

4) Silica (Silicon dioxide)

Silica can cause slight irritation of the skin and eyes when in contact. If inhaled, it may be toxic to the lungs and the upper respiratory tract. Repeated or prolonged exposure to the substance can produce target organs damage. In case of exposure or contact, immediately flush eyes with plenty of water for at least 15 minutes. Use soap and water for the skin, and cover the irritated skin with an emollient. Get medical attention if irritation persists.

Waste Disposal:

The aspirated extraction and washing buffers should be placed in a brown glass bottle with a minimum of 1/20th volume 10N NaOH. This bottle should be kept under the fume hood and should be disposed of as hazardous waste.

N/B
For more and elaborate information on all chemicals refer to the Materials Data Safety Sheets information available from the internet or laboratory manuals.