DETECTION OF LEISHMANIA ANTIGEN FROM BUCCAL SWABS IN KALA-AZAR PATIENTS USING KATEX METHOD

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature……Date……...

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This thesis has been submitted for examination with our approval as University supervisors.

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DEDICATION

This thesis is dedicated to my family; my dear wife Christine Mutindi, our beloved two children Diana Mueni Musau and Brian Muia Musau who stood by me all through my entire course, my parents, siblings and friends for their moral support and encouragement.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

ABLC	Abelcet
AIDS	Acquired Immunodeficiency Syndrome
CCR	Centre for clinical research.
CIC	Circulating immune complexes
CI	Confidence interval
CL	Cutaneous Leishmaniasis
DALYs	Disability adjusted life years
DAT	Direct agglutination test
DLR	Diagnostic Likelihood Ratio
DNA	Deoxyribonucleic acid
DND*i*	Drugs for neglected disease *initiative*
ELISA	Enzyme linked immunosorbent assay
ESM	Excretory, Secretary and Metabolic
FAST	Fast agglutination screening test
FPC	Finite population correction
H & E	Haematoxylin & Eosin
HIV	Human immunodeficiency virus
HMS	Hypereactive malarial splenomegaly
ICT	Immunochromatographic test
IFAT	Immunofluorescence Antibody Test
FRET	Fluorescence resonance energy transfer
IHA	Indirect Hemagglutination Antibody
ITS	Internal transcribed spacer
IQR	Interquartile range
IRG	Internal research grant
IV	Intra-venous
KATEX	Kala-azar latex agglutination based test
k-D	kilo-Daltons

KEMRI	Kenya Medical research Institute
LAMP	Loop-mediated isothermal amplification
LD	Leishmania donovani
Ld-rGBP	*L. donovani* **-** recombinant gene B protein
NASBA-OC	Nucleic acid sequence based assay-oligochromatography
NPV	Negative predictive value
PM	Paromomycin
PCR	Polymerase chain reaction
PKDL	Post-kala-azar dermal leishmaniasis
PPV	Positive predictive value
PTI	Prothrombin time index
QT-NASBA	Quantitative nucleic acid sequence-based amplification
RDTs	Rapid Diagnostic Tests
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rGBP	recombinant gene B protein
rK39	recombinant 39amino acid antigen
SA	Soluble antigen
SD	*Standard deviation*
SSG	*Sodium stibogluconate*
SSU rRNA	*Small Sub-unit ribosomal ribonucleic acid*
SOP	*Standard operating procedure*
STATA	Statistics and data
TDR	Research & Training in Tropical Diseases
TSS	Tropical splenomegaly syndrome
UV	Ultra-violet
UK	United Kingdom
µL	Microliter's
VL	Visceral leishmaniasis
WHO	World health organization

ABSTRACT

Visceral leishmaniasis (VL) or kala-azar is a chronic protozoan infection in humans that is fatal unless treated and is associated with significant global morbidity and mortality. Confirmation of visceral leishmaniasis (VL) diagnosis requires microscopic examination to visualize the causative agent *Leishmania Donovani* in spleen or bone marrow aspirate. Kala-azar latex agglutination based test (KATEX) antigen test for VL in buccal swabs has not been evaluated locally and could offer a significant advantage in screening patients suspected of VL. The study was conducted after approval from KEMRI Scientific Ethical Review Unit (SERU). Buccal swabs were obtained from VL cases and controls patients presenting at Kimalel Health Centre, Baringo County, Kenya. Latex agglutination based test (KATEX) was used to detect parasite antigen in buccal swabs. The study evaluated the ability to use KATEX, a simple non-invasive diagnostic test to detect leishmania antigens in buccal swabs, determine the ability of the kit to detect leishmania antigens in buccal cells of kala-azar patients and compare the sensitivity and specificity of KATEX - buccal assay using microscopy as the gold standard. Eighty eight samples were analyzed using statistics and data (STATA) software version 13.2 standard edition, including 44 VL and 44 non-VL patients. KATEX kit was able to detect visceral leishmaniasis antigens from buccal swabs giving a sensitivity of (81.8%; 95%CI: 67.3% to 91.8% and specificity of (79.5%; 95%CI: 64.7 –90.2%). Buccal swab test assay using KATEX is an easy test to perform and promising non-invasive based antigen detection test which may be useful for screening kala-azar patients and could be applied in the diagnosis of VL. It is a functional assay that warrants a larger study with a larger sample size for the purpose of evaluating the utility of the test in diagnosing visceral leishmaniasis.

CHAPTER ONE

INTRODUCTION

1.1 Background

Visceral leishmaniasis (VL) or kala-azar is a chronic protozoan infection in humans associated with significant global morbidity and mortality. The etiological agent is a haemoflagellate protozoan *Leishmania donovani*, an obligate intracellular parasite that resides and multiplies within macrophages of the reticulo-endothelial system. Kinetoplastid flagellates of the genus Leishmania are causative agents of leishmaniasis; a group of protozoan diseases transmitted to mammals, including human beings, by phlebotomus sandflies. At least 21species and subspecies of Leishmania have been recorded as being infective to humans, many of which cause extensive morbidity and, in some cases, mortality (Chance *et al.*, 1985). The same vector is responsible for the spread of the disease but the species vary; Phlebotomus spp (in Europe, North Africa, Eastern Africa, the Middle East, and Asia) or Lutzomyia spp (from Latin America to northern Argentina).

The global burden of leishmaniasis caused 2.4 million disability adjusted life years (DALYs) loss and 59 000 deaths in 2001 (Clive *et al*., 2003). It has been estimated that there are likely, over 500,000 cases of VL per year. Over 90% of cases are found in three regions, Sudan /Ethiopia/ Kenya/Uganda, India /Bangladesh/ Nepal, and Brazil with as many as 100,000 deaths every year. Leishmaniasis remains a public health problem in about 30 sub-counties in Kenya. The disease occurs in two forms: visceral leishmaniasis (kala-azar) and cutaneous leishmaniasis (Oriental sore). There is inadequate information on the prevalence, burden and spatial distribution of the disease, which is distributed mainly in arid and semi-arid regions. Visceral leishmaniasis is endemic in the Rift Valley and Eastern regions, with small foci in North Eastern. In the Eastern Region, foci have been documented in Isiolo, Kitui, Machakos, Makueni, Marsabit, Mwingi and Tharaka counties. In Rift Valley, the disease is more common and is found in Baringo, Pokot, Turkana, Samburu, Kajiado and Laikipia sub-counties (Mebrahtu *et al*., 1987; Mebratu *et al*., 1988). The exact status of the problem in North Eastern region and the northern parts of the country is not well understood due to

inaccessibility and problems associated with diagnosis. However, the disease has been reported sporadically in Mandera and Wajir counties in North Eastern region. (National guidelines for health workers prevention, diagnosis and treatment of visceral leishmaniasis (kala-azar) in Kenya 2017).

In Kenya, Visceral leishmaniasis (VL) is endemic in Kitui, Machakos, West Pokot, Meru, Turkana and Baringo Counties. In Baringo, the disease has a focal distribution in the dry, hot areas. Both the disease incidence and its severity are linked to poverty; malnutrition was found to be associated with a 8.7 times higher risk for VL (Marleen *et al*., 1999).

The symptoms of VL include prolonged fever, anaemia, splenomegaly, pancytopenia, and weight loss/wasting. These symptoms are often mimicked by malaria , schistosomiasis ,brucellosis , leptospirosis , typhoid fever , tuberculosis ,chronic hepatitis ,liver cirrhosis , lymphomas and leukaemias ,AIDS ,malnutrition and hypereactive malarial splenomegaly (HMS) formerly known as tropical splenomegaly syndrome (TSS), all widely prevalent in the VL-endemic regions. Therefore proper diagnostic methods for VL must be available, accurate, simple and affordable for the population for which they are intended. This represents the "tip of the iceberg," as the majority of cases are asymptomatic and many symptomatic cases may not be recognized if proper diagnosis is not done.

The gold standard for VL diagnosis is laboratory demonstration of parasites by microscopy in smears and/or cultivation in culture parasites (Andresen *et al.*, 1996). The specimen for smears and cultures are obtained by aspiration of the organs known to harbor the parasites e.g. spleen, bone marrow and lymph nodes. The sensitivity of microscopy varies, being higher for spleen (93–99%) than for bone marrow (53–86%) or lymph node (53–65%) aspirates (Babiker *et al.* 2007). However, the aspiration of tissue samples is often painful and requires experienced/well trained clinicians and specialized laboratories with skilled personnel (Kager *et al*., 1983). The use of culture, though sensitive is a method that requires specialized laboratory settings, is laborious and takes two to four weeks before detection .For these reasons, other indirect immunological methods of diagnosis have been developed. They include the antibody based direct agglutination test (DAT), and Immunochromatographic test (ICT).

Although these tests are readily available, they are not the standard diagnostic tests due to their insufficient sensitivity and specificity.

Kala-azar latex agglutination based test (KATEX) kit, an antigen assay based on latex agglutination test using urine has previously been studied. However, this test has not been validated and is laborious. The kit detects a heat-stable low molecular weight carbohydrate antigen in urine.The antigen has been characterized and found to be a low molecular mass (5–20 kD) glycoconjugate (Sarkari *et al.,* 2002).The molecule is unique to *Leishmania* (absent from other Kinetoplastidae, such as *Trypanosoma brucei* or *T. cruzi*), but present in all species of *Leishmania.* Animal experiments and early results suggest that it's a promising diagnostic tool, and its results correlate well with the splenic parasite status. Preliminary studies using urine of infected animals and patients have demonstrated the presence of leishmanial antigen in active VL, and its disappearance after successful treatment (Sundar *et al*., 2005).

1.2 Statement of the Problem

Visceral Leishmaniasis is a fatal disease which needs to be diagnosed early and treated. Diagnosis of VL is demonstration of parasites in the spleen, bone marrow or lymph node, a procedure which is not easily accesible, invasive and poses a great risk such as active bleeding. This calls for accurate, easily accesible and easy to use non-invasive diagnostic test.

1.3 Justification of the study

The gold standard for diagnosis of VL is by microscopic examination of splenic aspirates or bone marrow for amastigotes. However, this procedure is too invasive and requires highly skilled clinician. The processing and reporting of the results also requires skilled laboratory personnel. Non invasive methods of obtaining bio specimens are very useful for diagnostics and clinical trials, as well as epidemiological screening studies. Exfoliated buccal epithelial cells are a promising alternative source of genomic DNA as they have a high yield. Among different non invasive sampling techniques, buccal cells have the advantages of ease of collection and storage (Strauss *et al*., 2004). The presence of *L. donovani* nucleotides in buccal epithelia has been confirmed by restriction digestion and sequencing (Manisha *et al.,* 2011). The molecular diagnosis of

VL by PCR on blood samples and PCR is accurate, but it is invasive and requires phlebotomists, which may not be feasible in large epidemiologic studies. Also, subjects are often reluctant to provide blood samples, which reduces participation rates. Therefore, efforts have been made to develop non invasive, simpler, and sensitive techniques. Thus there is need to simplify diagnostic procedures by use of a non invasive immunological detection of leishmania antigens in easily collectable specimens such as buccal swabs.

Buccal swab collection also eliminates the risks associated with splenic aspiration or venous blood collection for serological diagnosis. This is a proof of concept pilot study aimed at investigating the possibility to detect leishmania antigen in buccal cells of VL patients using KATEX agglutination assay as a simple non-invasive diagnostic test for the diagnosis of *L. donovani.* It is hoped that, its success will warrant for a larger study with a larger sample size for the purpose of evaluating the utility of the test in diagnosing visceral leishmaniasis.

1.4 Research questions

- What is the posibility of detecting leishmania *donovani* antigens in buccal cells using the KATEX assay Kit?
- What is the likelihood of utilizing detection of *leishmania donovani* antigens in buccal cells, for the diagnosis of Visceral Leishmaniasis?

1.5 Objectives

1.5.1 General objective

To evaluate the ability of a simple non-invasive KATEX diagnostic tests to detect leishmania antigens in buccal cells.

1.5.2 Specific objectives

- To determine the ability of KATEX assay kit to detect Leishmania antigens in buccal cells of kala azar patients.

- To compare sensitivity and specificity of KATEX - buccal assay with the gold standard microscopy test.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology of Visceral Leishmaniasis

Visceral leishmaniasis (VL), is a vector-borne disease caused by replication of parasites in macrophages, mononuclear phagocytic system. It is caused by the *Leishmania donovani* complex, which includes *L. donovani* and *L. infantum*. It is endemic in large areas of the tropics, subtropics and the Mediterranean Basin. VL is a systemic disease and is fatal if left untreated. The transmission characteristics of VL differs in different geographical regions; in the Mediterranean Basin, Brazil and parts of Africa, the dog is the main reservoir and visceral leishmaniasis (VL) is zoonotic; while in the Indian subcontinent and parts of Africa, it is anthroponotic (Pankaj *et al.,* 2010).

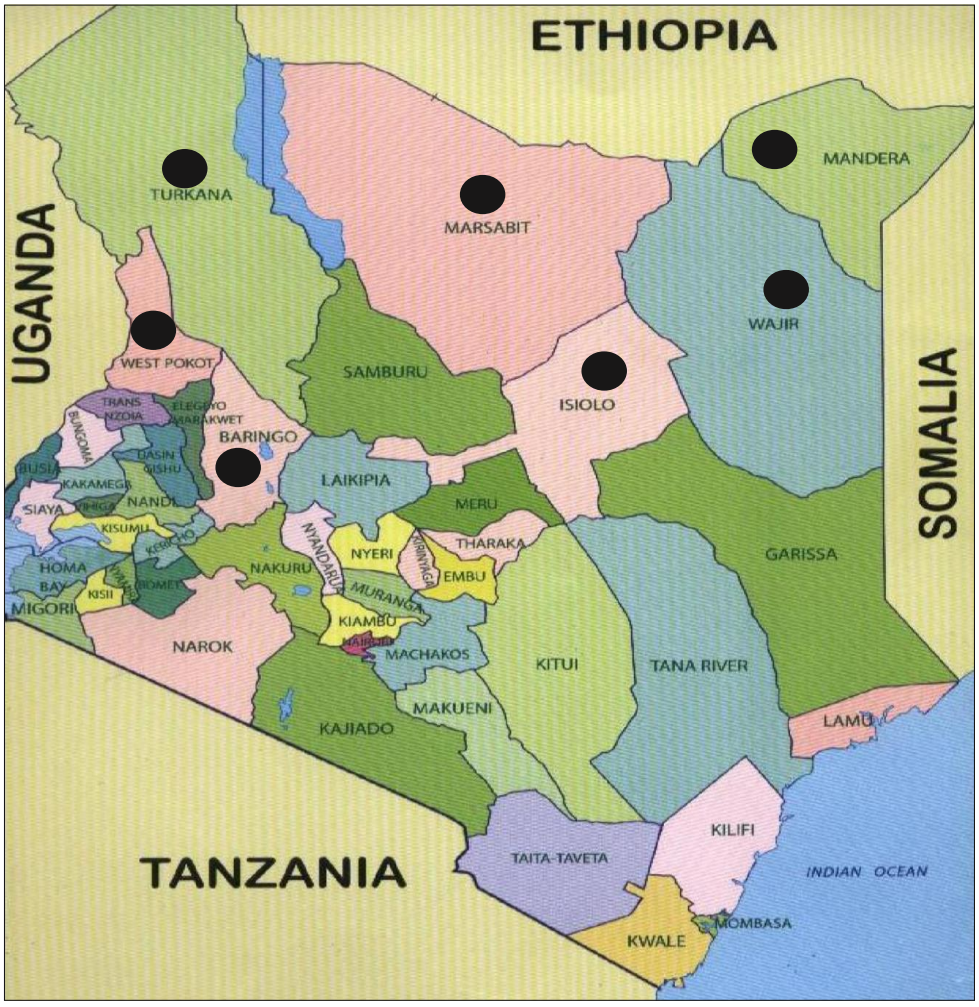
Clinical features of VL can be easily mistaken for other febrile illnesses such as malaria and enteric fever. Reliable laboratory methods become mandatory for accurate diagnosis. Early case detection followed by adequate treatment is central to the control of VL.Patient management, screening of asymptomatic infections, surveillance including verification of elimination, and epidemiological studies are some of the areas where diagnostic tests play a major role. Ideally, a test should make the distinction between acute disease and asymptomatic infection, as most of the antileishmanial drugs are toxic (Pankaj *et al.,* 2010).

2.2 Geographical Distribution of Leishmania Species.

Human leishmaniases are found on all continents, except Antarctic and Australia. Approximately 350 million people live in endemic areas, there by comprising populations at risk, and annual incidence is estimated at 1–1.5 million cases of cutaneous leishmaniasis plus 500,000 cases of visceral leishmaniasis; overall prevalence is 12 million people. Most of the affected countries are in the tropics and subtropics: more than 90% of the world's cases of visceral leishmaniasis are in India, Bangladesh, Nepal, Sudan, and Brazil, 90% of all cases of mucocutaneous leishmaniasis occur in Bolivia, Brazil, and Peru, whereas 90% of all cases of cutaneous leishmaniasis occur in Afghanistan,Brazil,Iran,Peru,SaudiArabia,andSyria(http://www.who.int/leishmaniasis/en/).

Five hundred phlebotomine species are known to cause Leishmaniasis. Only 31 have been positively identified as vectors of the *Leishmania* pathogenic species and 43 as probable vectors (Killick-Kendrick *et al*., 1999). Among them, some vectors such as Phlebotomus Phlebotomus papatasi and P. Paraphlebotomus sergenti can only be infected by one Leishmania species, whereas Lutzomyia longipalpis is a permissive vector, able to transmit different Leishmania species. Twenty species described as pathogenic for humans belong to the Leishmania genus and are divided into two subgenera (Leishmania in the Old World and Viannia in the New World. The Leishmania subgenus is composed of several species or species complexes Leishmania donovani complex, L. mexicana complex, L. major, L. tropica, and the Viannia subgenus contains species of the L. braziliensis complex (L. braziliensis), (L. peruviana), and the L. guyanensis complex (L. guyanensis and L. panamensis) (Hide *et al*., 2009). In Kenya, the causative agent of visceral leishmaniasis is *Leishmania donovani*, which is transmitted by two different sandfly species depending on the region: *Phlebotomus martini* and *P. orientalis*.

Visceral leishmaniasis is endemic in semi-arid and arid areas of Rift Valley, Eastern and North Eastern regions of Kenya. It is estimated that about 2500 (hospitals record estimates) cases occur annually, the majority of whom are children aged >5 years and young adults; some 6.81 million people are at risk of the infection. The most important transmission foci remain Baringo, Isiolo, Marsabit, Pokot, Turkana and Wajir counties (Figure 2.0). The disease foci may change to areas previously not known to be endemic as a result of climate change and population movements. There have been several outbreaks of the disease in Kenya; the most recent was confirmed in Isiolo, Marsabit and Wajir counties in, 2008, 2011, 2013 and 2014.



Counties endemic for visceral leishmaniasis

Figure 2.0: Map of kenya showing distribution of endemic counties (National guidelines for health workers prevention, diagnosis and treatment of visceral leishmaniasis (kala-azar) in Kenya 2017.

2.3 The Life Cycle of the *Leishmania* Parasite

Leishmania parasites are transmitted to their host by the bite of an infected female phlebotomine sand fly (Psychodidae family, Phlebotominae subfamily), which needs a blood meal to produce its eggs (Fig. 2.1). The sand fly vectors are primarily infected when feeding on the blood of an infected individual or a vertebrate reservoir host. Many mammal species could act as a reservoir host, for example, rodents or dogs (Ashford *et al*., 2000). During feeding, host macrophages, containing *amastigotes* are ingested by the vector. These parasite forms, round and nonmotile (3–7 m in diameter), are released

into the posterior abdominal midgut of the insect, where they transform into *promastigotes* to begin their extracellular life cycle in the vector. This form is motile, elongated (10–20m), and flagellated. The promastigotes then migrate to the anterior part of the alimentary tract of the sand fly where they multiply by binary fission.Approximately 7 days after feeding, the promastigotes undergo metacyclogenesis and become infectious (metacyclic promastigotes). They are released into the host together with saliva when the sand fly lacerates the skin with its proboscis during feeding. The sand flies usually feed at night while the host is asleep. These metacyclic promastigotes are taken up by host macrophage, where they metamorphose into the amastigote form. They increase in number by binary fission within the phagolysosome until the cell eventually bursts, then infect other phagocytic cells and continue the cycle. In cases of visceral leishmaniasis, all organs, containing macrophages and phagocytes, can be infected, especially the lymph nodes, spleen, liver, and bone marrow.

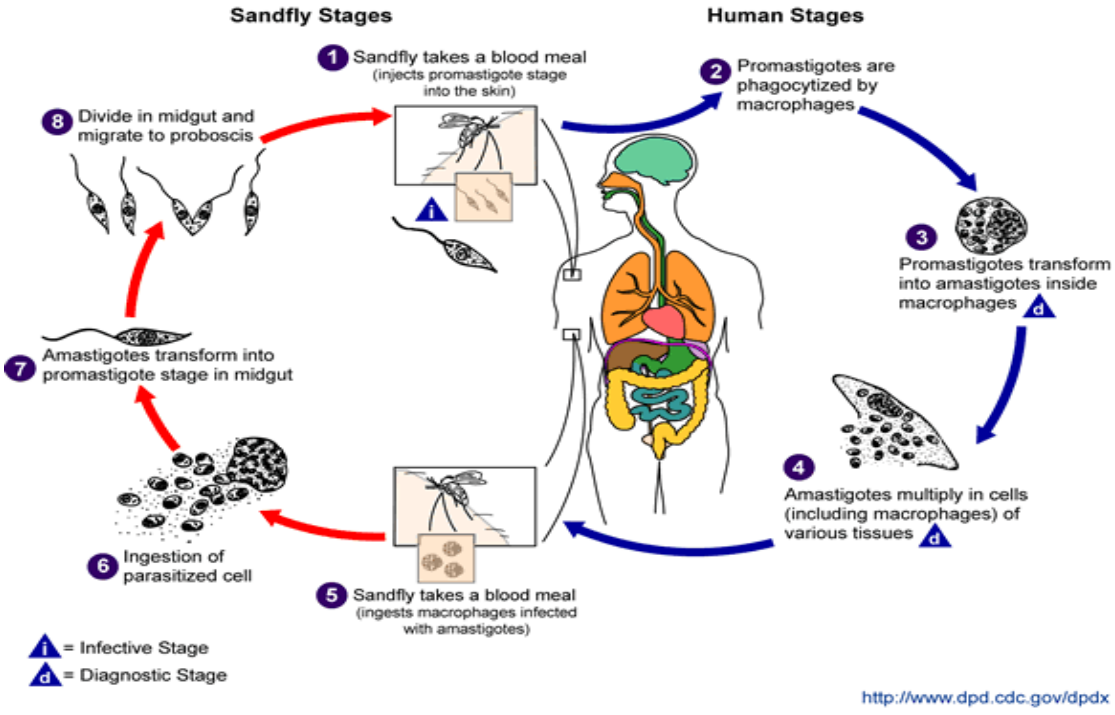


Figure 2.1: Transmission life cycle of Leishmania (http://www.dpd.cdc.gov/dpdx).

2.4 Symptoms and Treatment of Leishmaniasis

A high rate of infected people remain asymptomatic, but for others, the infection by *Leishmania* can produce very different clinical symptoms.People infected with visceral leishmaniasis usually become sick within several weeks or months (rarely as long as years). It is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anemia. *Leishmania* species responsible for this form mainly belong to the *Leishmania donovani* complex.

Lipid-associated amphotericin, i.e., liposomal amphotericin B (AmBisome), amphotericin B lipid complex (ABLC, Abelcet) and amphotericin B colloidal dispersion (Amphocil), has been one of the most important developments in the chemotherapy of leishmaniasis. In these formulations, deoxycholate has been replaced by other lipids that mask amphotericin B from susceptible tissues, thus reducing toxicity, and are preferentially taken up by reticuloendothelial cells, thus targeting drug delivery to the parasite and increasing efficacy. Three lipid formulations are commercially available, but their cost is prohibitive (Davidson *et al*.,1991 & 1994).

In India, all three formulations, with comparable efficacy, have been used, with AmBisome being the safest (Davidson *et al*., 1994). *Paromomycin*, an aminoglycoside, is well tolerated and effective for VL, but less so for CL (Jha *et al.,* 1998). The search for an effective oral antileishmanial drug spans two decades. *Allopurinol*, the azoles, rifampicin, and atovaquone showed activity in experimental systems, but proved disappointing in clinical trials. Oral *miltefosine*, an alkyllysophospholipid, originally developed as an anti-cancer agent, is now approved for the treatment of VL in India (Sundar *et al*., 2002). In several clinical trials, miltefosine cured more than 90% of patients with only minor gastrointestinal side effects such as vomiting in about half of the patients and less commonly diarrhea. An asymptomatic transient rise in hepatic transaminases occurs during the second week of treatment, returning back to baseline values on continued treatment. It induces rapid cure, with a majority of patients becoming afebrile within the first week, quick regression of spleen, and recovery of blood counts.

However, due to the risk of teratogenicity, Miltefosine should not be given to child-bearing age women except if contraception can be secured during and after treatment.

Oral *sitamaquine*, an 8-aminoquinoline derivative, has been shown to have clinically significant antileishmanial activity.

2.4.1 Treatment for visceral leishmaniasis

2.4.1.1 First-line treatment – combination therapy

The Ministry of Health recommends sodium stibogluconate (SSG) and paromomycin (PM) combination therapy as the first-line treatment for primary VL in Kenya unless contraindicated otherwise. Primary visceral leishmaniasis Combination therapy includes sodium stibogluconate (pentavalent antimonials)/SSG at 20 mg/kg per day intramuscularly or intravenously plus paromomycin 15 mg [11 mg base] per kg body weight per day intramuscularly) for 17 days.

Monotherapy treatment Sodium stibogluconate (SSG) as a monotherapy can still be used in health facilities in endemic areas in situations where paromomycin is not available or is contraindicated (hearing impairment or concomitant renal disease). The treatment as monotherapy is SSG 20 mg/kg per day intramuscularly or intravenously for 30 days.

2.4.1.2 Second-line treatment - Liposomal amphotericin B (AmBisome)

Liposomal amphotericin B is an efficacious treatment against visceral leishmaniasis with a much improved safety profile as compared to the amphotericin B deoxycholate formulation. Mild infusion reactions (fever, chills and rigor) and back pain may occur in some patients. Transient nephrotoxicity or thrombocytopenia is also occasionally seen. AmBisome comes in vials of 50 mg and needs to be reconstituted and diluted in 5% dextrose and given over a period of 30–60 minutes as an intravenous infusion. The recommended dose in Kenya is 3–5mg/kg body weight per daily dose by infusion given over 6–10 days up to a total dose of 30 mg/kg.

2.5. Leishmania Diagnosis

2.5.1 Parasitological

This is usually confirmed by direct Microscopy through demonstration of amastigotes in tissue smears mostly from the spleen, bone marrow, or lymph nodes. The use of microscopy in the diagnosis of VL offers the benefits of high specificity and the possibility of grading the parasite on a logarithmic scale (0–6+) in splenic smears. But like all microscopic procedures, it suffers from variability of detection sensitivity and the inevitable need for an expert microscopist.

The amastigotes are readily seen in smears or touch preparations of infected tissue stained with Giemsa's stain, preferably at *p*H 7.2 rather than the *p*H 6.8 normally used in haematology. Sections of tissue stained more conventionally, with H & E, are much more difficult to interpret. To ensure that the visualized structures are amastigotes, rather than other "dot"-like structures (*e.g.*, *Histoplasma* spp¸ platelets), an experienced observer should look for the characteristic size (2-4 mm in diameter), shape (round to oval), and internal organelles, the nucleus and kinetoplast. With Giemsa staining, the cytoplasm typically takes pale blue and the nucleus and kinetoplast take purple-pink colour.

According to Kenyan national guidelines for VL, demonstration of parasite amastigotes by microscopic examination of smears from splenic aspirates is needed to confirm diagnosis and initiate treatment. Splenic aspiration is an invasive procedure that is highly sensitive (93.1–98.7%) but carries a small but significant risk of major bleeding. It should be performed by experienced clinicians in reference hospitals or research centers, and is therefore not suitable for use in first-line health services or district hospitals.

Blood transfusion must be locally available in case of need. In addition, splenic aspiration is not possible in non-cooperative children, difficult in those with mild splenomegaly, and contra-indicated in persons with active bleeding, thrombocytopenia, severe anemia, as well as in pregnant and moribund patients. Identification of amastigotes and grading in stained smears of splenic aspirates requires expertise and well-trained microscopists (Mbui *et al*., 2013).

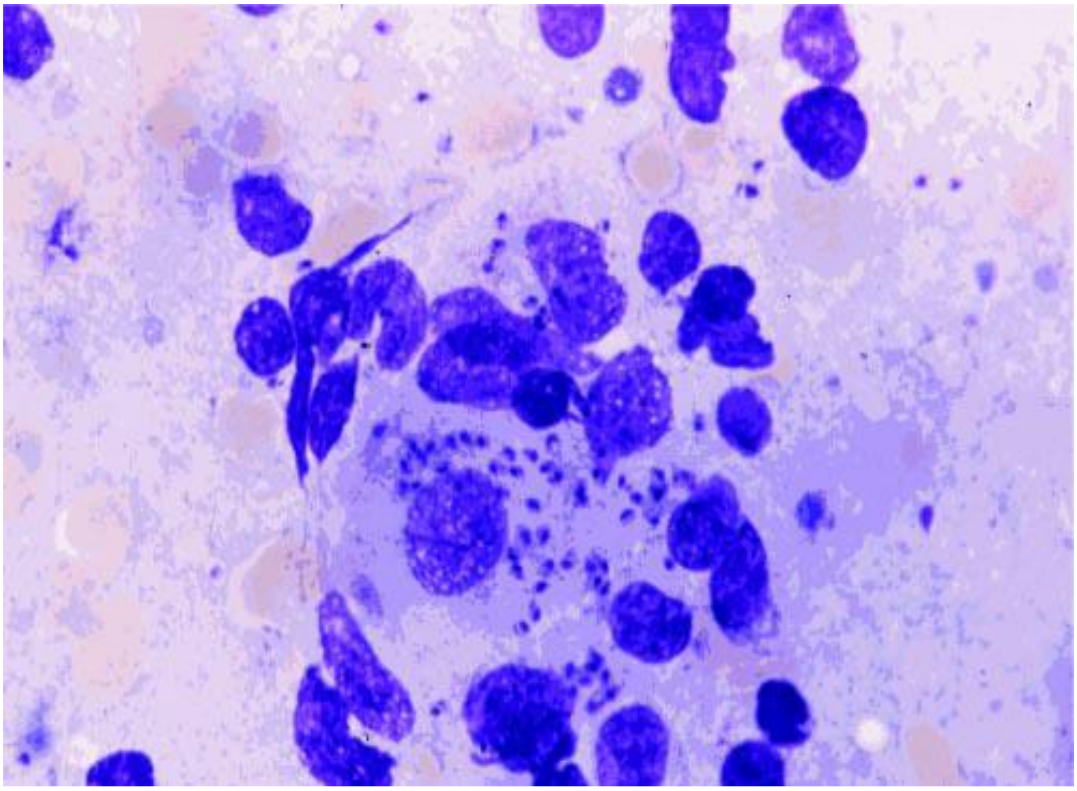


Figure 2.2: Geimsa stained bone marrow aspirate smear from a kala-azar patient, showing numerous extracellular as well as intramacrophage amastigote forms of the *Leishmania donovani*.X1000 (Indian J Med Res 123, March 2006).

2.5.2 Immunological

2.5.2.1 Direct Agglutination Test (DAT)

Direct Agglutination Test (DAT) is a semi-quantitative test that uses microtitre plates in which increasing dilutions of patient's serum or blood are mixed with stained killed promastigotes of *L.* Donovan (El Harith *et al*., 1986; El Harith *et al*., 1987). If specific antibodies are present, agglutination is visible with the naked eye after 18 hours. The test has been extensively validated in most endemic areas. Thirty studies were included in a recent meta-analysis, showing sensitivity and specificity estimates of 94.8% (95%CI: 92.7-96.4) and 97.1% (95%CI: 93.9-98.7), respectively (Chappuis *et al*., 2006b). The performance of DAT was neither influenced by the region nor by the Leishmania species.

Direct Agglutination Test is simpler than many other tests but requires equipment and materials such as micropipettes and microtitre plates as well as well-trained laboratory technicians and regular quality control. The storage of antigen, once dissolved, at 2-8°C and the prolonged incubation time are other drawbacks. The fast agglutination screening test (FAST) is a simplified (single serum dilution at 1:800 or 1:1600 cut-off) and more rapid (2-3 hours) version of the DAT, and its diagnostic accuracy seems comparable (Hailu *et al.* 2006; Schoone *et al*., 2001; Silva *et al*., 2005).

2.5.2.2 rK39-Based Immunochromatographic Test (ICT)

Recombinant Kinetoplastid 39 antigen (rK39) is a 39-amino acid repeat that is part of a kinesin-related protein of *L. chagasi*. The repeat is conserved within the *L. donovani* complex (Burns *et al*., 1993). RK39-based ELISA showed excellent sensitivity (93 - 100%) and specificity (97-98%) in numerous VL endemic countries (Badaro *et al*., 1996, Braz *et al*. 2002, Burns Jr. *et al*. , 1993, Kurkjian *et al*., 2005, Maalej *et al.,* 2003, Ozensoy *et al*., 1998, Qu *et al*. , 1994, Singh *et al.* 1995;Zijlstra *et al.,* 1998; Zijlstra *et al.,* 2001). The test was then developed into an ICT (dipstick) format more suitable for field use. A meta-analysis that included 13 validation studies of rK39 ICT showed sensitivity and specificity estimates of 93.9% (95%CI: 87.7-97.1) and 95.3% (95%CI: 88.8-98.1), respectively (Chappuis *et al.*, 2006b).

Recently, the excellent diagnostic performance of rK39 ICT was confirmed in India and Nepal (Chappuis *et al*. 2006a; Sundar *et al*., 2006a; Sundar *et al*., 2006b; Sundar *et al*., 2007). However, rK39 ICT was shown to be less accurate in East Africa (Diro et al., 2007; Ritmeijer *et al*., 2006; Veeken *et al*., 2003; Zijlstra *et al*., 2001). Due to several reports of lower test sensitivity in endemic regions such as East Africa, a negative rk39 may not be reliably exclude diagnosis thus additional parasitological test are necessary (WHO/TDR 2011). For reasons that remain unclear, Sudanese patients seem to develop lower titres of antibodies against rK39 than do Indian patients, though the kit used might be a factor, as other brands of ICT performed better in this region (Ritmeijer *et al*., 2006).

2.5.2.2.1 Advantages and disadvantages of rK39 RDTs

Advantages

1. rK39 RDTs enable individual patients to be tested at the bedside.
2. Tests are individually packaged and easy to store/transport.
3. Little training and no laboratory equipment are needed.
4. Results are available in 10–20 minutes.
5. Results are clear and easy to read.
6. Enables decentralized screening of VL even at community level.
7. Easy to use in field setting during active case search or an outbreak.
8. Kits can be transported and stored at ambient temperature (up to 30 °C).

Disadvantages

1. As any other serological test, they cannot distinguish between active and past symptomatic or asymptomatic infections. Therefore interpretation must always be in combination with clinical case definition, and diagnosis of relapse must rely on parasitology.
2. In some cases (around 10–18%) the test will give a negative result even if the patient suffers from VL. In this case, another serological or parasitological test must be performed in VL suspected patients.
3. In patients with advanced HIV infection a negative result cannot preclude the diagnosis of VL.
4. Inability to predict response to treatment makes this test less suitable.
5. Significant proportion (12.5%) of healthy controls show reactivity in these tests and considerably low sensitivity (57.4%) has been reported in patient's co infected with human immunodeficiency virus (HIV).

2.5.2.3 KATEX Kit as an Antigen Detection Test for VL.

Kala-azar Latex (KATEX) is an agglutination based test which detects a heat-stable low molecular weight carbohydrate antigen in urine.The antigen has been characterized and found to be a low molecular mass (5–20 kD) glycoconjugate (Sarkari *et al.,* 2002).The molecule is unique to *Leishmania* (absent from other Kinetoplastidae, such as *Trypanosoma brucei* or *T. cruzi*), but present in all species of *Leishmania*. This kit unlike ICT has been shown to detect VL in HIV-VL coinfected patients. Soon after

infection, *Leishmania* parasites secrete/excrete antigens that accumulate in the body and then are secreted as waste products in blood and urine. Detection of these antigens may be used as a confirmatory test since the antigens indicate actual infection. The only commercially available antigen detection test is KAtex (Kalon Biological, UK); despite its simplicity (is a latex agglutination test) its widespread use has been limited by a variable sensitivity and specificity obtained in different studies, and the need to boil the urine before testing to avoid false positive results.

To improve it's sensitivity, the urine samples have to be put into tubes in a rack then immersed in boiling water to avoid false-positive reactions.Also it is difficult to distinguish between weakly positive results from negative results.This affects the test's reproducibility (Chappuis *et al*., 2006a). In addition, during boiling of samples using water bath, there is a high likelihood of cross-contamination. Several studies conducted in East Africa and the Indian subcontinent showed good specificity but low-to-moderate sensitivity (48-87%) (Chappuis *et al*., 2006a, Sundar *et al*., 2005, Sundar *et al*., 2007).

The latex format is better suited for use in remote rural areas in the tropics, where VL is mostly endemic. In its present format, the test is simple to use, economical and robust. Its main advantages being the fact that, the rapid capability of the test in diagnosis, especially when the patient is coming from far distances for the test. The performance of the test does not require electric appliance, most technicians are familiar with the latex format (which is routinely used for pregnancy tests) and they do not experience any difficulty in scoring the test (Zamil *et al*., 2001).

This method is useful in the diagnosis of disease in cases where there is deficient antibody production. Because of the conditions prevailing in endemic area, any sophisticated method cannot be employed on a wider scale hence the need for a simple and accurate test with good sensitivity and specificity. Its correlation with active VL makes it superior to other diagnostic test that uses body fluids. KATEX kit is specific for it does not give any cross reaction with toxoplasmosis, tuberculosis, and brucellosis hence indicating that, the KATEX kit is more valuable only for VL diagnosis. Antigen detection tests are more specific than antibody detection tests as they avoid cross-reactions and can distinguish active disease, subclinical and past infections (Attar *et al*., 2001).

2.5.2.4 Enzyme Linked Immunosorbent Assay.

Enzyme linked immunosorbent assay *(*ELISA) is a valuable tool and one of the most sensitive tests for the serodiagnosis of visceral leishmaniasis. The test is useful for laboratory analysis or field applications and to screen a large number of samples at a rapid pace. With the advances in automation, ELISA can be performed easily and is adaptable for use with various antigens such as whole cytoplasmic (soluble antigen, SA), purified antigens such as fucosemanose (Palatnik *et al.*, 1995).The sensitivity and specificity of ELISA is greatly influenced by the antigen used. Beside the most commonly used soluble promastigote antigen, several antigenic molecules have been reported and their negative and positive predictive values (NPV & PPV) compared (Maalej *et al.*, 2003).

An excretory, secretary and metabolic antigens released by *L. donovani* promastigotes (Ld-ESM) into a protein free medium was used for the serodiagnosis of VL by ELISA (Martin *et al*., 1998). This antigen has been reported to be 100 per cent specific and sensitive, the PPV was 99.99 per cent and NPV was 95.45 per cent. Lately, a variety of recombinant antigens have been developed. *A L. major* gene B encoding a hydrophilic protein (gene B protein, rGBP) expressed on the surface of both promastigotes and amastigotes and characterized by an amino acid repeating motif of 5.5 copies of a 14-amino acid sequence has been identified and shown to be expressed in *L. donovani* also. The protein encoded by *L. donovani* gene B homologue (Ld-rGBP) contains up to 22 copies of a repetitive element in which 9 out of 14 residues are completely conserved between the two species.

An ELISA using this antigen is reported to be specific for *L. donovani* infections only (Jensen *et al*., 1999). Another recombinant protein rORFF of *L. infantum* origin has been developed by Raj *et al., 1999)* for diagnosis of VL in India. The ORFF protein is encoded in the LD1 locus of chromosome 35 of *L. infantum*. An ELISA based test using this antigen was found to be highly sensitive and specific. Its sensitivity and specificity was compared with DAT and soluble antigen (SA) ELISA. The sensitivity of rORFF ELISA was found to be significantly more than Direct Agglutination Test.

2.5.3 Molecular detection test

Molecular methods based on the polymerase chain reaction (PCR) amplification of a Leishmania DNA fragment have since been developed. PCR has been proved to be most sensitive and specific technique, albeit limited to tertiary care hospitals and research laboratories. The specificity of the PCR can be adapted to specific needs by targeting conserved region of the gene. Gene amplification through the PCR has several advantages compared to traditional techniques, because of its extremely high sensitivity, rapidity and the ability to be performed with a broad range of clinical specimens (Tavares *et al*., 2003).

Various gene targets nucleic acids can be used in PCR. The important gene targets are 18S-rRNA, small subunit rRNA (SSU rRNA), a repetitive genomic sequence of DNA the miniexon (spliced ladder) gene repeat, the b-tubulin gene region, gp63 gene locus, internal transcribed spacer (ITS) regions; micro-satellite DNAs such as maxi- and minicircles of kinetoplast DNA (da Silva ES *et al*., 2004). Modified form of PCR such as nested PCR has proved its predictive values in diagnosis of PKDL.

In a study, nested PCR was positive in 27 of 29 (93%) samples while only 20 of 29 (69%) samples were positive in the primary PCR assay. Using PCR methodology, it is no more essential to undergo invasive methods such as bone marrow, splenic punctures, lymph node biopsy, liver biopsy or collect large volumes of blood samples. Even a few drops of blood on filter paper may be sufficient (Gannavaram *et al*., 2004). However, several assays require pre –PCR, PCR and post-PCR handlings thus not always available in endemic regions, such as sequencing, restriction digests, or melting curve analysis, which are required to reveal polymorphisms within the amplified fragment.

The internal transcribed spacer 1 of the ribosomal DNA repeat unit (rDNA-ITS1) has previously been exploited for Old World species discrimination (Schönian *et al*., 2001) using restriction fragment length polymorphisms, reverse hybridization assays and melting curve analysis (Talmi *et al*., 2010).There are an estimated 20 to 200 identical copies in the Leishmania genome, making it a good target for analyzing low parasite quantities. A recent development in PCR technology (fluorogenic probes and automation) takes care of non-specific amplification, speed and inter-operator variability. In this technique, two fluorogenic dyes, a reporter dye, and a quencher dye,

are attached to 5' and 3' ends of the probes. The real-time PCR is used qualitatively and quantitatively, as the fluorescence is directly proportional to the number of amplicons or the parasite load in the given specimen (Monroy *et al.*, 2002).

The automization of real-time PCR with comprehensive portable units has made these tools field friendly. The multiplex PCR can be used whenever, double or mixed infections are suspected.

PCR-based assays currently constitute the main molecular diagnostic approach of researchers and health professionals. Several distinct PCR formats are available that may broadly be classified into "mid-tech," "hightech," and "low-tech" approaches. Mid-tech approaches are probably the most widely used, and comprise conventional PCR assays, in which PCR amplicons are resolved by electrophoresis (eventually after cleavage with restriction enzymes, i.e., PCR and restriction fragment length polymorphism analysis (PCR-RFLP) and visualized after ethidium bromide staining (Singh *et al*., 2005).

These assays are performed with several pieces of laboratory equipment (e.g., a thermocycler, a power supply, an electrophoresis tank, a UV transilluminator, and a camera) available in any standard molecular laboratory and are generally time-consuming (which may considerably alter the cost of analysis, depending on the personnel costs). High-tech approaches are methods in which PCR products are analyzed during their amplification (real-time PCR) after staining with SYBR-green I dye or hybridization with fluorogenic probes (e.g., TaqMan or fluorescence resonance energy transfer (FRET) (Espy *et al***.,** 2006). In this case, assays are performed with a single all-in setup, and the detection of fluorescence is done within a closed tube, decreasing the risk of laboratory contamination by amplicons. Applications are rapid and of high throughput, but equipment is comparatively expensive, and working costs remain high (e.g., according to our own estimation at the Instituut voor Tropische Geneeskunde, Antwerp, Belgium, the cost per sample analyzed is $12 [U.S. dollars] for FRET-based assays versus $2.5 for PCR-RFLP). Low-tech approaches refer to simplified PCR methods for use in laboratory settings with minimal equipment. Simplification can potentially be done at the two main steps of the PCR protocol: target amplification and detection of the PCR products. Loop-mediated isothermal

amplification (LAMP) represents a promising avenue for both steps: it requires only a simple water bath for amplification, and detection can be done visually by using SYBR-green dye, which turns green in the presence of amplified products and remains orange in its absence. The method was claimed to be 100 times more sensitive than conventional PCR in the detection of *Trypanosoma brucei* (Kuboki *et al*., 2003).

Recently epitope specific PCR and oligochromatographic dipstick assay has been reported for Visceral Leishmaniasis (VL) and PKDL detection with sensitivity to detect less than one parasite. Real-time PCR has made quantification of parasite burden possible, with a high degree of complex-specific diagnostic accuracy for clinical samples. Quantitative nucleic acid sequence-based amplification (QT-NASBA) detects RNA in a background of DNA and may thus serve to measure viable parasites which might significantly increase assay sensitivity and decrease required sample volume. Leishmania DNA in buccal swabs from VL patients has been demonstrated by the presence of *L. donovani* nucleotides in buccal Epithelia (Manisha *et al*., 2011). PCR-based assays offer a reliable alternative for the demonstration of parasites in clinical samples because they allow a high sensitivity and specificity (up to 100%) detection of the Leishmania parasite irrespective of species or genus (Reithinger *et al*., 2007).

In a recent report from Kenya, the *Leishmania* OligoC-TesT showed a sensitivity of 96.4% and a specificity of 88.8%, while the sensitivity and specificity of the NASBA-OC were 79.8% and 100%, respectively. These findings indicate high sensitivity of the *Leishmania* OligoC-TesT on blood while the NASBA-OC is a better marker for active disease. These techniques remain restricted to referral hospitals and research laboratories due to cost and need of skilled personnel therefore need to evaluate non-invasive and easy to use diagnostic tests such as leishmania antigen detection in buccal

cells which ch can serve as an alternative test that can be used in the field.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was conducted at Kimalel Health Centre in Baringo County (Figure 2.0), treatment center, located in the Rift Valley province, Kenya. It is situated 280 km north of Nairobi County and located 10 km from Marigat town ship along Marigat-Kabarnet road. The area is endemic for Kala-azar, a fatal disease if untreated. Rainfall is sparse and it ais semiarid low land. The Ethnicity comprises of Tugens, Njemps and pokots. The villages on the borders of Kimalel include Koriema, Patkawanini on the north and Kimorok on the south. The physical facility comprise outpatient lounge, kala-azar ward, delivery/maternity ward, kitchen, newborn ward, laboratory, minor theatre, pharmacy, store, staff houses and an ambulance.

3.1.2 Study Component

The study had two components: 1) The field component which was carried out at Kimalel Health centre in Baringo County and 2) the laboratory component which took place at the Centre for Clinical Research in KEMRI Immunology Laboratory and involved testing the buccal swab samples collected at Kimalel Health centre.

3.2 Study Population

The study population comprised of patients who were evaluated at the KEMRI clinical research station at Kimalel Health Centre in Baringo County, a treatment Centre for VL in Kenya. Patient's presenting with symptoms of VL defined by a history of fever 14 days with clinical splenomegaly were recruited while endemic controls, defined as patients presenting to the health center with no fever and no prior history of Kala azar but living in VL endemic area were enrolled to participate in the study. The age bracket in both cases was 5 to 60 years.

3.2.1 Inclusion criteria

1. Male / female aged 5 to 60 years patients presenting with VL symptoms and a positive splenic aspirate test by microscopy.

2. Patients aged 5 to 60 years with no fever and no prior history of Kala azar but living in VL endemic area
3. Willing to give informed consent.

3.2.2 Exclusion criteria

1. Male / female aged below 5 years and above 60 years
2. Unwilling to give informed consent.

3.3 Study design

A survey was conducted for subject's meeting the eligibility criteria with consecutive sampling used to recruit participants.

3.3.1 Sample size

There is inadequate information on the prevalence of visceral leishmaniasis in Kenya according to national guidelines for health workers prevention, diagnosis and treatment of visceral leishmaniasis (kala-azar) in Kenya 2017 therefore unable to come up with a statistical driven sample size. Considering that, the maximum number of VL cases by microscopy seen at Kimalel is less or equal to (N~90) in a year, a survey was conducted within the study period (March 2015 to July 2016) and I managed to recruit 44 VL cases and 44 endemic controls.

3.4 Specimen Collection

3.4.1 Splenic Microscopy procedure

Informed consent was sought from the subject's after which baseline clinical data (symptoms, vital signs, weight, height, spleen and liver size) and pre-liminary laboratory tests were collected in all participants before obtatining the splenic aspiration. Five milliliter's of blood were drawn to perform hemoglobin, leucocyte and platelet counts and prothrombin time index (PTI).

After the laboratory results were ready and deemed ideal to obtain splenic aspirate (Appendix V), splenic enlargement was measured below the costal margin along its axis of enlargement by clinician, two slides were prepared for splenic aspirates then splenic aspirate was obtained, and taken to the laboratory

The slides were air dried, fixed with methanol, stained with 10% Giemsa stain for 10 minutes and then examined. Diagnosis of VL was confirmed by demonstration of parasites (amastigotes) in splenic smears and parasite density was graded after which buccal samples were collected for cases before treatment.

3.4.2 Buccal swab collection procedure.

The buccal swabs were collected from confirmed VL cases (positive) by microscopy and controls (negative) using a sterile buccal swab according to standard operating procedure (SOP) following manufacturers' instructions. Briefy the kit was removed from the packaging without handling the actual swab followed by collection cheek cells c from the subjects mouth by rolling the tip of the collection swab firmly for about 20 seconds on the inside of one cheek several times in each direction. The specimens were briefly air dried then placed directly into the receptacle which contained a desiccant for proper drying of the swab to avoid other microbes.

3.4.3 Testing procedure.

This entailed, two step methods that involved the harvesting of the antigens from the buccal swab and their detection using the KATEX kit (Attar *et al*., 2000).

3.4.3.1 Heat Induced Epitope Retrieval.

To expose the leishmania antigens from the buccal cells, 100 - 500 µL of citrate buffer PH 6.0 was dispensed in a 2 mL eppendorf tube.The swab was then placed in the tube and rotated at least five times. The swab was pressed against the side of the tube to ensure that most of the liquid remained in the tube. The tube with retrieved antigens was then vortexed for 60 seconds and heated at 98°C for 5 minutes then allowed to cool at ambient temperature before carrying out the test (Sundar *et al*, 2005).

3.4.3.2 Test kit.

The test kit consisted of polystyrene (800-nm diameter) latex particles coated with polyclonal antibodies (against the leishmanial antigen),*Leishmania* culture supernatant diluted in saline as a positive control, and buffered saline as a negative control (both were preserved with sodium azide and ready to use). The kit also contained a reusable black glass slide with three equal circles (reaction zone) drawn on it for performing the agglutination (Kalon Biologic Ltd, Aldershot, United Kingdom). After each use, the

glass slide was rinsed with water and the surface was wiped with paper towel. With one test kit, it is possible to test 100 samples including positive and negative controls.

Figure 3.1: Illustration of KATEX Kit

3.4.3.4 KATEX kit testing procedure.

All reagents stored at 4°C were brought to ambient temperature. The latex particles were mixed by inverting the reagent tube up and down before use. Fifty (50) µL of the retrieved antigens were dispensed on to a reaction zone on the glass slide and one drop of test latex added .This was stirred to obtain a homogenous mixture which covered the whole surface of the reaction zone. The glass slide was tilted with a rotating action in clockwise and anticlockwise directions continuously for two minutes, and the results (agglutination or no agglutination) read (Attar *et al*., 2001)

3.4.3.4.1 Quality assurance

A one day protocol training workshop was organized at the Kimalel Health Centre in Baringo during the study preparation phase. Standard Operating Procedures were prepared and clinicians trained on collection of buccal swabs. Laboratory technicians had refresher training on reading splenic aspirates. Leishmania culture supernatant diluted in saline as a positive control and buffered saline as a negative control (both were preserved with sodium azide and ready to use) were incorporated when carrying out the Katex test.

3.5 Ethical considerations

The study was conducted after Ethical clearance was obtained through the KEMRI Ethics Committee. Detailed information was made available for potential study participants in their language. A consent form was completed only after the patient had understood the points enumerated in the information sheet. Eligible patients were included after signing the informed consent form (or parent/guardian's for minors).Patients diagnosed with VL were treated according to the Kenyan VL national guidelines. Standard clinical care and free provision of VL diagnostic tests and drugs to all patients (whether or not enrolled in the study) were guaranteed by the participating centers.

3.6 Data management and statistical analysis

The Results of both the microscopic examination of spleen aspirate and buccal swabs and demographics were recorded in log books and later entered in Microsoft excel. A review on completeness of the data was performed with consistency checks done on the data set. Any deviations were counter checked against the log books which were the source documents. Data analyses was done using STATA software version 13.2 standard edition (*http://www.stata.com*). Continuous data were summarized using mean and standard deviation or median and inter-quartile range where appropriate while binary data were summarised using proportions. The calculations for specificity (defined as the number of individuals with a negative test among the total number of individuals with a negative reference standard) and sensitivity (defined as the number of individuals with a positive test among the total number of individuals with a positive reference standard) were also calculated. The usefulness of these tests (sensitivity and specificity)

was estimated using positive diagnostic likelihood ratio (DLR+) which is calculated as (Sensitivity/1-Specificity) as a proxy determinant of how useful a test is with a value further away from +1 implying more usefulness of the test while a value of +1 indicating that the test is not useful. The parameter estimates for both the specificity and sensitivity were presented alongside their binomial exact 95% confidence intervals. All the buccal swabs from both cases (VL splenic aspirate positive patients by microscopy) and endemic controls were tested using KATEX. The results were recorded as positive when agglutination was observed or negative when no agglutination was observed.

CHAPTER FOUR

RESULTS

4.1 Buccal Swabs tests results for VL Cases and Endemic controls using KATEX.

A total of 88 patients aged between 5–60 years, including 44 VL and 44 non-VL patients were included in the study between March 2015 to July 2016. All the buccal swabs from both cases (VL splenic aspirate positive patients by microscopy) and endemic controls were tested using KATEX.

4.1.1 Comparison between VL Cases and KATEX kit test results by Gender.

A total of 44 cases were recruited in the study. Of these, 34 were male and out of this, 27 samples were true positive (79.4%) while 7 were False Negative (20.6%) compared to 10 females who had 9 true positives (90%) and 1 false negative (10%) reflecting 75 % of males who were true positive against 25% females and 87.5% of males who were false negatives against 12.5% of females.

4.1.2 Endemic controls KATEX kit test reults by Gender.

A total of 44 endemic controls were recruited in the study.Of these, 26 were male and out of this, 21 were true negative (80.8%) with 5 samples turning to be false positive (19.2%) compared to 18 females who had 14 true negatives (77.8%) and 4 false positives (22.2%).

4.1.2.2 Comparison of Demographics between VL Cases and Controls.

By gender, overall male group n = 60(68.2%) with VL cases representing 34(77.3%) and Controls 26(59.1%) while females n= 28(31.8%) with VL cases representing 10(22.7%) and Controls 18(40.9%). By age, the VL cases; Mean (SD) was 17(11) with the median (IQR) being 15(10 to 22) while for Controls, Mean (SD) was 26(15) with the median (IQR) being 28(13 to34) with overall Mean (SD) of 22(14) and IQR Mean (SD) of 18(11 to 28). (Table 4.1).

Table 4.1: Comparison of Demographics between VL Cases and Controls.

		Overall, n=88	**VL cases n=44**	**Controls n=44**
Age (years)	Mean (SD)	22 (14)	17 (11)	26 (15)
	Median (IQR)	18 (11 to 28)	15 (10 to 22)	28 (13 to 34)
Gender	Male, n (%)	60 (68.2%)	34 (77.3%)	26 (59.1%)
	Female, n (%)	28 (31.8%)	10 (22.7%)	18 (40.9%)
Abbreviations: IQR – Interquartile Range; SD – Standard deviation; VL – Visceral leishmaniasis.				

Of the 44 VL Cases included in the analysis, 36 had a positive splenic aspirate (WHO parasite grading: 1–2: n= 4; 3–4: n= 20; 5–6: n =12) detected by KATEX while 8 (WHO parasite grading 1-2: n=5, 3-4: n=3 and 5-6: n=0) were not detected by KATEX. (Table 4.2).

Table 4.2: Comparison between Microscopy grading and KATEX results.

		KATEX		
		+ve(Agglutination)	**-ve(No-Agglutination)**	
Grading	1+	0	5	5 (11.4%)
	2+	4	0	4 (9.1%)
	3+	10	3	13 (29.5%)
	4+	10	0	10 (22.7%)
	5+	7	0	7 (15.9%)
	6+	5	0	5 (11.4%)

4.2 KATEX test Performance

4.2.1 Sensitivity and Specificity of KATEX Screening results for VL and endemic controls.

Sensitivity (ability of a test to identify those patients with the disease) is calculated using the following formula: sensitivity = [number of samples with true-positive results/ (number of samples with true-positive results + number of samples with false-negative results)] × 100%. The Specificity (ability of a test to identify patients without the disease) is calculated as the number of samples with true-negative results/ (number of samples with true-negative results + number of samples with false-positive results) x 100%).

Compared to microscopy, the buccal swabs from 44 cases of confirmed VL patients were positive by KATEX in 36 patients (sensitivity = 81.8%; 95%CI: 67.3% to

91.8%).Out of 44 endemic controls, 35 were negative (specificity = 79.5%; 95%CI: 64.7 –90.2%).

Table 4.3: Sensitivity and Specificity of KATEX Screening results for VL and endemic controls.

		Microscopy Result	
		Positive (+)	Negative (-)
Katex Test Results	Cases	36	9
	Controls	8	35
	Total	44	44

Positive predictive value (Defined as the number of individuals with a positive reference standard among the total number of individuals with a positive test) n= 36(80.0%); 95%CI: 65.4% to 90.4% and Negative predictive values (Defined as the number of individuals with a negative reference standard among the total number of individuals with a negative test) n = 35(81.4%); 95% CI: 66.6% to 91.6%(Table4.4).

Table 4.4: Sensitivity, Specificity, Positive and Negative predictive values of KATEX Screening in Buccal Swabs for VL and Non VL in Kimalel health center, Baringo County, Kenya.

	Index test result	**VL cases, n=44**	**Non VL n=44**	**Sensitivity [95% C.I]**	**Specificity [95% C.I]**	**PPV [95% C.I]**	**NPV [95% C.I]**	**DLR+**
KATEX	Positive	36	9	81.8% [67.3% to 91.8%]	79.5% [64.7% to 90.2%]	80.0% [65.4% to 90.4%]	81.4% [66.6% to 91.6%]	4.0
	Negative	8	35					

Abbreviations: PPV = positive predictive value, NPV = negative predictive value, CI = Confidence Interval, DLR+ = positive Diagnostic Likelihood Ratio; VL – Visceral leishmaniasis.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

This study evaluated the ability of a simple non-invasive KATEX diagnostic tests to detect leishmania antigens in buccal cells, determine the ability of KATEX assay kit to detect Leishmania antigens in buccal cells of kala azar patients and to compare sensitivity and specificity of KATEX - buccal assay with the gold standard microscopy test. Demonstrating the presence of the infecting organism, or a surrogate marker of infection, is often crucial for effective clinical management and for selecting other appropriate disease control activities. To be useful, diagnostic methods must be accurate, simple and affordable for the population for which they are intended. They must also provide a result in time to institute effective control measures, particularly treatment. (TDR Diagnostics Evaluation Expert Panel, 2010).

The findings of this study revealed that, viseral leishmania antigens were detected from buccal swabs giving a sensitivy of 81.8 % and specificity of 79.5%. These findings are consistent with previous studies done on urine samples in Brazil, Nepal, Sudan and Yemen which indicated that, the test works well regardless of the geographical origin of samples.

Evaluation of KAtex kit in Nepal based on antigen detection in urine showed low sensitivity (47.4%) against excellent specificity 98.7% and in Brazil, a study showed that, 16 of 25 confirmed kala-azar showed a positive KAtex sensitivity 64%, specificity 95%.(AbdolSamad Mazloumi Gavgani1 *et al.*, 2008)**.** Compared to the findings of this study, the sensitivity was high in buccal swabs with a slightly lower specificity. Sensitivity for rK39 antigen using saliva was 83.3% and 82.5% in ELISA and ICT technique, whereas specificity was found to be 90.5% and 91.5% respectively by ELISA and ICT in endemic healthy controls (Prajapti *et al.*, 2013). However, these test in saliva were antibody based thus being less specific than antigen based diagnosis which is more specific.

In another study done for evaluating urinary antigen-based latex agglutination test in the diagnosis of kala-azar (Rijal *et al.*,2004), the sensitivity of KAtex was found to be low in confirmed VL cases although the specificity was excellent amongst a control group of patients with similar symptoms in whom kala-azar was ruled out.Similarly, series of kala-azar patients contained a high number with low parasite intensity of parasitological grade of between 1+ and 2+ (Rijal *et al*., 2004) differing from the findings of this study where by, high number had high parasite intensity of between 3+ and 4+.

Detection of leishmania antigen in buccal swab would serve as a good quality diagnostic test fit for purpose which will provide accurate results and therefore of paramount importance in reducing the burden of infectious diseases due to its specificity. The lack of access for Good diagnostic tests contributes to the enormous burden of ill health in the developing world, where infectious diseases are the major causes of death (Rosanna *et al.,* 2010). For infections such as Visceral Leishmaniasis, early diagnosis using accurate diagnostic tests such as antigen detection in buccal swabs will play an important role in preventing the development of long-term complications or in interrupting transmission of the infectious agent. In a broader context, diagnostic tests can have multiple uses, including: patient management, especially when clinical symptoms are not specific for a particular infection (as is often the case); screening for asymptomatic infections; surveillance; epidemiological studies (for example, rapid assessments of disease burden or outbreak investigations); evaluating the effectiveness of interventions, including verification of elimination; and detecting infections with markers of drug resistance (TDR Diagnostics Evaluation Expert Panel.,2010).

The commonly used method for diagnosing visceral leishmaniasis (VL) has been by direct microscopic demonstration of parasites (amastigotes) in splenic or bone marrow aspirate, which is invasive and examining by direct microscopy. Detection of antigens in patient serum is also done but this is made complex by the presence of high levels of antibodies, circulating immune complexes (CIC) and auto-antibodies, all of which may mask immunologically important antigenic determinants or competitively inhibit the binding of antibodies to free antigen. This may explain why

no antigen detection assay for VL is routinely in use to-date, despite a number of reports describing the existence of circulating antigens and immune complexes in VL (Sehgal *et al.,* 1982; Galvao-Castro *et al*., 1984; Azazy *et al*., 1994., 1997). In this study, KATEX kit was used to detect VL antigens in buccal swabs and the results compared using microscopic examination of spleen aspirate, one of the recommended reference standards for VL diagnostic evaluation studies (Boelaert *et al.,* 2007).

Molecular methods have been successfully used in VL diagnosis, demonstrating high sensitivity and specificity. Leishmania DNA in buccal swabs from VL patients has been demonstrated by the presence of *L. donovani* nucleotides in buccal Epithelia (Manisha *et al*., 2011). PCR-based assays offer a reliable alternative for the demonstration of parasites in clinical samples because they allow a high sensitivity and specificity (up to 100%) detection of the Leishmania parasite irrespective of species or genus (Reithinger *et al*., 2007).However, their implementation poses a challenge because they are not cost-effective, they need well-established laboratories, trained and skilled staff. The sensitivity and specificity of serological methods, such as DAT, IHA, IFAT, ELISA and ICTs may be variable in different endemic areas worldwide. However, their main drawback is that, antileishmanial antibodies may be detectable for a long period after recovery thus the need to use non-invasive and cost-effective antigen diagnostic test which can be used by less skilled personnel in the diagnosis of VL in the endemic areas.

The advantages of using buccal swabs over urine in detection of leishmania antigens is that, the performance of the test does not require sophisticated electric appliance and that most technicians can perform the test with ease (Zamil *et al.,* 2001) is simple to use, economical, robust and easy to perform.Also, it, s easier to obtain buccal swabs than urine from children who are the most affected by VL and the test can be used as a test of cure since urine antigen detection by KATEX by other investigators demonstrated reduction of antigen following cure(Zamil *et al.,* 2001).

5.2 CONCLUSIONS

1. Kala-azar latex based agglutination test (KATEX) kit was able to detect Visceral Leishmania antigens from buccal swabs.

2. Compared to microscopy in a group of 44 participants with clinical manifestations suggestive of Visceral leishmaniasis and 44 endemic controls, the kit was able to detect Visceral Leishmania antigens in the buccal swabs giving a sensitivity of 81.8% and specificity of 79.5%.

5.3 RECOMMENDATIONS

1. Based on the results of this study, there is need for further evaluation of test specificity using buccal swabs from patients with other infections and sensitivity using larger sample size including the stability of antigen in buccal swab specimens and also use of test as a test of cure.

2. These trial results can be used to demonstrate the utility and potential impact of the diagnostic test and obtain data for regulatory submission and approval so that the test can be recommended useful for screening active kala-azar patients.

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APPENDICES

Appendix I: Informed consent form for adults

Study Principal Investigator: Mr Alfred M. Muia.

Co- Investigators: Dr.Margaret Mbuchi,Dr.Jesca O. Wesongah,Dr.Juma Rashid,Mr. Charles Magiri ,Dr.Samwel Odiwuor,Dr. Raymond Omollo,Dr. Monique Wasunna.

Kenya Medical Research Institute – CCR, Jomo Kenyatta University of Agriculture and Technology, MT. Kenya University and Drugs for Neglected Diseases *initiative* (DND*i*).

Study Title: Detection of Leishmania Antigen from Buccal Swabs in Kala-azar Patients Using KATEX Method.

Sponsor: Kenya Medical Research Institute – Internal Research Grant (IRG).

Introduction

We are studying the diagnosis of the disease kala-azar, which is common in this part of Kenya. You may or may not be suffering from this disease.

In this study we would like to develop a new diagnostic test for Kala Azar that uses cheek cells. In order to do this, we need to collect cheek swabs from both people suffering from and not suffering from the disease. It is important we collect samples from both groups to ensure that test can predict who has and does not have the disease. This will ensure that in future, appropriate treatment can be given to Kala Azar patients after cheek swab test. This study will include 73 subjects who have kala-azar and 73 who don't. With your permission, we would like to include you in this study.

Procedure during the study

You are being invited to join the study after routine testing in the hospital has been conducted to determine if you have or do not have Kala Azar and you are being considered for this study. If you are not suspected to have Kala Azar, you will volunteer for cheek swab collection. If you have been found to have Kala Azar, from your spleen or bone marrow, you will be requested to volunteer for cheek swab collection.

Benefits and dissemination of study findings.

There is no direct benefit of participation to you but the community may benefit from a new non-invasive diagnostic test which if effective, will replace the more invasive test (splenic or bone marrow aspiration). The study findings will be disseminated to research participants through the Clinical personnel at the Kimalel health centre who shall receive a study report at the end of the study.

Risks.

It is not expected that you will suffer significant harm by participating in this study. Your study records will be kept by the study team, here, at the Kimalel health centre, but it will be stored in a secure and locked cabinet to reduce the risk of loss of confidentiality.

Confidentiality.

Records relating to your participation as a research volunteer will remain confidential. The research records will be made available only to authorized study team members**.** The Clinical personnel at the health centres shall receive a study report who shall then in turn make it available to the Ministry of Health should they require it. The identity of individuals who volunteer for the study will be protected by use of a special code; names, national identity card numbers and other personal identifiers will not be used during data entry. The code will be used to identify the data gathered on the particular volunteer. Access to subjects' records will be granted to the sponsor, regulatory agencies and ethics committees for the purpose of validating data. The records may be reviewed by authorized personnel of the Kenya

Medical Research Institute (KEMRI) as part of their responsibility to ensure that research is safely and properly conducted.

Your name will not be used in any report or publication resulting from this study. We are therefore asking for your consent to use the test results for writing any report.

Your study records will be stored in a locked cabinet at KEMRI or a designated storage facility at Kimalel health centres for at least 3 years after formal discontinuation of the study. This is in line with regulatory requirements for the purpose of validating data.

Right to refuse or withdraw

Your participation is voluntary. If you do decide to participate and then change your mind later, you may do so, at any time, without losing any of your rights as a patient. In this event, the samples you gave us will be destroyed and the data will not be used in the data analysis. If you agree to participate in the study, we will ask you to read and sign the consent form.

Study approval

This study has been approved by the KEMRI Ethics Committee. The study will only perform tests approved by the Ethics Committee. Should any other tests be required for this or another study, permission must first be given from the Ethics Committee.

Do you have any questions? If you have any questions later, you can ask the doctors and nurses or contact the Principal Investigator

Name: Alfred M.Muia

Physical / Hospital address: Kimalel health centre, Baringo, Kenya. Telephone 0722 479803 for information or answers to questions concerning your rights as a research subject you may contact:

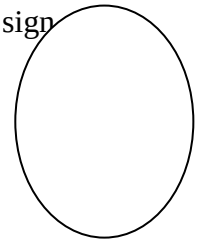
The Secretary of the Kenya National Ethical Review Committee, c/o Kenya Medical Research Institute,P.O. Box 54840-00200, Nairobi, Kenya, Tel. 020-2722541,0722205901,0733400003; E-mail address: erc@kemri.org

You will receive a copy of this consent form to keep after it is completed.

I consent voluntarily to participate in this research study. I attest that the information in these consent forms has been explained to me and that my questions and concerns have been satisfactorily addressed. I have also been informed that I can ask more questions at any time during the study and that I can also withdraw from the study at any time.

Printed name of volunteer

Thumb Print If volunteer is unable to sign



_____ _____

Volunteer's signature Date

_____ _____

Witness's Signature (If volunteer is unable to sign) Date

_____ _____

Signature of person administering Informed Consent Date

_____ _____ _____

Investigator's name Signature Date

WITNESS TO CONSENT INTERVIEW

Complete this section only if the volunteer is illiterate

On the date given next to my signature, I witnessed the "Informed Consent Interview" for the Research Study named above in this document. I attest that the

information in these consent forms were explained to the subject and the subject indicated that his/her questions and concerns were adequately addressed.

Printed Name of Witness _____

Signature of Witness _____ Date _____

Appendix II: Parental/guardian informed consent

Study Principal Investigator: Mr Alfred M. Muia.

Co- Investigators: Dr.Margaret Mbuchi,Dr.Jesca O. Wesongah,Dr.Juma Rashid,Mr. Charles Magiri ,Dr.Samwel Odiwuor,Dr. Raymond Omollo,Dr. Monique Wasunna.

Kenya Medical Research Institute – CCR, Jomo Kenyatta University of Agriculture and Technology, MT. Kenya University and Drugs for Neglected Diseases *initiative* (DND*i*).

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Procedure during the study

Your child is being invited to join the study after routine testing in the hospital has been conducted to determine if the child has or does not have Kala Azar before being considered for this study. If the child is not suspected to have Kala Azar, we seek your permission for the child to volunteer for cheek swab collection. If the child has been found to have Kala Azar, from his or her spleen or bone marrow, we seek your permission for the child to volunteer for cheek swab collection.

Benefits and dissemination of study findings.

There is no direct benefit of participation to you but the community may benefit from a new non-invasive diagnostic test which if effective, will replace the more invasive test (splenic or bone marrow aspiration). The study findings will be disseminated to research participants through the Clinical personnel at the Kimalel health centre who shall receive a study report at the end of the study.

Risks.

It is not expected that the child will suffer significant harm by participating in this study. The child study records will be kept by the study team, here, at the Kimalel health centre, but it will be stored in a secure and locked cabinet to reduce the risk of loss of confidentiality.

Confidentiality.

Records relating to your participation as a research volunteer will remain confidential. The research records will be made available only to authorized study team members. The Clinical personnel at the health centres shall receive a study report who shall then in turn made it available to the Ministry of Health should they require it. The identity of individuals who volunteer for the study will be protected by use of a special code; names, national identity card numbers and other personal identifiers will not be used during data entry. The code will be used to identify the data gathered on the particular volunteer. Access to subjects' records will be granted to the sponsor, regulatory agencies and ethics committees for the purpose of validating data. The records may be reviewed by authorized personnel of the Kenya Medical Research Institute (KEMRI) as part of their responsibility to ensure that research is safely and properly conducted.Your name will not be used in any report or publication resulting from this study. We are therefore asking for your consent to use the test results for writing any report. Your study records will be stored in a locked cabinet at KEMRI or a designated storage facility at Kimalel health centre for at least 3 years after formal discontinuation of the study. This is in line with regulatory requirements. This will enable the investigators to readily answer questions concerning a volunteer's participation in a research study.

Right to refuse or withdraw

The child's participation is voluntary. If you do decide the child to participate and then change your mind later, you may do so, at any time, without losing any of the rights as a patient. In this event**,** the samples obtained from the child will be destroyed and the data will not be used in the data analysis.

Study approval

This study has been approved by the KEMRI Ethics Committee. The study will only perform tests approved by the Ethics Committee. Should any other tests be required for this or another study, permission must first be given from the Ethics Committee.

Do you have any questions? If you have any questions later, you can ask the doctors and nurses or contact the Principal Investigator

Name: Alfred M.Muia

Physical / Hospital address: Kimalel health centre, Baringo, Kenya. Telephone 0722 479803

For information or answers to questions concerning your rights as a research subject you may contact:

The Secretary of the Kenya National Ethical Review Committee, c/o Kenya Medical Research Institute,P.O. Box 54840-00200, Nairobi, Kenya, Tel. 020-2722541,0722205901,0733400003; E-mail address: erc@kemri.org

I consent voluntarily for my child to participate in this research study. I attest that the information in these consent forms has been explained to me and that my questions and concerns have been satisfactorily addressed. I have also been informed that I can ask more questions at any time during the study and that I can also withdraw from the study at any time.

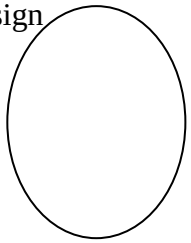
_____

Printed name of volunteer

_____ _____

Volunteer's signature Date

Thumb Print If volunteer is unable to sign



_____ _____

Witness's Signature (If volunteer is unable to sign) Date

_____ _____

Signature of person administering Informed Consent Date

_____ _____ _____

Investigator's name Signature Date

Appendix III: Assent form agreement for individuals 13 through to 17 years of age

Study Principal Investigator: Mr Alfred M. Muia.

Co- Investigators: Dr.Margaret Mbuchi,Dr.Jesca O. Wesongah,Dr.Juma Rashid,Mr. Charles Magiri ,Dr.Samwel Odiwuor,Dr. Raymond Omollo,Dr. Monique Wasunna.

Kenya Medical Research Institute – CCR, Jomo Kenyatta University of Agriculture and Technology, MT. Kenya University and Drugs for Neglected Diseases *initiative* (DND*i*).

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Sponsor: Kenya Medical Research Institute – Internal Research Grant (IRG).

Introduction

We are studying the diagnosis of the disease kala-azar, which is common in this part of Kenya. You may or may not be suffering from this disease. In this study we would like to develop a new diagnostic test for Kala Azar that uses cheek cells. In order to do this, we need to collect cheek swabs from both people suffering from and not suffering from the disease. It is important we collect samples from both groups to ensure that test can predict who has and does not have the disease. This will ensure that in future, appropriate treatment can be given to Kala Azar patients after cheek swab test.This study will include 73 subjects who have kala-azar and 73 who don't. With your permission, we would like to include you in this study.

Procedure during the study

You are being invited to join the study after routine testing in the hospital has been conducted to determine if you have or do not have Kala Azar. Regardless of whether you have tested positive or negative for Kala-Azar, you are invited to participate in the study. If you are willing to participate you will be requested to provide a small sample from the inside part of the mouth. To collect the sample a cotton swab will be rolled on the inside part of the cheek to collect cheek cells that will tested for Kala Azar.

Benefits and dissemination of study findings.

There is no direct benefit of participation to you but the community may benefit from a new non-invasive diagnostic test which if effective, will replace the more invasive test (splenic or bone marrow aspiration). The study findings will be disseminated to research participants through the Clinical personnel at the Kimalel health centre who shall receive a study report at the end of the study.

Risks.

It is not expected that you will suffer significant harm by participating in this study. Your study records will be kept by the study team, here, at the Kimalel health centre, but it will be stored in a secure and locked cabinet to reduce the risk of loss of confidentiality.

Confidentiality.

Records relating to your participation as a research volunteer will remain confidential. The research records will be made available only to authorized study team members**.** The Clinical personnel at the health centres shall receive a study report who shall then in turn made it available to the Ministry of Health should they require it. The identity of individuals who volunteer for the study will be protected by use of a special code; names, national identity card numbers and other personal identifiers will not be used during data entry. The code will be used to identify the data gathered on the particular volunteer. Access to subjects' records will be granted to the sponsor, regulatory agencies and ethics committees for the purpose of validating data. The records may be reviewed by authorized personnel of the Kenya Medical Research Institute (KEMRI) as part of their responsibility to ensure that research is safely and properly conducted.Your name will not be used in any report or publication resulting from this study. We are therefore asking for your consent to use the test results for writing any report. Your study records will be stored in a locked cabinet at KEMRI or a designated storage facility at Kimalel health centre for at least 3 years after formal discontinuation of the study. This is in line with regulatory requirements. This will enable the investigators to readily answer questions concerning a volunteer's participation in a research study.

Right to refuse or withdraw

Your participation is voluntary. If you do decide to participate and then change your mind later, you may do so, at any time, without losing any of your rights as a patient. In this event, the samples you gave us will be destroyed and the data will not be used in the data analysis.

Study approval

This study has been approved by the KEMRI Ethics Committee. The study will only perform tests approved by the Ethics Committee. Should any other tests be required for this or another study, permission must first be given from the Ethics Committee.

Do you have any questions? If you have any questions later, you can ask the doctors and nurses or contact the Principal Investigator

Name: Alfred M.Muia

Physical / Hospital address: Kimalel health centre, Baringo, Kenya. Telephone 0722 479803

For information or answers to questions concerning your rights as a research subject you may contact:

The Secretary of the Kenya National Ethical Review Committee, c/o Kenya Medical Research Institute,P.O. Box 54840-00200, Nairobi, Kenya, Tel. 020-2722541,0722205901,0733400003; E-mail address: erc@kemri.org

you will receive a copy of this assent form to keep after it is completed.

I assent voluntarily to participate in this research study. I attest that the information in these assent forms has been explained to me and that my questions and concerns have been satisfactorily addressed. I have also been informed that I can ask more questions at any time during the study and that I can also withdraw from the study at any time.

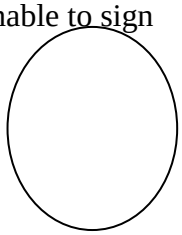
_____

Printed name of volunteer

_____ _____

Volunteer's signature Date

Thumb Print If volunteer is unable to sign



_____ _____

Witness's Signature (If volunteer is unable to sign) Date

_____ _____

Signature of person administering Informed Consent Date

_____ _____ _____

Investigator's name Signature Date

WITNESS TO ASSENT INTERVIEW

Complete this section only if the volunteer is illiterate

On the date given next to my signature, I witnessed the "Informed Assent Interview" for the Research Study named above in this document. I attest that the information in these assent forms were explained to the subject and the subject indicated that his/her questions and concerns were adequately addressed.

Printed Name of Witness _____

Signature of Witness _____ Date _____

Appendix IV: Parental/guardian permission form for minors aged 13–17 years.

Study Principal Investigator: Mr Alfred M. Muia.

Co- Investigators: Dr.Margaret Mbuchi,Dr.Jesca O. Wesongah,Dr.Juma Rashid,Mr. Charles Magiri ,Dr.Samwel Odiwuor,Dr. Raymond Omollo,Dr. Monique Wasunna.

Kenya Medical Research Institute – CCR, Jomo Kenyatta University of Agriculture and Technology, MT. Kenya University and Drugs for Neglected Diseases *initiative* (DND*i*).

Study Title: Detection of Leishmania Antigen from Buccal Swabs in Kala-azar Patients Using KATEX Method.

Sponsor: Kenya Medical Research Institute – Internal Research Grant (IRG).

Introduction

We are studying the diagnosis of the disease kala-azar, which is common in this part of Kenya. You child may or may not be suffering from this disease. In this study we would like to develop a new diagnostic test for Kala Azar that uses cheek cells. In order to do this, we need to collect cheek swabs from both people suffering from and not suffering from the disease. It is important we collect samples from both groups to ensure that test can predict who has and does not have the disease. This will ensure that in future, appropriate treatment can be given to Kala Azar patients after cheek swab test .This study will include 73 subjects who have kala-azar and 73 who don't. With your permission, we would like to include the child in this study.

Procedure during the study

Your child is being invited to join the study after routine testing in the hospital has been conducted to determine if the child has or does not have Kala Azar before being considered for this study. If the child is not suspected to have Kala Azar, we seek your permission for the child to volunteer for cheek swab collection. If the child has been found to have Kala Azar, from his or her spleen or bone marrow, we seek your permission for the child to volunteer for cheek swab collection.

Benefits and dissemination of study findings.

There is no direct benefit of participation to you but the community may benefit from a new non-invasive diagnostic test which if effective, will replace the more invasive test (splenic or bone marrow aspiration). The study findings will be disseminated to research participants through the Clinical personnel at the Kimalel health centre who shall receive a study report at the end of the study.

Risks

It is not expected that the child will suffer significant harm by participating in this study. The child study records will be kept by the study team, here, at the Kimalel health centres, but it will be stored in a secure and locked cabinet to reduce the risk of loss of confidentiality.

Confidentiality

Records relating to your participation as a research volunteer will remain confidential. The research records will be made available only to authorized study team members**.** The Clinical personnel at the health centres shall receive a study report who shall then in turn made it available to the Ministry of Health should they require it. The identity of individuals who volunteer for the study will be protected by use of a special code; names, national identity card numbers and other personal identifiers will not be used during data entry. The code will be used to identify the data gathered on the particular volunteer. Access to subjects' records will be granted to the sponsor, regulatory agencies and ethics committees for the purpose of validating data. The records may be reviewed by authorized personnel of the Kenya Medical Research Institute (KEMRI) as part of their responsibility to ensure that research is safely and properly conducted.Your name will not be used in any report or publication resulting from this study. We are therefore asking for your consent to use the test results for writing any report. Your study records will be stored in a locked cabinet at KEMRI or a designated storage facility at Kimalel health centre for at least 3 years after formal discontinuation of the study. This is in line with regulatory requirements. This will enable the investigators to readily answer questions concerning a volunteer's participation in a research study.

Right to refuse or withdraw

The child's participation is voluntary. . If you do decide the child to participate and then change your mind later, you may do so, at any time, without losing any of the rights as a patient. In this event, the samples obtained from the child will be destroyed and the data will not be used in the data analysis.

Study approval

This study has been approved by the KEMRI Ethics Committee. The study will only perform tests approved by the Ethics Committee. Should any other tests be required for this or another study, permission must first be given from the Ethics Committee.

Do you have any questions? If you have any questions later, you can ask the doctors and nurses or contact the Principal Investigator

Name: Alfred M.Muia

Physical / Hospital address: Kimalel health centre, Baringo, Kenya. Telephone 0722 479803

for information or answers to questions concerning your rights as a research subject you may contact:

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you will receive a copy of this consent form to keep after it is completed.

ASSENT STATEMENT

My mother and/or father or caregiver knows about this study and they want me to be in the study if I want to be part of it.

I want to be part of the study, but I can stop taking part in the study at any time I want to.

I know that the Principle or Co principle investigators can talk about this study with my parent(s), caregiver or me, but they will not talk about it with anyone else who is not working on the study unless myself, my mum and/or dad or caregiver says that it is OK.

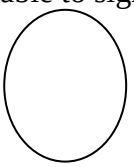
I will be given a copy of this signed assent form. I can call the study PI or any of the Co-PI,s any time if I have any questions.

I understand that I may at any time change my mind and withdraw myself from the study.

Subject's Signature /Mark/Fingerprint: _____Date: _____

Witness's Signature: _____Date: _____

Thumb Print If volunteer is unable to sign



Witness's Name: _____

_____

Volunteer's signature Date

_____ _____

Witness's Signature (If volunteer is unable to sign) Date

_____ _____

Signature of person administering Informed Consent Date

_____ _____ _____

Investigator's name Signature Date

Appendix V: Procedures for splenic aspiration.

The following requirements must be met before splenic aspiration can be done:

1. Splenomegaly must be present in a patient clinically suspected of visceral leishmaniasis (VL)

2. Haemoglobin of ≥ 5 gm/dl

3. Platelet count of ≥ 40 000/mm3

4. White blood cell count of at least ≥ 1.0 x 103/mm3

5. A prothrombin time difference of not more than 5 seconds compared with the normal control.

6. The patient should not have had a splenic aspiration within one week from the date of the current procedure.

7. No active bleeding.

8. No clinical jaundice (a possible marker of liver dysfunction).

9. No pregnancy.

10. Given the potential risk of this approach, the center on which spleen aspirate is performed should have access to blood supplies as well as blood transfusion and surgery facilities.

11. In patients less than 5 year old, splenic aspiration should be performed only by a clinician fully experienced with the procedure otherwise bone marrow is recommended from the iliac crest.

12. A patient is made to lie in the supine position with hands on the sides, the edge of the spleen is outlined with a ball pen.

13. The area of the spleen is cleaned by the clinician using spirit or any other antiseptic and a green towel (sterile, like the one used for bone marrow aspirates) placed over the splenic area.

14. A 21 gauge needle attached to a 5 ml syringe is inserted just under the skin over the middle of the spleen, and as suction is applied (about 1 ml) the needle is rapidly inserted into the spleen and withdrawn, with the needle remaining in the spleen only

a fraction of a second. Aim the needle cranially at an angle of 45° to the abdominal wall.

15. The small amount of splenic tissue and blood in the needle is expressed into the culture medium (NNN) or Schneider's with 10% overlay of fetal calf serum) and onto slides for the smears.

16. The splenic smears are stained with Giemsa or Leishman stain to demonstrate amastigotes.

NB: Bone marrow aspirate may be done but usually bone marrow smears contain fewer amastigotes and parasites are found in only 80–85% of cases.

The procedure should be handled under sterile conditions and therefore this must be observed by the attending clinician.

Instructions after the splenic aspirate

1. Patient to stay in bed strictly for 12 hours.

2. Observe blood pressure, pulse, temperature, and respiratory rate immediately after the aspiration then ½ hourly for 2 hours, 1 hourly for 4 hours then 4 hourly.

3. Suspect bleeding if there is falling blood pressure, rising pulse an normal or falling temperature; fix an IV line immediately of 5% dextrose alternating with normal saline. Take blood for grouping and cross matching immediately. Usually the majority of patients settle with IV fluids infusion within 12 hours.

4. Repeat aspiration should only be done after one week and not earlier.

Appendix VI: Preparation and staining of aspirates

Materials needed

Slides rack, staining rack or staining trough, 100% methanol, buffered solution (pH 7.2), filtered stock of Giemsa stain and tap water (filtered water preferred).

Fixation

1. Place the slides horizontally on the slide rack and leave to air dry.
2. Fix the slides by dipping them in 100% methanol for l minute. The methanol must be stored in a tightly closed bottle to prevent absorption of water.

Staining

1. Stain the slides with Giemsa stain 1:10 concentration; 1 ml of stock Giemsa stain to 9ml buffer solution pH 7.2. In the absence of buffer solution, filtered water can be used provided the pH is 7.2. The slides can either be stained in a staining trough or on a staining rack. When the stain concentration is 1:10 the staining time is 20 minutes.
2. At the end of the staining, rinse the slides briefly with tap water or filtered water and place them in vertical position on a slides rack to dry.

Reading slides

1. Examine at least 1000 microscope fields for amastigotes using x100 oil immersion lens. An artefact is more likely to be mistaken for a parasite if the microscopists are overloaded (more than 4 hours of microscopy per day), have poor light for the microscopes, or if dirty (unfiltered) Giemsa is used.