Comparison between DAT and rK39 used in the Diagnosis of Visceral Leishmaniasis and their Potential Role in the Diagnosis of the Disease Progress and PKDL

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بسم الله الرحمن الرحيم

(وَكَذَٰلِكَ مِبَالِغُهُمْ مِنَ الْعَلَمِ، إِنْ رَبُّكَ هُوَ أَعْلَمُ مِنْهُمْ ضَلٌّ عَنْ سَبِيلِهِ وَهُوَ أَعْلَمُ مِنْهُمْ أُهْتَدِى)

الآية 30 من النجم

(قَالَ الَّذِينَ آمَنُوا مَعَنِى لَنَا إِلَّا مَا عَلِمَتْنَا إِنَّكَ أَنتَ الْعَلِيمُ الْأَكْلِمُ)

الآية 32 من البقرة

صدق الله العظيم
Dedication

To my parents
To my wife
To my son
To my daughters
To my brothers
To my sisters
To my teachers
To all my friends
To my beloved country (Sudan)
To my beloved family
And to all those who supported during study
Acknowledgment

I would like to thank my supervisor Dr. Bakri Yousif Dean of the Faculty of Medical Laboratory Science for his supervision, advice and encouragement during this research. My gratitude is also for my co-supervisor Prof. Ahmed Abd Alla Mohamedani who guided and mentored me through this research and for her kind encouragement, continuous support and supervision throughout the study. My appreciations are due to Mr. Elhadi and Mr. Faisal. My thanks are also due Abu Eesa and Mrs. Lana M. Elamin. Thanks to everyone who helped me but I forgot to mention in these short lines.
Comparison between DAT and rK39 used in the Diagnosis of Visceral Leishmaniasis and their potential Role in the Diagnosis of the Disease Progress and PKDL

Hussien Ali Mohammed Ahmed Abd Allah

Abstract

Visceral Leishmaniasis (VL) is the most severe form of leishmania, second largest parasitic killer in the world (after malaria), responsible for high rates of infections each year worldwide, Sudan is the one of the most affected areas. This study aimed to compare and evaluate two common serological techniques were used (DAT and rK39) for diagnosis of visceral leishmaniasis using the gland puncture for L.Donavani bodies as the gold standard technique. It is prospective, descriptive, and health-based study conducted in Gedaref state in which 352 patients were subjected gland puncture for microscopic diagnosis of VL. The positive cases were serologically tested to evaluate both DAT and rK39 tests. The data were analyzed by using SPSS program, and results were provided as tables and graphs and discussed. Out of 352 cases, those with positive VL were 71 (20.2%), both DAT and rK39 tests showed a similar sensitivity (75%) in day zero of each. Among the cases males were 43 (60.6%), (85%) of patients were children while only 10 (14.1%) were adults. PKDL represented (10%) and all affected were males and children. Death during and on follow up were (11%), and mostly found among children. The serological methods used in the study; DAT and rK39 found to be reliable in the first diagnosis, because their sensitivity found to be (75%), but not trusted in follow up tests; due to their low sensitivity. The PKDL incidence found to be similar to WHO estimates in 2012 (10%).
مقارنة وتقييم اختبارات التراص المباشر والفحص السريع (rK39) في تشخيص الشمنيات الحشوية والشمانيات الجلدية النائية للكلازار. 

حسين علي محمد احمد عبدالله

ملخص الدراسة

يعتبر مرض الشمنيات الحشوية (الكلازار) من أكثر أنواع أمراض الشمنيات حدة، ومن أكثر الأمراض المزمنة القاتلة في العالم (بعد الملاريا)، وتسبب نسبة عالية من الإصابات سنوياً على مستوى العالم كما يعتبر السودان من أكثر الدول وفاءً به. هدفت الدراسة إلى مقارنة وتقييم كل من اختبار التراص المباشر واختبار الفحص السريع أر كي 39 في تشخيص الشمنيات الحشوية وذلك مقارنة باستعمال اختبار بزل العقدة المفاوية كعمودي ذهبي. هذه دراسة وصفية توقعية سريرية تم إجراؤها في ولاية القضارف وسط 352 من المرضى خضعوا لاختبار بزل العقدة المفاوية لتشخيص مرض الشمنيات الحشوية. العينات ذات النتيجة الإيجابية استعملوا في اختباري التراص المباشر واختبار أر كي 39 لتقسيم درجة حساسية هذه الاختبارات ومتابة مسار المرض. تم تحليل النتائج المتحصل عليها باستعمال برنامج الحزمة الإحصائية للدراسات الاجتماعية ومن ثم عرضت النتائج في شكل جداول وأشكال وتتم مناقشتها. من بين 352 مريض، وجد مرض الشمنيات الحشوية لدى 71 مريض (20.2%) بناءً على نتائج فحص عينات العقدة المفاوية واختباري التراص المباشر وأر كي 39 أظهرها نسبة حساسية متساوية (57%) وكان من بين ال71 مريض نسبة الذكور أعلى حيث مثلت 10.1% بينما الاناث مثلت (39.4%)، بينما كانت نسبة الأطفال (85.9%) ومتلك البالغون (14.1%) وأيضًا وجد في الدراسة أن نسبة الشمنيات الجلدية النائية للكلازار مثلت (10%) ووجدت في الأطفال الذكور فقط، وكانت نسبة الوفيات أثناء فترة العلاج والمتابة لديهم (11%) معظمهم كان و من الأطفال الاختبارات المعملية التي استخدمت في هذه الدراسة (اختبار التراص المباشر واختبار أر كي 39) أظهرت درجة حساسية متساوية بلغت (75%) مما يجعلها يمكن الاعتماد عليها في التشخيص لأول مرة، ولا يصحا في اختبارات المتاحة لضعف نسبة الحساسية، نسبة حدوث الشمنيات الجلدية النائية للكلازار وجدت مشابهة لتقديرات منظمة الصحة العالمية للعام 2012 في السودان.
## List of Contents

<table>
<thead>
<tr>
<th>No</th>
<th>CONTENTS</th>
<th>Page No</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dedication</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Acknowledgment</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>English abstract</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Arabic abstract</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>List of contents</td>
<td>VI</td>
</tr>
<tr>
<td></td>
<td>List of tables</td>
<td>IX</td>
</tr>
<tr>
<td></td>
<td>List of figures</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>List of abbreviation</td>
<td>XI</td>
</tr>
</tbody>
</table>

### CHAPTER ONE

1. **Introduction** 1
   - 1.1. General Introduction 1
   - 1.2. Problem identification and Justification 3
   - 1.3. Objectives 3
     - 1.3.1. General objective 3
     - 1.3.2. Specific objectives 3

### CHAPTER TWO

2. **LITERATURE REVIEW** 4
   - 2.1. Background 4
   - 2.2. Reservoir 5
   - 2.3. Vector 5
   - 2.4. History 5
   - 2.5. Life cycle 6
   - 2.6. Morphology 9
   - 2.7. Location in the human host 9
   - 2.8. Epidemiology of VL in Sudan 9
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9.</td>
<td>HIV-Liesshiamiaco infection</td>
<td>10</td>
</tr>
<tr>
<td>2.10.</td>
<td>Diagnosis of VL</td>
<td>11</td>
</tr>
<tr>
<td>2.10.1.</td>
<td>Clinical aspects and diagnosis</td>
<td>11</td>
</tr>
<tr>
<td>2.10.1.1</td>
<td>The main symptoms &amp; signs of VL</td>
<td>11</td>
</tr>
<tr>
<td>2.10.2.</td>
<td>Laboratory Diagnosis of VL</td>
<td>12</td>
</tr>
<tr>
<td>2.10.2.1.</td>
<td>Parasitological diagnosis</td>
<td>12</td>
</tr>
<tr>
<td>2.10.2.2.</td>
<td>Serology</td>
<td>12</td>
</tr>
<tr>
<td>2.10.2.2.1.</td>
<td>Direct Agglutination Test (DAT)</td>
<td>13</td>
</tr>
<tr>
<td>2.10.2.2.2.</td>
<td>Recombinant Kala-azar antigen rK39</td>
<td>14</td>
</tr>
<tr>
<td>2.10.2.2.3.</td>
<td>Indirect Fluorescent Antibody Test (IFAT)</td>
<td>18</td>
</tr>
<tr>
<td>2.10.2.2.4.</td>
<td>Leishmanin <strong>Skin Test (LST)</strong></td>
<td>18</td>
</tr>
<tr>
<td>2.10.2.2.5.</td>
<td>Enzyme-linked Immunosorbent Assay (ELISA)</td>
<td>19</td>
</tr>
<tr>
<td>2.10.2.2.6.</td>
<td>Formal gel test</td>
<td>19</td>
</tr>
<tr>
<td>2.10.2.2.7.</td>
<td>Latex agglutination test</td>
<td>19</td>
</tr>
<tr>
<td>2.11.</td>
<td>Culture</td>
<td>20</td>
</tr>
<tr>
<td>2.12.</td>
<td>Molecular probes (DNA detection method)</td>
<td>20</td>
</tr>
<tr>
<td>2.13.</td>
<td>Clinically suspected cases</td>
<td>21</td>
</tr>
<tr>
<td>2.14.</td>
<td>Confirmed cases</td>
<td>21</td>
</tr>
<tr>
<td>2.15.</td>
<td>The choices for obtaining the parasite</td>
<td>21</td>
</tr>
<tr>
<td>2.16.</td>
<td>Treatment</td>
<td>21</td>
</tr>
<tr>
<td>2.16.1.</td>
<td>Initial non-responder</td>
<td>22</td>
</tr>
<tr>
<td>2.16.2.</td>
<td>Drugs under investigation</td>
<td>22</td>
</tr>
<tr>
<td>2.16.3.</td>
<td>Supportive treatment for patients</td>
<td>22</td>
</tr>
<tr>
<td>2.17.</td>
<td>Follow up</td>
<td>23</td>
</tr>
<tr>
<td>2.17.1.</td>
<td>Case definition according to follow up criteria</td>
<td>23</td>
</tr>
<tr>
<td>2.17.1.1.</td>
<td>Non responder</td>
<td>23</td>
</tr>
<tr>
<td>2.17.1.2.</td>
<td>Slow responder</td>
<td>23</td>
</tr>
</tbody>
</table>
CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Study design

3.2. Study area

3.3. Study population

3.4. Study duration

3.5. Inclusion criteria

3.6. Exclusion criteria

3.7. Samples and samples size

3.7.1. Lymph node aspiration (collection)

3.7.2. Collection of blood samples on filter paper (execution of the DAT)

3.7.3. Collection of Peripheral blood samples for (rK39)

3.8. Materials and Equipments

3.8.1. Equipments

3.8.2. Reagents

3.9. Methods

3.9.1. Gland puncture for L.D.bodies

3.9.2. Performing the DAT assay

3.9.3. Procedure of rk39

CHAPTER FOUR

4. RESULTS AND DISCUSSION
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1.</td>
<td><strong>Results</strong></td>
<td>30</td>
</tr>
<tr>
<td>4.1.1.</td>
<td>Result of suspected cases</td>
<td>30</td>
</tr>
<tr>
<td>4.1.2.</td>
<td>Gender</td>
<td>31</td>
</tr>
<tr>
<td>4.1.3.</td>
<td>Age</td>
<td>32</td>
</tr>
<tr>
<td>4.1.4.</td>
<td>TOC</td>
<td>33</td>
</tr>
<tr>
<td>4.1.5.</td>
<td>PDKL with patients</td>
<td>34</td>
</tr>
<tr>
<td>4.1.6.</td>
<td>PDKL with Gender</td>
<td>35</td>
</tr>
<tr>
<td>4.1.7.</td>
<td>PDKL with Age groups</td>
<td>36</td>
</tr>
<tr>
<td>4.1.8.</td>
<td>The mortality</td>
<td>37</td>
</tr>
<tr>
<td>4.1.9.</td>
<td>Mortality with gender</td>
<td>38</td>
</tr>
<tr>
<td>4.1.10.</td>
<td>Mortality with Age</td>
<td>39</td>
</tr>
<tr>
<td>4.2.</td>
<td><strong>Discussion</strong></td>
<td>41</td>
</tr>
</tbody>
</table>

**CHAPTER FIVE**

5. **CONCLUSIONS AND RECOMMENDATIONS**

5.1. Conclusions

5.2. Recommendations

**REFERENCES**
List of Tables

<table>
<thead>
<tr>
<th>Details</th>
<th>Page No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table (1): LeishmaniaDonovanibodies in Day zero.</td>
<td>29</td>
</tr>
<tr>
<td>Table (2): Age distribution according to sex</td>
<td>31</td>
</tr>
<tr>
<td>Table (3): The Test Of Cure among patients with VL at day 22.</td>
<td>32</td>
</tr>
<tr>
<td>Table (4): Distribution of PKDL according to sex</td>
<td>34</td>
</tr>
<tr>
<td>Table (5): Distribution of PKDL according to age</td>
<td>35</td>
</tr>
<tr>
<td>Table (6): Shows the distribution of VL Mortality according to gender following</td>
<td>37</td>
</tr>
<tr>
<td>Table (7): shows the distribution of VL outcome according to age.</td>
<td>38</td>
</tr>
</tbody>
</table>
## List of figures

<table>
<thead>
<tr>
<th>Details</th>
<th>Page No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure (1): Life cycle of visceral leishmaniasis.</td>
<td>8</td>
</tr>
<tr>
<td>Figure (2): rK39 immunochromatographic dipstick test for kala-azar</td>
<td>15</td>
</tr>
<tr>
<td>Figure (3): Frequency of Gender (sex distribution)</td>
<td>30</td>
</tr>
<tr>
<td>Figure (4): PKDL among patients with VL</td>
<td>33</td>
</tr>
<tr>
<td>Figure (5): Mortality among treated outcome of patients with VL cases (n 71)</td>
<td>36</td>
</tr>
<tr>
<td>Figure (6): Follow up of DAT an rK39 (from day 0 to day 180) among patients with positive VL according to gland puncture test</td>
<td>39</td>
</tr>
</tbody>
</table>
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Stands for</th>
</tr>
</thead>
<tbody>
<tr>
<td>VL</td>
<td>Visceral Leishmaniasis</td>
</tr>
<tr>
<td>PKDL</td>
<td>Post Kala-azar Dermal Leishmaniasis</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>LRG/Sudan</td>
<td>Leishmaniasis Research Group/Sudan</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>DPDx</td>
<td>Division of Parasite Disease</td>
</tr>
<tr>
<td>RES</td>
<td>Reticule Endothelial System</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebro Spinal Fluid</td>
</tr>
<tr>
<td>CL</td>
<td>Cutaneous Leishmaniasis</td>
</tr>
<tr>
<td>MCL</td>
<td>Muco Cutaneous Leishmaniasis</td>
</tr>
<tr>
<td>MSF</td>
<td>Medicines Sans Frontiers</td>
</tr>
<tr>
<td>DAT</td>
<td>Direct Agglutination Test</td>
</tr>
<tr>
<td>rK39</td>
<td>Recombinant Kala-azar antigen</td>
</tr>
<tr>
<td>IFAT</td>
<td>Indirect Fluorescent Antibody Test</td>
</tr>
<tr>
<td>LST</td>
<td>Leishmanin Skin Test</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FGT</td>
<td>Formal Gel Test</td>
</tr>
<tr>
<td>LATEX</td>
<td>Latex Agglutination Test</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra-acetic Acid</td>
</tr>
<tr>
<td>ICDDR B</td>
<td>International Center for Diarrheal Diseases and Research, Bangladesh</td>
</tr>
<tr>
<td>Kdna</td>
<td>Kinetoplast DNA</td>
</tr>
<tr>
<td>LDbodies</td>
<td><em>Leishmania Donovani</em> bodies</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>NNN</td>
<td>Novy McNeal Nicolle</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster deviation</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed Type Hypersensitivity</td>
</tr>
<tr>
<td>SSG</td>
<td>Sodium Stibogluconate</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo-dalton</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
</tbody>
</table>
1. Introduction

2. General Introduction:

Leishmaniasis is a vector-born disease that is caused by obligated intra macrophage protozoa of the genus Leishmania (Herwald, 1999) which multiply in the phagocytic mononuclear system cells (Leishman, 1903). Natural transmission of leishmania is carried out by a certain species of sand fly of the genus phlebotomus [Old World] or Lutzomyia [New World] (Leishman, 1903).

The disease is characterized by both diversity and complexity; it is caused by more than 20 leishmanial species and is transmitted to humans approximately 30 different species of phlebotomine sand flies which are endemic in large areas of the tropics, subtropic and the Mediterranean basin (Herwald, 1999).

Leishmaniasis consists of four main clinical syndromes: cutaneous leishmaniasis; muco–cutaneous leishmaniasis [also known as espundia]; visceral leishmaniasis [also known as kala-azar; or known as blackness]; and post kala-azar dermal leishmaniasis [P.K.D.L.]. In cutaneous leishmaniasis the patient generally present with one or several ulcers or nodules in the skin. Different species of leishmania can infect macrophages in the dermis, with variable clinical presentations and prognosis (Arevalo et al., 2007) (Dedet and Pratlong, 2003). The ulcers heal spontaneously—although slowly—in immune competent individuals, but can cause disfiguring scars. In muco-cutaneous leishmaniasis, patients suffer from progressively destructive ulcerations of the mucosa, extending from the nose and mouth to the pharynx and larynx. These lesions are not self-healing and are usually seen months or years after episode of cutaneous leishmaniasis, when the macrophages of the naso-oropharyngeal mucosa become colonized. Leishmania braziliensis is responsible for most cases of muco-cutaneous leishmaniasis. VL is a systemic disease that is fatal if
left untreated and is caused by the *Leishmaniadonovani* complex – *L.donovani* sensu stricto in East Africa and the Indian subcontinent and *Liesenmannia infantum* in Europe, North Africa and Latin America (Lukes et al., 2007) (Mauricio et al., 2000). There are two types of VL, which differ in their transmission characteristics: zoonotic VL is transmitted from animal to vector to human and anthropoontic VL is transmitted from human to vector to human. In the former, humans are occasional hosts and animals mainly dogs, are the reservoir of the parasite (Alvaret al., 2004). Zoonotic VL is found in areas of *L.infantum* transmission whereas anthropoontic VL is found in areas of *L.donovani* transmission.

PKDL is characterized by amacular, maculo-pupular or nodular rash and is a complication of VL that is frequently observed after treatment in Sudan and more rarely in other East Africa countries and in the Indian subcontinent (Zijlstreet et al., 2003). It can also occur in immunosuppressed individuals in *L.infantum*-endemic areas. The interval between treated VL and PKDL is 0-6 months in Sudan and 6 months to 3 years in India. PKDL cases are highly infectious because the nodular lesions contain many parasites (Bengal, 1992), and such cases are the putative reservoir for anthropoontic VL between epidemic cycles.
1.1. **Problem identification and Justification:**

Visceral leishmaniasis is one of the most common diseases in Sudan, particularly in Gedaref Area. Due to the high incidence of VL in the area of the study, more efforts are needed for improvement and evaluation of the techniques used in the diagnosis. Serological methods are more practical and need little training. Follow up of cases using serological methods is logical.

1.2. **Objectives**

1.2.1. **General objective:**

To compare and evaluate two commonly serological techniques used for diagnosis of the visceral leishmaniasis.

1.2.2. **Specific objectives:**

- To parasitologically diagnose V.L by Lymph gland puncture using microscopically examination.
- To determine the using of DAT and rK39 in parasitological.
- To assess the performance of DAT and rK39 in Diagnosis of VL and follow up.
- To assess the incidence of PKDL.
- To evaluate the DAT and rK39 in Test of Cure (TOC) and follow up.
- To determine the sensitivity and specificity of the DAT and rK39.
- To assess the incidence of the disease in Gedaref area.
- To suggest a policy for VL diagnosis.
2. Literature Review:

2.1. Background:

The group of diseases known as leishmaniasis is caused by obligate intracellular protozoa of the genus *Leishmania* (Leishman, 1903). Natural transmission of leishmaniasis is carried out by a certain species of sandfly of the genus *Phlebotomus* (Old World) or *Lutzomyia* (New World). These are present in three different forms: (i) visceral leishmaniasis (VL), (ii) cutaneous leishmaniasis, and (iii) mucocutaneous leishmaniasis. The visceral form, also known as black sickness or kala-azar in Asia, is characterized by prolonged fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anemia, pancytopenia, and hypergammaglobulinemia and is complicated by serious infections. It is the most severe form of the disease and, left untreated, is usually fatal. Although confirmed cases of VL have been reported from 66 countries, 90% of the world's VL burden occurs on the Indian subcontinent and in Sudan (Desjeux., 1999) (Bora, D. 1999) (Seaman et al., 1996)( WHO. 1995). After recovery, some patients (50% in Sudan and 1 to 3% in India) develop post-kala-azar dermal leishmaniasis (PKDL), which requires prolonged and expensive treatment (Ramesh and Mukherjee, 1995) (Zijlstra et al, 1992). PKDL patients also play an important role in VL transmission (Thakur and Kumar, 1992). VL is typically caused by the *Leishmanialdonovani* complex, which includes three species: *L. donovani*, *Leishmania infantum*, and *Leishmania chagasi*. The clinical features of VL caused by different species are different, and each parasite has a unique epidemiological pattern. On the Indian subcontinent, the disease is almost exclusively caused by *L. donovani*. The initial report of *Leishmaniatropica* causing VL in India (Sackset al., 1995) was refuted by us and others (Sundar et al., 2001)(Singhet al., 1996). *L. infantum* is responsible for VL in children in the Mediterranean basin. However, due to increasing prevalence of human immunodeficiency virus (HIV) infection in this region, HIV-VL co-infection in the adult population is being reported frequently. *L. chagasi* causes VL in children in Latin America, where lymphadenopathy is a dominant clinical feature. *L. tropica*, the causative organism of Old World cutaneous leishmaniasis, is reported to produce visceral disease in nonimmune persons (Magillet et al, 1993). Similarly, visceralization by *Leishmaniaaamazonensis*, has also been reported (Herwaldt, 2001). Clinical
manifestations of all forms of VL change from time to time, and this is the case more so in AIDS patients (Berheet et al, 1992) (Desjeux, 1999) (Marsden and Jones, 1985) (Munoz-Rodriguez, 1997). (Thakuret et al., 2001).

2.2. Reservoir:

Natural infection was found in 2 rodent species (Arvicanthisniloticusluctosus, the Nile grass rat) and Acomysalbigena (the spring mouse) and 2 carinvores (Genetag.Senegalensis(the Senegal genet in the civet family) and Felis Phillips (the Sudanese equivalent of cat family). It was also suggested that other animals such as hyenas and jackals could not be ruled out as potential reservoirs. Extensive work by Leishmaniasis Research Group/Sudan (LRG/Sudan; Elnaem et al), using simple and sophisticated techniques failed to detect the parasite in a wide range of wild and domestic animals. The role of anthroponotic transmission cannot be ruled out(El-Hassan and Khalil, 2000).

2.3. Vector:

Sandfly species and subspecies of Phlebotomus in the Old World (Asia, Africa and Europe) and Lutzomyia in the New World (Central and South America) are the only proven vectors of Leishmania (WHO, 1990).In the Central and South America Lutzomyialongipalpis the main vector for transmission of Kala-azar. In the Mediterranean region, Phlebotomusperniciousus and Phlebotomusariasiare important vectors while in China PhebotomuschinensisandPhlebotomusalexandriare the proven vectors. In East Africa including Sudan Phlebotomus martini and Phlebotomusorientalisare considered as vectors for the diseases. In India, for the anthropologic form of Kala-azar the proven vector is Phlebotomusargentipes.Besides these many other species have been implicated as vectors of leishmaniasis(WHO, 1990; Swaminathanet al., 1942).

2.4. History:

The term leishmaniasis refers to various clinical syndromes caused by obligate intracellular protozoa of the genus Leishmania. Sir William Leishman (British Army Pathologist) reported about the parasite in 1903 and in the same year the discovery
was verified by Captain Charles Donavan (Professor of Physiology at Madras University in India). Henceforth the parasite was named as the Leishman-Donovan body or \textit{Leishmania}Donovani (Gibson, 1983).

Kala-azar carries a high mortality rate ranging from 80\% to 100\% and even with treatment, case fatality rates in excess of 10\% are common (Salam \textit{et al.}, 2009). Leishmaniasis development depends on several risk factors such as malnutrition, immunosuppression, age, immunological status and genetic factors (Assimina\textit{et al.}, 2008). The risk of Kala-azar was highest for people in the 3 to 14 years and 15 to 45 years age groups (Bern \textit{et al.}, 2005). Kala-azar mainly affects infants and young children and male sex predominance (Uzair \textit{et al.}, 2004). Kala-azar is typically caused by the \textit{Leishmaniadonovani} complex, which includes three species: \textit{L. donovani}, \textit{Leishmaniainfantum}, and \textit{Leishmaniachagasi}. On the Indian subcontinent, the disease is almost exclusively caused by \textit{L. donovani}. The initial report of \textit{Leishmaniatropica} causing Kala-azar in India was refuted by us and others. \textit{L. infantum} is responsible for Kala-azar in children in the Mediterranean basin (Begum \textit{et al.}, 2002).

It is transmitted to man by phlebotomus (sandfly) in the Old World and Lutzomyia in the New World Sergentomyia is another vector found in Baluchistan (Brito\textit{et al.}, 2000; EI-Hassan \textit{et al.}, 1995). Occasional nonvector transmissions also have been reported through blood transfusions, sexual intercourse, organ transplants, excrements of dogs, and sporadically outside endemic areas. Congenital Kala-azar was described first in 1926 by Low and Cooke (Christophet \textit{et al.}, 1999). The incubation period is 3-6 months, though 10-24 years is also reported (Arias \textit{et al.}, 1996). The disease is characterized by fever, hepatosplenomegaly, anaemia, leucopenia and hypergammaglobulinemia. Serious complications are cancrumoris, dysentery, pneumonia, anaemia, agranulocytosis, jaundice, severe haemorrhage and anasarca. Pulmonary tuberculosis may occur with Kala-azar (Cruz \textit{et al.}, 2006).

\textbf{2.5. Life cycle:}

The life cycle of Leishmaniaspecies is commonly viewed as consisting simply of two different morphological stages: the intracellular amastigote in the vertebrate host and the extracellular promastigote in the invertebrate host (Molyneux and Killick-Kendrick, 1987; Hommel, 1999). Briefly, the female sandfly picks up infected cells from the skin with their blood meal. The amastigotes are released in the midgut of the insect, transform to
the procyclic stage and start multiplying actively without penetrating the hemocoele. After few days, numerous procyclic conquer the gut of the insect. Then the elongated procyclic promastigote attach to the midgut epithelium by inserting their long flagella between the microvilli that line the midgut. They migrate to the cardiac valve, where they transform into short, spherical, non-dividing promastigotes. Then the parasites are released from the midgut and penetrate the pharynx (proboscis) as metacyclic promastigotes, also termed paramastigote. From proboscis the metacyclic promastigotes are ousted to the new mammalian host. Metacyclic promastigotes enter the skin of the vertebrate host when the infected sandfly takes its blood meal. It may inoculate 10-200 promastigotes into the dermis. Within the macrophages and related cell types, they rapidly transform into amastigotes, remain within the phagocytic vacuole, where they develops and multiply, at some stage this infected cell, which may harbour up to 20 or more amastigotes, bursts and released free amastigotes, which infect other cells. Infected macrophages move from the skin to other tissues, infecting the spleen, liver and bone marrow, while certain parasites exhibit a specific tropism for each given host e.g. viscerotropism or dermatotropism. The features that control this tropism has not yet been elucidated but are thought to include, both host, and parasite genetics as well as the status of immunity (Molyneux and Killick-Kendrick, 1987; Hommel, 1999). Most leishmaniasis are zoonotic (transmitted to humans from animal, reservoir hosts), and humans become infected only when accidentally exposed to the transmitting sandflies. However, in the anthroponotic form (those transmitted from human to human through the sandfly vector), humans are probably the sole reservoir host (WHO, 1996). The reservoir of L. infantum and L. chagasi usually dog, but in several Old World and New World foxes, rats, opossum or raccoon dogs may also act as reservoirs in some areas. The reservoir of L. donovani is mainly humans (Hommel, 1999), although other species are incriminated in Africa e.g. Arvi canthis niloticus in Sudan (El-Hassan et al., 1995). Domestic dog is incriminated as a reservoir host of American cutaneous leishmaniasis caused by L. braziliensis, L. panamensis, and L. peruviana (Reithinger & Davies, 1999). Gerbils (Psammomys obesus) have been implicated as a reservoir host of L. major in different Asian countries (Elbibhariand El-Hassan, 1987; Rioux et al., 1990; El-Sibae et al., 1993). Also Psammomys obesus is suspected as a reservoir host of L. major in Egypt (Morsy et al., 1996).
Figure (1): Life cycle of visceral leishmaniasis. Image and information courtesy of Division of Parasite Disease DPDx 2013
2.6. Morphology:

In man this organism is always in leishmania stage, appearing as an ovoidal or rounded body, measuring about 2-3µl in length, and living interacellularly in monocytes, polymorph nuclear leukocytes or endothelial cells in preparations stained with Giemsa or Wright, the cytoplasm is pale blue in colour and there is limiting membrane. Lying within the cytoplasm is a relatively large nucleus which stained red, while at right angles to it is a deep red or violet red, like body (Para basal body).

In well-stained specimens a dot-like blepharoplast may be seen near the Para basal body, as well as a delicate thread connecting the two organisms and axoneeme which arises from the blepharoplast and extends to the anterior top. An elongated slender form, so called (trouped form), may also be observed in man, having pointed or rounded ends, with the nucleus and kinetoplast closely associated.

In cultures in NNN medium leptomonas forms develop which have a single flagellum and possess marked motility. The average length of fully developed organism is 15-25µl and the breadth is 1.5-3.5 µl while the flagellum measures 15-28µl in length.

Short, broad, flagellated forms also occur, while rounded lepotomonas form, 4-5 µl in diameter, but with along flagellum may also be present,(Molyneux D.H, et al.,1987).

2.7. Location in the human host:

The natural habitat of L. donovaniin man is the reticuloendothelial system (RES) of the visceral, especially of the spleen, liver, BM, intestinal mucosa and mesenteric lymph nodes. The parasite may be found in endothelial cells of the kidneys, suprarenal capsules, lungs, meninges and in cerebrospinal fluid (CSF). Not infrequently the organisms are present in considerable numbers in macrophages in the intestinal wall and also been found in faces, urine, circulating blood, and in nasal secretions of patients (WHO, 1990).

2.8. Epidemiology of VL in Sudan:

Sudan is considered as one of the most important areas of leishmaniasis in the world where sharp epidemics involving thousands of people with many deaths were recorded. All forms of leishmaniasis i.e. CL, MCL, VL and PKDL occur in Sudan. This group of diseases causes serious economic loss in the country, both in terms of the disability of affected individuals and in the cost of treatment, especially as most of
those with leishmaniasis are on low incomes and live in rural areas. Visceral leishmaniasis (VL) is the one of the most important endemic diseases in the country and is known to occur in the Sudan since 1904 when Neave (1904) described the first patient in the country. The main endemic area is in the eastern part of the country, from the banks of the White Nile in the West to the Ethiopian border in the East, and from Kassala in the North towards Malakal in the South. Other smaller foci have also been described in Kapoeta in Equatoria and parts of Kordofan and Darfur provinces. Occasional severe outbreaks occur, like the one in the southern Fung in Blue Nile province in 1956-1960, which caused thousands of death (Sati, 1958). An outbreak of kala-azar was reported in Khartoum among displaced people (de Beer et al, 1990). Also, epidemics have occurred in recent years in war zones of southern Sudan where about 100,000 people died of leishmaniasis since 1984 (Seaman et al, 1996). Recently Roberts et al. (2000) reported that more than 10% of the population in southern Sudan died from visceral leishmaniasis over the past 5 years. Zeese and Frank (1987) estimated that of the total number of 1300 patients reported annually in the Sudan, more than 75% were treated in the hospitals of Gedaref and Hawata, a small rural town ∼100km south to Gedaref, situated along the Rahad River. L. donovanisensolatois incriminated as the aetiological agent of VL (Hoogstraal and heyneman, 1969; El- Hassan et al., 1995

2-9 HIV-Leishmaniaco infection

Atypical clinical presentations of VL in HIV-infected patients pose a considerable diagnostic challenge. In fact, the clinical trial of fever, splenomegaly, and hepatomegaly is found in less than half of such patients, though more so in patients with low CD4 counts (<50 CD4 cells/mm3). In these patients, leishmaniasis can present with gastrointestinal involvement (stomach, duodenum, or colon); ascites; pleural or pericardial effusion; involvement of lungs, tonsils, and skin; and even as widely disseminated disease, the diagnostic principles remain essentially the same as those for non-HIV-infected patients. The presence of amastigotes may be demonstrated in buffy coat preparation. Sometimes the presence of amastigotes in unusual sites may be demonstrated (e.g., amastigotes may be present in specimens from bronchoalveolar lavage, pleural fluid, or biopsy specimens from the gastrointestinal tract). For HIV patients, the sensitivity of antibody-based
immunologic tests like the IFA test and ELISA is low and the parasite load is quite heavy in these patients, the presence of leishmania amastigotes in the bone marrow can often be demonstrated, but there are well-described instances in the literature where amastigotes were not demonstrable on bone marrow, though they were found at unexpected locations like the stomach, the colon, or the lungs. PCR analysis of the whole blood or its buffy coat preparation may prove a useful screening test for these patients, obviating the need for traumatic procedures (WHO, 1996)

2.10. Diagnosis of VL:

Clinical diagnosis.
Laboratory diagnosis.

2.10.1. Clinical aspects and diagnosis:

2.10.1.1. The main symptoms & signs of VL are:

- Prolonged, irregular fever with or without rigors.
- Enlarged spleen, which is soft at the start of the disease later it can become hard.
- Weight loss that may progress to wasting.
- Enlarged lymph nodes.
- Anemia that is secondary to the chronic illness, with iron deficiency features.
- Cough.

About half of the Sudanese Kalaazar patients have hepatomegaly, nasal bleeding, diarrhea and vomiting. Few patients show oedema and jaundice. Other signs and symptoms are insomnia, arthralgia, ascites, uveitis. Patients become gradually ill over a period of a few months, and nearly always die if not treated.

2.10.2. Laboratory Diagnosis of VL:

After taking a proper history and performing a thorough clinical examination the following laboratory test can be of help in eliciting the diagnosis:

- Parasitological diagnosis (microscopical).
• Serological diagnosis.
• Culture
• Molecular

2.10.2.1. Parasitological diagnosis:
This is a minimally invasive laboratory diagnosis with variable sensitivities on aspiration from spleen, bone marrow [BM] and lymph node. It is the gold standard in the diagnosis in almost all settings. The time from sample taking to diagnosis is less than 60 minutes. With minimum training laboratory technicians/laboratory assistants can master reading the slides. The sensitivity of microscopy on lymph node ranges from 52-65%, while BM sensitivity can reach up to 75% (Kirl, 1956) (Satti, 1958). Although splenic aspiration sensitivity is 90-95%, Negative slides are not a proof of absence of the parasites; low parasites density can be missed microscopically.

1.10.2.2. Serology:
Several immunological blood tests that identify antibodies against leishmania are available:-

• Direct Agglutination Test (DAT).
• Recombinant Kala-azar antigen (rK39).
• Indirect Fluorescent Antibody Test (IFAT).
• Leishmania Skin Test (LST).
• Enzyme-Linked Immunosorbent Assay (ELISA).
• Formal Gel Test.
• Latex Agglutination Test (Katex).

2.10.2.2.1. Direct Agglutination Test (DAT):
It is a highly specific, sensitive and simple test that can be easily performed under field circumstances. This technically not very difficult but training and standardization are needed to be executed. The test measures the serological responses to a surface born antigens of whole parasite. Improvement of DAT for field study has been achieved in 1988 by Harith et al; which revealed a specificity of 98.8% and sensitivity of 100%. Significant titre for the test is ≥1:60. It has been realized that the DAT can remain positive for up to five years following treatment.
of VL. Some individuals in endemic areas can have significant titers without previous history of clinical disease (sub clinical infection). In 1986, El-Harith and his co-workers at the KIT developed a Direct Agglutination Test (DAT) for the detection of anti-\textit{Leishmania} antibodies in serum samples from humans and dogs suspected for visceral leishmaniasis. The DAT is a simple test with a high sensitivity and specificity, easy-to-perform and does not require specialised equipment. Results can be read visually within 18 hours. The test is the first line diagnostic tool in many countries; a problem with the DAT is the limited stability of the used aqueous antigen for which cold storage is required (Medicines sans forentieres, 1999). A freeze-dried antigen has been developed by KIT Biomedical Research that remains stable at ambient temperature (\(20^\circ\) - \(45^\circ\) C) for at least 1 year and compares well under laboratory conditions (Meredith \textit{et al} 1995) and under field conditions (Zijlstraet \textit{et al}., 1997) (Oskamet \textit{et al}., 1999) with the standard aqueous antigen. The DAT test should only be performed and interpreted by appropriate trained operators. The diagnosis visceral leishmaniasis may never be based only on the results of the DAT. The results of the DAT should be judged judiciously in connection with clinical, epidemiological and other diagnostic data. Sensitivity and specificity of the DAT were determined on a limited number of healthy subjects, patients with visceral leishmaniasis and patients with various other diseases. Therefore 100% sensitivity and specificity are not claimed and cross-reactions cannot be fully excluded. The cut-off value of the test must always be determined using appropriate local endemic controls.

\textbf{2.10.2.2.2. Recombinant Kala-azar antigen (rK39):}

It is cloned of 39 amino acids repeats of a gene found in \textit{L.chagasi}. In Brazil, antibodies against rK39 antigen could be detected in about 98% of cases. The rK39 in the dipstick format is a rapid and easy to perform diagnostic test. In India a reported sensitivity of 100% and specificity of 98%. This test has potential (simple to read, rapid) but has had limited field testing in Sudan.

Further, it is costs about ($1/test), depending on the number of tests ordered. New improved test are under evaluation.

Now a rapid dipstick test based on the recombinant K39 protein is available for rapid diagnosis of kala-azar. rK9is an epitope apparently conserved on
amastigotes of Leishmania species that cause visceral infection; by use of laboratory ELISA testing, circulating anti-K39, IgG is detectable in 95%-100% of patients who have kala-azar, irrespective of geographic region. Using rK39 antigen-impregnated nitrocellulose strips developed for field conditions, fingersprick-obtained blood and serum samples tested from Indian subjects demonstrated a positive anti-K39 immunochromatographic reaction in 362 patients with aspirate-proven kala-azar; with an estimated sensitivity of 100% and a specificity of 97%. The strip testing proved simple to perform and yielded results within five minutes (Herwald, 1999). The rapid diagnostic test when evaluated in comparison to the diagnostic performances of DAT, based both on freeze-dried and liquid antigens, on parasitologically confirmed Kala-azar and Post-Kala-azar Dermal Leishmaniasis (PKDL), the sensitivity of the tests was almost 100%, rK39 was found, to be more sensitive. It was concluded that these tests are comparable to parasitology in terms of their sensitivity and can replace parasitology as the basis for a decision to treat visceral leishmaniasis at peripheral health centers in endemic areas. Considering the field evaluation results, the expert committee under the Chairmanship of Dr. S.P. Agarwal, considered that the rapid dipstick test based on rK39 can be introduced into the Kala-azar elimination programme.

The recombinant antigen is a 39-amino acid (rK39) cloned in Escherichia Coli, from the C terminus of the kinesin protein of Leishmania major in India. The rK39 rapid diagnostic test has undergone extensive evaluation and has been found to be highly sensitive and specific in the diagnosis of both VL and PKDL. Kala-azar Case Definition for enrolling a subject for dipstick testing is as follows, a case presenting to a clinician with a fever of more than two weeks duration, with splenomegaly and not responding to the full course of anti-malaria, should be subjected to rK39 dipstick.
Immunochromatographic strip rK39 test for detection of VLA recombinant antigen, rK39 has been shown to be specific for antibodies in patients with Kala-azar caused by members of the *L. Donovani* complex (Badaro et al., 1996; Bern et al., 2000; Burns et al., 1993). This antigen, which is conserved in the kinesin region, is highly sensitive and predictive of the onset of acute disease. The antigen is derived from *L. chagasi*, which in the United States is used for veterinary purposes, thought it is not approved for human use. High antibody titers in immunocompetent patients with Kala-azar have been demonstrated. This antigen has been reported to be 100% sensitive and 100% specific in the diagnosis of Kala-azar and PKDL by ELISA (Kumar et al., 2001; Singh et al., 1995). Another important fact of anti-rK39 antibody is that the titer correlates directly with the disease activity, indicating its potentiality for use in predicting response to chemotherapy. It was previously shown that anti-rK39 antibody titers were 59-fold higher than those of antibody against CSA at the time of diagnosis, and with successful therapy, it fell sharply at the end of treatment and fell further during follow-up monitoring. In patients who experience disease relapse, the titer rose steeply again (Kumar et al., 2001). The diagnostic and
The prognostic utility of rK39 for HIV-infected patients has also been demonstrated (Houghton *et al.*, 1998). Because of the conditions prevailing in areas of endemicity, any sophisticated method cannot be employed on a wider scale. There is a need for a simple rapid and accurate test with good sensitivity and specificity, which can be used without any specific expertise. A promising ready to use immune chromatographic strip test based on rK39 antigen has been developed as a rapid test for use in difficult field conditions. The recombinant antigen is immobilized on a small rectangular piece of nitrocellulose membrane in a band form and goat-anti-protein A is attached to the membrane above the antigen band. After the finger is pricked, half a drop of blood is smeared at the tip of the strip, and the lower end of the strip is allowed to soak in 4 to 5 drops of phosphatebuffered saline, placed on a clean glass slide or tube. If the antibody is present, it will react with the conjugate (protein A colloidal gold) that is predried on the assay strip. The mixture moves along the strip by capillary action and reacts with rK39 antigen on the strip, yielding a pink band. In the strip of patients who are infected, two pinkish lines, appear in the middle of the nitrocellulose membrane the upper pinkish band serves as a procedural control. In the first extensive field trial in 323 patients, we found the strip test to be 100% sensitive and 98% specific (Sundar *et al.*, 1998). Several studies from the Indian subcontinent reported the test to be 100% sensitive (Bern *et al.*, 2000; Sundar and Rai, 2002; Sundar *et al.*, 1998). However, when evaluated in Sudan, the sensitivity of the test was only 67%. In the Sudan study, all the parasitologically confirmed Kala-azar patients who tested negative by the rK39 strip test showed IgG against rK39 by micro ELISA at lower titers (Zijlstra et al., 2001). In a study done in southern Europe, the rK39 strip test results were positive in only 71.4% of the cases of Kala-azar (Jelinek *et al.*, 1999). These differences in sensitivity may be due to differences in the antibody responses observed in different ethnic groups (Singh *et al.*, 1995). When tested for PKDL, the test had 91% sensitivity (Salotra et al., 2001). High levels of specificity (97 to 100%) have been reported uniformly for this test; however, with a later version of the rK39-treated strips, some (12.5%) healthy endemic control subjects also tested positive (Sundar and Rai, 2002). While such reactions might be considered to be false positive, these probably represent subclinical infections: PCR assay for *L. donovani* was positive in a few of these cases (Salotra et al., 2001; Sundar and Rai, 2002). Anti-rK39 IgG may be present in serum for an extended period after successful treatment for Kala-azar; thus, patients with suspected relapse of Kala-azar with a past history of infection
would not be candidates for diagnosis by strip testing. Another drawback of this format is that an individual with a positive rK39 strip test result may suffer from an illness (malaria, typhoid fever or tuberculosis) with clinical features similar to those of Kala-azar yet be misdiagnosed as suffering from Kala-azar. Besides these limitations, the rK39 ICT strip test has proved to be versatile in predicting acute infection. It is the only available format for diagnosing Kala-azar with acceptable sensitivity and specificity. It is also inexpensive and simple (Sundar and Rai, 2002).

2.10.2.2.3. Indirect Fluorescent Antibody Test (IFAT):

It is one of the most sensitive tests available. The test is based on detecting antibodies, which are demonstrated in the very early stages of infection and are undetectable 6-9 months after cure. If the antibodies persist in low titers, it is good indication of a probable relapse. Titers above 1:20 are significant and above 1:128 are diagnostic. There is a possibility of a cross reaction with trypanosomal sera. However, this can be overcome by using Leishmania amastigotes as the antigen instead of the promastigotes (Williams et al., 1995). Although this test is more sensitive (96%) and specific (98%) than soluble antigen ELISA, it is cumbersome and not suitable for field conditions (Sassi et al., 1999).

2.10.2.2.4. Leishmanin Skin Test (LST);

Delayed type hypersensitivity (DTH) or T-cell-mediated immunity is a group-specific immune response. The Montenegro skin test is a test for DTH specific to leishmaniasis, but its role is limited (Weigl et al., 1987)(Meheus et al. 1013). In this method, 0.5 ml of phenol-killed whole parasites (5 × 10^7 promastigotes) is injected on the volar aspect of the forearm of the patient. After 48 to 72 hrs, the size of induration is measured and compared with the size of induration produced by injection of a phenol-saline control in the other forearm. Presently, there is no available standardized leishmanin reagent. All leishmanins are said to be alike and nonspecific. The test is negative in acute cases of VL due to the absence of DTH and is positive only in cases where kala-azar has been cured (Weigl et al., 1987) (Desjeux et al., 2012). No cross reactions occur with Chagas disease, but some cross-reactions are found with cases of glandular tuberculosis and lepromatous leprosy. LST is usually
used as an indicator of the prevalence of cutaneous and mucocutaneous Leishmaniasis in human and animal populations and successful cure of VL (Weigle et al., 1987. Manson et al., 1987) During active Kala-azar disease there will be no or negligible cell mediated immune response.

2.10.2.2.5. Enzyme-linked immunosorbent assay (ELISA);

Enzyme-linked immunosorbent assay (ELISA), is specific 100% with sensitivity reaching 98%. The antigen is prepared from promastigotes of *L. donovani* and the test can be carried out on plasma, serum, blood spots or whole blood.

2.10.2.2.6. Formal gel test;

It is advantage is that; cheap and simple to perform. Serum obtained from 5 ml of blood is mixed with one drop of 40% formaldehyde. A positive reaction is shown if the mixture solidifies and forms a white opaque precipitate within 20 min. A positive test cannot be detected until 3 months after infection and becomes negative 6 months after cure. The test is not specific since it is based on detection of raised levels of IgG and IgM which also result from other infection such as African trypanosomiasis, malaria and schistosomiasis.

2.10.2.2.7. Latex agglutination test;

This has been developed to detect the leishmania antigen in the patients-urine. It is simple, rapid, economical and most suitable for use in remote areas. The importance of this test is that, the antigen level starts to decline very quickly after treatment so it can detect active cases, and a good test of recovery. Antigen detection is more specific than antibody-based immunodiagnostic tests (El-Harith et al., 1988). This method is also useful in the diagnosis of disease in cases where there is deficient antibody production (as in AIDS patients). De Colmenares et al. from Spain have reported two polypeptide fractions of 72-75 kDa and 123 kDa in the urine of kala-azar patients. The sensitivities of the 72-75-kDa fractions were 96%, and the specificities were 100%. Besides, these antigens were not detectable within 3 weeks of anti-kala-azar treatment, suggesting that the test has a very good prognostic value (El-Harith et al., 1988). A new latex agglutination test (KATEX) for detecting leishmanial antigen in urine of patients with VL has showed sensitivities between 68 and 100% and a
specificity of 100% in preliminary trials. The antigen is detected quite early during the infection and the results of animal experiments suggest that the amount of detectable antigen tends to decline rapidly following chemotherapy. The test performed better than any of the serological tests when compared to microscopy. Large field trials are under way to evaluate its utility for the diagnosis and prognosis of VL (Lukes et al., 2007).

2.2.3. Culture:
One hundred micro liters of BM aspirate dilution was cultured in NNN medium at 27 C and examined by light microscope every week for promastigote forms before sub culturing with fresh medium. Subcultures are performing for 4 weeks before a negative result is returned.

2.2.4. Molecular probes (DNA detection method);

Diagnostic tests of VL based on serology are amongst other draw-backs, made less reliable due to cross-reaction with other microorganism. Competition ELISA is making use of monoclonal antibodies and is more specific but cannot distinguish between past and present infection. Recently molecular probes, using Kinetoplast DNA (Kdna), ribosomal RNA (rRNA), mini exon derived RNA (med RNA), and genomic repeats have been highly diagnostic due to a much higher sensitivity and specificity. Due to the limitations inherent in techniques used for detection of parasites, new approaches to the detection of parasites, such as DNA hybridization, have been attempted since the early 1980s. Although these methods had considerable sensitivity (detecting as few as 50 to 100 parasites) (Sundwe et al.,), their potential use in routine diagnosis is hampered by the complex procedure of hybridization. The development of PCR has provided a powerful approach to the application of molecular biology techniques to the diagnosis of leishmaniasis. Primers designed to amplify conserved sequences found in minicircles of KDNA of leishmaniasis of different species were tested in various tissues of relevance. Such a target was eminently suitable because the kinetoplast is known to possess thousands of copies of
minicircle DNA. In recent years, PCR-based diagnostic methods with a wide range of sensitivities and specificities have been described (Dedet al., 2003).

2.2.3. Clinically suspected case:

Any patient who lives in (or traveled to) an endemic area (Gedaref, Upper Nile, Unity, Sennar, Blue Nile, South Kordfan and Darfour States) presenting with fever of more than two weeks duration in whom malaria was ruled out, with one of the following signs: Splenomegaly, wasting and/or lymphadenopathy.

2.2.4. Confirmed case:

There are two accepted ways of confirming Kala-azar in a clinically suspected case either by parasitology or serology.

Parasitology is the gold standard diagnostic method in suspected/relapsed cases. A positive Direct Agglutination Test (DAT) with a titer of \( \geq 1:60 \) combined with a negative Leishmania skin test (LST) in a clinical suspect is accepted as a confirmation test, after repetition of the lymph node/bone marrow/splenic aspirate after 10-14 days. It has been clearly appreciated that the DAT test can be positive in some individuals in the endemic areas.

2.2.5. The choices for obtaining the parasite as follow:

The first choice will be either from lymph node as it is safe, easy and can be done by paramedicals, or from BM; it is painful, need specific needle and trained personnel.

The second option is from a spleen; it is most sensitive procedure but it is the most hazardous one, need an experienced medical doctor and should be performed in hospital settings, where blood transfusion is available, because bleeding is a life-threatening complication.

2.2.6. Treatments:

Accurate parasitological diagnosis is essential in leishmaniasis to determine the correct treatment. Some infections, especially simple cutaneous lesions due to L. major, are often self-healing and induce immunity to reinfection and treatment of these is generally not recommended, unless the lesions do not heal within 6-9 months.

In case of chronic lesions due to L. tropica the treatment is based on pentavalent antimonials intramuscularly or intravenously at 10-20 mg/kg/day until
cure. Treatment of other forms, such as VL and MCL infections, mainly relies on the pentavalent antimonials sodium stibogluconate (Pentostam) or meglumine antimoniate (Glucantime), the first-line drugs except when resistance exists, and the usual dose is 20 mg/kg/day for 30 days. They are expensive and need to be given by injection. The second-line drugs in case of resistance - amphotericin B and pentamidine, used in cases unresponsive to antimonials, need careful management to avoid serious side effects. They are used intravenously over several days on alternate days from 0.1 mg/kg/day up to 1 mg/kg/day with a maximum total dose of 3g. For VL, aminosidine, alone or in association with pentavalent antimonials, has shown good efficacy but it is still under evaluation. Amphotericin B, included in liposomes, has proven to be very efficient but its use is still limited and expensive (Baily & Nandy, 1994; Desjeux, 1996; WHO, 1998).

2.2.6.1 Initial non-responder:

Extended SSG treatment; 20 mg/kg daily IM or IV up to 30 days total until two consecutive TOC is negative. Follow up after 3 months. Always check the ECG at regular intervals. If no response consider second line drugs (Ambosome).

First relapse: SSG 20 mg/kg/daily for 30-60 days.
Second relapse: Ambisome (Dose as above).
Third relapse; the patient should be thoroughly investigated for concurrent disease (e.g. T.B). Combination therapy should be contemplated.

Load transfusion is available because bleeding is a lifethreatening complication.

2.2.6.2 Drugs under investigation:

- Paromomycin (Aminosidine) PM.
- Miltefosine.

2.2.6.3. Supportive treatment for patients with VL:

Care under direct medical supervision & observation:
1- Nutritional support.
2- Multivitamins.
3- Iron sulphate/gluconate & Folic acid.
4- Tinidazole for parasitic infections.
5- Malaria prophylaxis in special circumstances.
6- Care for concurrent infection (Tuberculosis; HIV / AIDS).
7- Blood transfusion is not generally needed.

**2.2.7 Follow up:**
Follow up after treatment is very important every 3 to 6 months and self-reporting whenever symptoms return.

**2.2.7.1. Case definition according to follow up:**

**2.2.7.1.1. Non responder:**
A patient who shows no/poor clinical response after 30 days of SSG treatment and presents with a parasitological smear of grade 4+ or more.

**2.2.7.1.2. Slow responder:**
A patient who shows no clinical response after 30 days of SSG treatment and presents with a parasitological smear grade 1+, 2+, 3+, If after a second course of treatment, such a patient is still parasitologically positive (even 1+) he should be considered a non-responder.

**2.2.7.1.3. Relapse case:**
A patient with clinically and parasitologically confirmed VL within 6 months of successful treatment of VL.

**2.2.8. Test of Cure (TOC):**
The TOC is an aspiration of lymph node, BM or spleen at day 30 of treatment in primary Kala-azar patients, to obtain proof of cure (to demonstrate the presence/absence of parasites).

**2.2.8.1. Criteria of cure:**
Primary VL: clinical cure with no fever, absence/reduction in the size of the spleen, weight gain, and increase in level of hemoglobin and serum albumin levels.
2.2.9. Control:

- Early detection by serological diagnosis and treatment of infected persons, especially in areas where humans are the only or important reservoirs of infection.
- Personal protection from sand fly bites:
  - Using insect repellants, although in hot humid conditions they are of limited use due to profuse sweating.
  - Avoiding endemic areas especially at times when sand flies are most active.
- Vector control by the use of light traps, sticky paper traps, or residual insecticide spraying of houses and farm buildings where this is possible or alternatively using insecticide patients in slow-release mollifiable solution.
- Destruction of stray dogs and infected domestic dogs in areas where the main reservoir hosts are dogs.
- Elimination and control of rodents in areas where these are the sources of human infection.
- Wherever possible construct human dwellings away from the habitats of animal reservoir hosts where sand flies are known to breed, e.g.: rodent burrows or rocks where canines live (WHO, 2000).
3. MATERIALS AND METHODS

3.1. Study design:
This is a comparative prospective, descriptive health facility study.

3.2. Study area:
Gadaref area is located in South East Sudan; it is characterized by wide spaces of cultural multi-tribes. Most of populations are farmers of low or medium socio-economic status or medium and little have agricultural projects.

There are four educational and public hospitals, one private clinic in addition to 10 rural hospitals and many health centers. There are six Kala-azar centers (Spanish Project in Bazora and Um Alkhair area, MSF in Tabark Allah, Ahmed M.Elhassan Center and Kassab Center in Gadaref and belongs to the Institute of Endemic Diseases-Khartoum). The study was conducted in the Spanish Project in Bazora and Um Alkhair area.

3.3. Study population:
Patients were admitted to Gadarefrural hospitals for new and suspected cases from many places to Kala-azar center; in this study specially in SpanishProject (Bazora and Um Alkhair). Study individual were included the outpatient, they were diagnosed for VL awaiting TOC then followed up until six months after treatment.

3.4. Study duration:
This study was conducted between March 2011 and October 2011.
3.6. **Inclusion criteria:**
- Both sexes.
- All ages.
- New cases.

3.7. **Exclusion criteria:**
- Known patients (coming to follow up).
- HIV patients
- T.B patients.

3.8. **Sampling and sample size:**
The study individuals were including all outpatients which suspected for VL they were 352 suspected cases.

3.8.1. **Lymph node aspiration:**
Lymph node aspirate were taken for diagnostic purposes during the first clinical episode of VL and again at 22 days after the start of treatment. When a relapse episode was suspected, samples are taking during the Active phase for each sampling point.

3.8.2. **Collection of blood samples on filter paper (Execution of the DAT):**
- One drop of blood (obtained from finger-prick) on Whatman No3 filter paper; this is essential, do not use other types, was collected.
- The blood were leaved to dry on the filter papers.
- The filter paper samples were stored in a cool dry place preferably at –20°C.
- With a paper puncher machine 1 round were punched out of blood stored on the filter paper prior to use.
- 125µl physiological saline was added to the first column of a V-shaped micro-titre plate.

3.8.3. **Collection of Peripheral blood samples (rK39):**
After sterilization of skin about by ethanol.
Introduced 5ml sterile syringe was pushed in veins blood was collected 2.5 ml for application rk39 tests in a tube containing an anticoagulant (EDTA or
heparin). Serum was obtained by centrifugation of the blood samples at 2,000 ×g for 15 min.

3.9. Materials and Equipments:

3.9.1. Equipment:
The following equipment were used:

- Cotton.
- Slides.
- Disposable syringes.
- Gloves.
- Centrifuge.
- Compound microscope.
- Tubes.
- Oven.
- Autoclave.
- Disposable Petri dish.
- Yellow Tips.
- Blue Tips.
- Automatic pipette 1ml.
- Automatic pipette 10-100.
- V-shaped microtitre plates.
- Filter paper what man 3.
- Plaster.
- Gauze.
- Plastic box.

3.9.2. Reagents:
The following reagents are used:

- Ethanol.
- Giemsa stain.
- Cedar oil.
- Leishmania freeze-dried antigen.
- 2-mercapto ethanol.
- Distilled Water (D.W).
3.10. Methods:

3.10.1. Gland puncture for L.D.bodies:

- Commonly from less hazardous clear lymph groups (inguinal, trochelear) were taking.
- The node was grasped between thump and finger after sterilization.
- Introduced 5ml sterile syringe was pushed in to the lymph node and was withdrawn the liquid in site.
- On clean non scratched slides were aspirated and transferred.
- Thin films were makes by spread the liquid on slides.
- The films were fixed with absolute ethanol for 2 min.
- The films were stained by the Giemsastain for 10 min.
- The films were dried.
- The films were examined microscopically by using ×100 oil immersions.

3.10.2. Performing the DAT assay:

- 1 round of blood was added on filter paper (equivalent to 5 µl blood) into these wells (of the first column) and elute the blood for 8 hours (or overnight) at 4 °C.
- 50 µl serum diluents fluids were pipette to all other wells of the plates.
- 50 µl of the eluted blood were eluted in the adjacent well, mix and transfer again 50 µl to the next well. The first dilution in the second well is 1:100; the dilution in the third well is 1:200.
- Up to well 11 were repeated and gave a 1:51200 final dilutions.
- Well 12 were used as a negative control well.
- 50 µl antigens were added to each well.
- The plates were tipped gently on all sides in order to mix the content.
- The plates were leaved at ambient temperature for approximately 18 hours.
- The micro-titer plates were interpreted against a white background.
- The end-titer is the last serum dilution that still agaves a clear agglutination.
3.10.3. Procedure of rk39:

The aluminium package was opened and all the material was taken out; do not leave the material exposed to humidity and high temperature. In tropical conditions, use the test within 15 minutes after opening the aluminium package. The device was taken, and it horizontally on a flat surface, and the patients name was written on the label. The ampoule of buffer was opened, and 1 drop of buffer was added to the first well (conjugated well, made with a color line), and 4 drop of the second well (wash well). Allow to stand for 1 minute. The serum was drawn from the tube into the pipette in the same manner. The entire volume of blood, serum (8-12) were added, by squeezing the pipette gently, to the first well (conjugate well, made with a color line). The upper end of the pipette was stirring and allows standing for 1 minute. Then the pipette was discarded into a suitable waste container. The IT device apart was pulling, out of the dipstick holder and was labeled. Then placed the wells on a flat surface, the legs of the dipstick holder was inserted into the holes beside the conjugated well (with color line) so that the end was reaching the bottom of the conjugated well. 5-10 minutes were allowed. Then the (serum/conjugate) mixture should be completely soaked up. The dipstick was transferred to the second well (wash well) and allow to stand for 10 minutes (when using serum, allow to stand for 5 minutes only). The reaction field should then be completely clear of blood serum. The control band must be clearly visible. The dipstick was removed from the wash well and click it back in to the clear plastic piece. Then closed the well with the well cover, break theme off, and legs off from the clear plastic piece. And discard them in to a suitable waste container. The reaction was readied and interpreted the results.

3.10.4 Statistical analysis:

Data of the study was analyzed with Statistical Package for Social Science (SPSS).

3.10.5. Ethical issues and approvals:

3.10.6. Ethical clearance of the study was taken from Ministry Of Health (MOH) Gedaref State. Data and samples were collected after complete consent was obtained from each volunteer and guardian.
CHAPTER FOUR

4.RESULTS AND DISCUSSION

4.1 Results

This study was conducted to compare and evaluate two immunological diagnostic techniques (DAT and rk39), and follow up until 6 months (in day 0, day 22, day 90, and day 180) for TOC.

4.1.1. Result of suspected cases:

In the present study, lymph node smears of 352 patients suspected to have leishmaniasis. Out of the 352 patients, 71 lymph node smears had detected amastigotes in microscopic examination, and demonstration of parasites in smears from it based on the microscopy was used as the gold standard; Table (1).

Table (1): Leishmania Donovani bodies in Day zero in study group.

<table>
<thead>
<tr>
<th>LD bodies , Day zero</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>71</td>
<td>20.2</td>
</tr>
<tr>
<td>Negative</td>
<td>281</td>
<td>79.8</td>
</tr>
<tr>
<td>Total</td>
<td>352</td>
<td>100.0</td>
</tr>
</tbody>
</table>
4.1.2. Sex:

The ratio between Males and Females was 1.53:1. The majority of patients were Males 43(60.6%) while the remaining minorities 28(39.4%) were Female; Figure (1).

![Gender](image)

**Figure (1):** Frequency of sex distribution among the study group.
4.1.2. Age:

Patients were divided into two groups Children and Adults. The majority of the patients were children 86% while 14% of them were adults; Table (2)

Table (2): Age distribution according to sex

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Children</td>
<td>Adults</td>
</tr>
<tr>
<td>Female</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>Male</td>
<td>37</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>10</td>
</tr>
</tbody>
</table>
4.1.3. TOC:

The test of cure was of the all patients were completed cure in day 22 by using L.D.bodies detection by lymph node aspiration; Table (3)

Table (3): The Test of Cure among patients with VL at day 22.

<table>
<thead>
<tr>
<th>LD bodies, Day 22</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>0</td>
<td>0.00%</td>
</tr>
<tr>
<td>Negative</td>
<td>71</td>
<td>100.0%</td>
</tr>
<tr>
<td>Total</td>
<td>71</td>
<td>100.0%</td>
</tr>
</tbody>
</table>
4.1.4. PDKL with patients:

The majority of patients after treatment were healthy 90% while 10% of them were PKDL; figure (2).

![Pie chart showing PKDL among patients with VL in the study group.](image)

**Figure (2):** PKDL among patients with VL in the study group.
4.1.4. PDKL with Sex:

**Table (4): Distribution of PKDL according to sex**

<table>
<thead>
<tr>
<th>Sex</th>
<th>PKDL healthy</th>
<th>PKDL</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>28</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>Male</td>
<td>36</td>
<td>7</td>
<td>43</td>
</tr>
<tr>
<td>Total</td>
<td>64</td>
<td>7</td>
<td>71</td>
</tr>
</tbody>
</table>

$P$-value = 0.024
4.1.5. PKDL with age groups:

The estimation of PKDL was 100% in children patients while in adults was 0%;

Table (5)

<table>
<thead>
<tr>
<th>PDKL</th>
<th>Age groups</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Children</td>
<td>Adults</td>
</tr>
<tr>
<td>Healthy</td>
<td>54 (88.5%)</td>
<td>10 (100.0%)</td>
</tr>
<tr>
<td>PDKL</td>
<td>7 (11.5%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Total</td>
<td>61 (100.0%)</td>
<td>10 (100.0%)</td>
</tr>
</tbody>
</table>
4.1.6. The mortality:

The responsibility of patients during the follow up showed that died represented 11% of the studied group, while 89% of them were alive; figure (3).

**Figure (3):** Mortality among treated outcome of patients with VL cases (n 71)
4.1.7. Mortality with sex:

Mortality among treated outcome of patients with VL cases shows death represented 11% in the same ratio from male to female Table (6)

Table (6): The distribution of VL Mortality according to sex.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Mortality</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dead</td>
<td>Live</td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>Male</td>
<td>4</td>
<td>39</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>63</td>
</tr>
</tbody>
</table>

P-value = 0.516
4.1.8. Mortality with Age:

Mortality among treated outcome of patients with VL was 63% children and 37% was adults; Table (7)

**Table (7):** The distribution of VL outcome according to age.

<table>
<thead>
<tr>
<th>Mortality</th>
<th>Age group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Children</td>
<td>Adults</td>
</tr>
<tr>
<td>Dead</td>
<td>5 (8.2%)</td>
<td>3 (30.0%)</td>
</tr>
<tr>
<td>Live</td>
<td>56 (91.8%)</td>
<td>7 (70.0%)</td>
</tr>
<tr>
<td>Total</td>
<td>61 (100.0%)</td>
<td>10 (100.0%)</td>
</tr>
</tbody>
</table>
Figure (4): Follow up of DAT an rK39 (from day 0 to day 180) among patients with positive VL according to gland puncture test
4.2 Discussions:

Leishmaniasis affects the poorest people on the globe, and is associated with malnutrition, population displacement, poor housing, a weak immune system and lack of resources. It is linked to environmental changes such as deforestation, building of dams, irrigation schemes and urbanization. An estimated 1.3 million new cases and 20,000 to 30,000 deaths occur annually (WHO, 2014).

In the present study, lymph node smears were performed for 352 patients suspected to have leishmaniasis. Out of all suspected cases, 71 lymph node smears had detected amastigotes in microscopic exam, and demonstration of parasites in smears from it based on microscopy was used as the gold standard technique, the assessment of the incidence out of the studied group were (20.2%) and calculated incidence in Gedaref state were 51/1000.

In 2012 a study was carried out by Médecins Sans Frontières (MSF) had diagnosed and treated patients with visceral leishmaniasis (VL) in a study in Tabarak Allah Hospital, eastern Gedaref State. The VL-specific mortality rate was estimated at 0.9/1000 per year. The medical teams examined 551 individuals referred for a history of fever of at least two weeks. Out of these, 16 were diagnosed with primary VL. The overall incidence of VL over the past year was 7.0/1000 persons per year, or 7.9/1000 per year when deaths possibly or probably due to VL were included. Overall, 12.5% (11,943/95,609) of the population reported a past VL treatment episode.

This agrees with study in different regions of Sudan are known to be endemic for VL with an annual incidence of 86/1000 causes in Gedaref State (Zijlstra EEI, Elhassan, 2001), the high incidence of the study area particular in children 41/1000 due to the reason that children of poor family generally suffered from malnutrition, and different regions of Sudan are known to be endemic for VL with an annual incidence of 86/1000 causes in Gedaref State (Zijlstra and EL Hassan, 2001). Consequently their immunity is hampered increasing the risk of getting kala-azar infection and developing severe disease (Desjeux, 2004). Moreover adult population in the endemic locality might develop protective immunity from previous infection that reduces the chance of re-infection (Gregory et al., 2008).

In the present study, out of 71 cases, 43 (60.6%) were male and 28 (39.4%) were female; giving a male to female ratio of 1.54:1 (Figure 1), this a computable with
Other studies from home also recorded almost similar ratio between male to female ratio. Bangladesh and (ICDDRB, 2003) found male to female ratio of 1.2:1 and 1.04:1 respectively. Several studies were also consistent as showing male to female ratio of 1.38:1 (Sarker et al., 2003), 1.8:1 (Musa et al., 1996), 2:1 (Talukder et al., 2003) and 2.4:1 (Shamsuzzaman et al., 2003). Similar finding was reported from abroad where ratio between male to female was 1.3:1 (Antonio et al., 2002). In a study, from Sudan, it was reported that like many countries males are almost twice (1.8:1) more likely to be affected by Kala-azar than females (Zijlstra et al., 1994). Males are infected more often than females, most likely because of their increase exposure to sand flies due to professional activate (Berman, 1997).

Kala-azar affects various age groups depending on the infecting species, geographic location, and disease reservoir and host immuno-competence. In Indian type of Kala-azar, children between 5 and 15 years of age are affected more (Gradoniet al., 1995). In our study, the majority of cases (85.9%) were in the children age group (Table 1). Almost similar findings were also found in many studies; Ali and Ashford (1994) from Ethiopia in a study found 142 cases of Kala-azar, where 58% children below 15 years. In study by Nuttalet al., (2002) observed that majority patients of Kala-azar were in the age group of 5-15 years. In other study on 65 patients, over 60 cases of V.L were under 20 years of age (Talukder et al., 2003). On the other hand, higher numbers (86%) of V.L cases were found under 5 year’s children in Pakistan during the period between 1985-1995, (Rab and Evans, 1995).

The higher incidence of kala-azar in 352 in current study (20.2%) and the 51/1000 in Gedaref state while in children were 41/1000 in Gedaref area, this agree with study in different regions of Sudan are known to be endemic for VL with an annual incidence of 86/1000 causes in Gedaref State. The two serological (DAT and rK39) tests identified 53 of 71 positive VL patients with sensitivity of 75% and specificity of 79.8% at day zero in the same of each, while sensitivity decreases within the following day which makes it incapable of determining complete cure. Several studies in Sudanese patients kala-azar have show specificity estimates of the DAT between 72% and 99%, which may result (Zijlstra EEI, Elhassan, 2001). Negative result in a test with high sensitivity is useful for ruling out disease. A high sensitivity test is reliable when its result is negative, since it rarely misdiagnoses those who have the disease. A test with 100% sensitivity will recognize all patients with the disease by
testing positive. A negative test result would definitively rule out presence of the disease in a patient.

The rk39 test also showed similar findings of DAT with sensitivity of 75% and specificity of 79.8%; and 18 (25%) negative samples rk39 and positive for LD bodies by smear, these findings agree with observations reported by (Zijlstra et al. 2001), when testing serum samples from VL patients in Sudan the sensitivity of rk39 was 67% in compared with DAT. In addition to, Sundar et al. (1998), reported the sensitivity of peripheral blood samples was 100% in Indian patients for detection of L. D.bodies. These results were compatible with the study in Nepal by Bern et al. (2000).

In the present study the mortality and follow up showed that; few cases found to have PKDL (Post-Kala-azar dermal leishmaniasis) representing 10% all of them were male children, also death represented 11% of the studied group, which also mostly found among children. This is similar to WHO’s report in 2012 which mentioned that, PKDL occurs mainly in East Africa and on the Indian subcontinent, where up to 50% and 5-10% of patients with kala-azar, respectively, could develop the condition. It usually appears 6 months to 1 or more years after kala-azar has apparently been cured. But it can occur earlier. People with PKDL are considered to be a potential source of kala-azar infection (Desjeux p, et al. 2001), and the present study was acceptable to the study was performed by MSF; the crude mortality rate over the mean recall period of 409 days was 0.13/10’000 people per day. VL was a possible or probable cause for 19% of all deaths.

In the other study, the median total cost for one VL episode in Gadaref State was estimated to be US$450. Despite the free provision of VL drugs at public hospitals, households bore 53% of the total cost of VL with one episode of VL representing 40% of the annual household income (Meheus F and. Abuzaid AA, et al in 2013) were reported. This might indicates that, there is still economic burden upon households represented in nutrition or other expenses like travel. Several expert meetings have recommended that a greater focus on PKDL was needed, PKDL is thought to be a reservoir for transmission of VL, and thus, adequate control of PKDL plays a key role in the ongoing effort to eliminate VL. In north-east Sudan Reeken (2003) was compared an rK39 with DAT and splenic aspirate (as gold standard technique) for the diagnosis of kala-azar in 77 patients. The sensitivity of the rK39 test compared with splenic aspiration was 92% (46/50) the specificity 59% (16/27)
while in this study was compared an rK39 with DAT and puncture gland aspirate (as gold standard technique) for the diagnosis of kala-azar in 71 patients, the sensitivity of the DAT and rK39 test compared with L.D.bodies was 75% in the same of each. The DAT and rK39 specificity were 79.8% (53/71). In the present study compare with the diagnostic protocol used by Médecins Sans Frontières (MSF) the sensitivity of rK39 test was 75% and specificity 79.8% same as MSF results sensitivity was 93% (50/54) and specificity 70% (16/23). Compared with splenic aspirates, the sensitivity of a DAT was 100% (50/50), but its specificity only 55% (15/27), the sensitivity was 84% (42/50), the specificity 85% (23/27). The rK39 dipstick is a good screening test for kala-azar; but further development is required before it can replace the DAT as a diagnostic test in endemic areas of the Sudan.
CHAPTER FIVE

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion:

According to the study findings, it was concluded that,

The serological methods used in this study were Direct Agglutination Test (DAT) and rK39 found to be reliable in first diagnosis, because the diagnosis of Leishmania-infection could be a policy in laboratory-diagnosis if it is sensitivity more than 70%, but in follow up was very poor. Gland puncture test still the gold standard and more suitable in the field for diagnosis of visceral leishmaniasis.

Visceral leishmaniasis in Gadarefareas and according to findings of the current study showed rate of 20.2% out of suspected group (352 cases).

The study also concluded that. Treatment and follow up showed that, few cases found to have PKDL (post-Kala-azar dermal leishmaniasis) representing 10% all of them were male children, also death represented 11% of the studied group, which also mostly found among children.

The PKDL incidence found to be similar to WHO in 2012 (10%) in Sudan.
5.2 Recommendations:

This study that recommended the following:

Designing and applying an interventional program to control the disease with a planned target of decreases in the rate of incidence, some issues can be considered as criteria of the program, as follows:

1. Long-term studies of serological techniques should be conducted to monitor the decrease/increase of VL incidence to provide continuous evaluation and monitoring.

2. Further efforts to determine the cut-off point of DAT technique in Sudan especially in first day of diagnosis and it should be adapted according to degree of VL severity in the areas.

3. Activating the awareness campaigns, fighting the sand fly and using bed nets and other methods should be in complementary with managing the disease.
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Chapter one

INTRODUCTION
Chapter Two

LITERATURE REVIEW
Chapter Three

MATERIAL AND METHODS
Chapter Four

RESULTS AND DISCUSSION
Chapter Five

CONCLUSIONS AND RECOMMENDATIONS
REFERENCES