PARASITOLOGICAL AND SEROLOGICAL STUDIES ON VISCERAL LEISHMANIASIS IN THE SUDAN

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Supervisor

Dr. Seyda H. El Safi
M.B.B.S, M. Sc, PHD
To my family
الخلاصة

انتمى هذه الدراسة لمعرفة بعض المناهج الطفيلية والمثلى لمرض الليشمانيا الحشوية في السودان. 

أجريت دراسة الناحية الطفيلية لتقييم استخدام زراء الطفيل في العمل التشخيصي للطفيليات الحشوية في السودان. درست 44 عينة نتاج الامراض من مرضى يشتبه في أصالتهم بالمرض، والذين راجعوا مستشفى سوبا التعليمي، ومستشفي المناطق الحارة بإمدرمان في الفترة ما بين سبتمبر 1993 – ديسمبر 1993 م. كل عينة اجريت لها 6 مزارع، حيث قوّرت نتائج الزراعات (العطاء الطفيلي) في الاستثمار في عينة 44 البالغة، وذلك نتائج عزل الطفيلي عن طريق الزراعات. 

والملاحظ أن نسبة نجاح الزراعات كانت حوالي 90% من العينات ذات الشراذ الشاذة، ونسبة 41% من العينات ذات الشراذ السالبة. من خلال ذلك ثبت أن عزل الطفيلي عن طريق الزراعات هو أكثر حساسية من طريقة الشراذ. وذلك لمعرفة وجود الطفيلي بالإضافة إلى أن الطفيلي السبب للفشل في المزارع غير صعب للغاية في نموه في المزارع في العمل حيث يوصى بإجراء الشراذ، وعمل الزراعات في حالة التشخيص المبدئي للمرض.

Evans

لقد استعملت 6 أنواع من الوسائط الزراعية لمعرفة الأفضل منها للعزل الأولي للطفيلي. مزرعة Blood Agar Base اعطت أكثر نسبة (89%) تعلّمتها مزرعة Sloppy. مزرعة NNN بنسبة (37%) ثم مزرعة RPMI اعطت نسبة 26%، وخري مزرعة Schneider’s Dorsophilıa اعطت نسبة 11% . مزرعة Evans Sloppy كانت الأفضل كذلك في كونها تحتوي أكبر نمو ممكن للطفيلي معها مقاومة للتلتوح الميكروبى الذي يعد من المشاكل الرئيسية هذه المزرعة تحافظ على نمو الطفيلي لفترة أكثر من 8 أشهر. وعليه قد أثبت ان مزرعة Evans Sloppy هو أفضل المزارع على الاطلاق في نمو وحفظ ونقل الطفيلي.

Formol Gel

فم اختياره على أنه اختيار مصل تشخيصي للليشمانيا الحشوية حيث جرب على 64 عينة مصل أخذت من مرضى كان الفحص المجهري لهم إيجابى، هذا الاختيار أعطى درجة حساسية عالية قدرت بـ92% (59 عينة من اصل 64) كما اختبرت عملية تفاعل الاختبار مع امراض أخرى كالكلاهيات والتابوفيود والسال والبلهارسيا، وعلى انتهاء والبرقان. وقد أعطت كل العينات المأخوذة من هذه الامراض نتائج سالبة ماعدا في حالة عمي انتهارى التي تفاعل كامل. هذا الاختبار يساعد في إعادة ويمكن إجراءه بأخذ عينات بسيطة مماثلة إلى تقليل تكلفة التشخيص. هذا الاختبار ينتمي في تشخيص الليشمانيا الحشوية ويعمل تطبيقه في جميع مستويات الرعاية الصحية الأولية.
ABSTRACT

This study was designed to investigate some parasitological and serological aspects of VL in the Sudan.

The parasitological study was performed to evaluate the use of in vitro culture in the parasitological diagnosis of VL in the Sudan. Bone marrow samples from 44 VL suspects presenting to Soba Teaching Hospital and the Tropical Hospital in Omdurman between September and December 1993 were studied. Each sample was inoculated into 6 different culture media. The occurrence of growth and its intensity were noted and compared with the smear results. Of the total 44 cases 14 were smear positive and 19 samples were isolated by culture in the different media. Growth was obtained in 61.5% (8 of 13) of the smear negative samples and in 90% (9 of 10) of the smear positive samples. The results showed that in vitro culture is significantly more sensitive than direct microscopy for the detection of Leishmania parasites in VL suspects (p<0.5). In addition the parasite responsible for VL in the Sudan is not intrinsically difficult to grow. It is recommended that for the initial diagnosis of VL both in vitro culture and direct microscopy should be employed.

Six culture media were used to identify the best medium for the primary isolation of Leishmania donovani responsible for VL in the Sudan. Evans sloppy medium showed the highest detection rate (89%) followed by Blood Agar Base No.2 (58%), NNN (37%), RPMI supplemented with 20% foetal calf serum (26%), Hank's solution (26%) and Schneider's Dorsophilida medium (11%). Evans sloppy medium was also the best medium in terms of intensity of growth and resistance to contamination which was a major problem; it maintained growth of the parasite for more than 8 months. It has been concluded that Evans sloppy medium is the best medium for the primary isolation, storage and transportation of the parasite.

The formol gel test was evaluated as a serodiagnostic test for VL. It was performed on sera from 64 smear positive patients. A sensitivity of 92% (59 of 64) was obtained. Cross-reactivity with malaria, typhoid, tuberculosis, schistosomiasis, onchocerciasis and viral hepatitis was tested. All samples gave negative results except onchocerciasis which caused an incomplete reaction. It is simple to perform, requires a small amount of serum and involves negligible cost. The test is valuable in the serodiagnosis of VL and
can easily be integrated in the most peripheral levels of primary health care
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Thanks are due to my family who were patient all through the days of hurry and worry.
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CHAPTER 1

INTRODUCTION AND LITRETURE REVIEW

PART I  VISCERAL LEISHMANIASIS

1. General Introduction

Leishmaniasis is a worldwide disease presenting in various clinical pictures differing in severity and health impact. It is far more abundant and of greater public health importance than was previously recognized (WHO 1990).

Visceral leishmaniasis (VL) is focal both in time and space and results of incidence studies can not be extrapolated to wider areas. Also many cases go unreported or undiagnosed. However rough estimates based on the scarce data available indicate that about 350 million people in the world are at risk of acquiring leishmaniasis and that 12 million are currently infected. It has been suggested (WHO 1993) that there may be as many as 400,000 new cases of leishmaniasis registered each year throughout the world. The number of new cases is of the order of 1.5 million of which 500000 are VL cases. These estimations take account of poor reporting from rural areas and the lack of obligatory reporting from endemic countries. VL causes large scale epidemics and the
number of cases varies between years. During 1991 there were large epidemics in India and the Sudan. The number of cases in India alone may have been of the order of 250000. Since case-fatality was generally between 5% and 10% in those diagnosed and treated (rising to 14% in the Sudan) and 100% in those who do not get treatment, the WHO report (1993) estimated that VL may have killed 750000 people in 1991.

2. Historical background

Leishman (1903) identified intracellular bodies in the viscera of fatal cases of kala-azar (KA) in India. He noted their morphological similarity to trypanosomes. In the same year Donovan (1903) made similar observations in India. A year later Marchland and Ledingham confirmed the presence of the parasite in China (Lainson and Shaw, 1987). In 1903 Laveran and Mesnil thought the parasite was a piroplasma and named it Piroplasma donovani but Ross (1903) changed it to Leishmania donovani in honour of the two who first described it. Then Nicolle in 1908 established the trypanosomatid nature of the species by demonstrating its flagellate stage when grown in vitro on blood agar medium (Lainson and Shaw, 1987).
3. Epidemiology
Visceral leishmaniasis may be endemic, sporadic or epidemic. Outbreaks have been associated with the movement of people; silent and subclinical infection is common (WHO 1990). Males are affected twice as common as females. In endemic foci of VL young children are affected most. Sporadic VL occurs in non-indigenous people who enter an endemic area. During an epidemic people of all ages are affected except those who have acquired immunity from previous exposure. VL is widely distributed in the tropics and subtropics (Manson-Bahr and Bell, 1987) (see Fig.1). It occurs in the Mediterranean, Central Asia, China, the Middle East, India, Africa and South and Central America. The climatic conditions of the various foci of VL range from arid to tropical humid and the terrain and altitude are equally variable (Adler, 1964).
3.1 Parasite

Visceral leishmaniasis is caused by a protozoan parasite of the genus *Leishmania* classified under the subgenus *Leishmania donovani complex* (Lainson and Shaw, 1987). This includes several subspecies. *L. donovani* was described by Laveran and Mensil in 1903. In 1908 Nicolle isolated *L. infantum* in *in vitro* culture. *L. chagasi* was later described by Cunha and Chagas in 1937. In addition there are several other possible species which until now are still undefined or of uncertain status. Of interest is *L. archibaldi* described in man and rodents in the Sudan. It is close to *L. donovani* and has been claimed to cause Sudanese VL, cutaneous and mucocutaneous leishmaniasis.

The parasite occurs in 2 morphological forms: amastigote and promastigote (Manson-Bahr and Bell, 1987). The amastigote stage is the form in the mammalian host; it is an ovoid or round body 2-3µm length living intracellular in monocytes, polymorphonuclear cells or endothelial cells. Amastigotes are also
known as Leishman-Donovan (L-D) bodies. The promastigote stage is the form in the sandfly host as well as in culture media; it has a flagellum 15-28µm in length and is very motile. This form is elongated with an average length of 15-20µm and has a diameter of 1.5-3.5µm (see Fig.2).

![Leishmania stages](image)

**Fig. 2** Leishmania stages

The amastigote forms of the parasite are ingested with the blood when a female sandfly feeds on an infected mammalian host. In the sandfly they immediately transform into promastigote which multiply and move forward to contaminate the wound caused by a new bite. The development in the fly takes 5-10 days and is synchronous with its gonadotrophic cycle. The promastigote
are inoculated by the sandfly bite into a capillary or the dermal tissues. They are engulfed by macrophages; inside the macrophages they convert to amastigotes which start dividing to spread and colonize fresh macrophages (Lane and Muiter, 1987).

3.2 Reservoir

A wide range of mammals act as a reservoir for the different subspecies of the *Leishmania donovani* complex. Kala-azar caused by *L.infantum* and *L.chagasi* is a zoonosis and its main reservoir is dogs and wild canids including foxes, jackals and rodents (Manson-Bahr and Bell, 1987). *L.donovani* is the cause of VL in India where the disease is anthroponotic. In East Africa the disease is caused by *L. donovani* as well but is zoonotic.

3.3 Vector and transmission

The transmission of VL is dependent on ecology and so the conditions resulting in infection of man vary both in time and place. Transmission can be domestic or peridomestic (WHO 1990). VL is transmitted from one mammalian host to another including man by sandflies of different species - *Phlebotomus* in the Old World and
Lutsomyia in the New World (Manson-Bahr and Bell, 1987). Sandflies are small hairy flies with long hairy legs. The females feed on blood which is needed for the maturation of their eggs. Their breeding sites are dark damp places rich in organic matter. Sandflies rest during daylight and become active at dusk. They become infective 5-10 days after a blood meal and remain so for the rest of their life, a few weeks in nature.

Although VL is mainly transmitted by the sandfly, other modes of transmission have been reported. Congenital (Lowe and Cooke, 1926) and direct transmission (Satti, 1962) as well as transmission by blood transfusion (Woodruff, 1987) have been reported. Accidental inoculation in the laboratory has led to the development of full blown VL (Manson-Bahr, 1959).

4. Clinical features and treatment (Old World)

The incubation period ranges from 10 days to over one year (Manson-Bahr and Heisch, 1956) but can usually be stated as 2-6 months (Napier, 1946; Maegraith, 1980). Subclinical cases outnumber clinical cases by about 5:1 in some countries (Kenya, Italy). VL is occurring in increasing number in people with immunosuppression due to AIDS or chemotherapy (WHO 1990; Davidson, 1991).
Visceral leishmaniasis is characterised by an insidious onset especially in endemic areas (Manson-Bahr and Bell, 1987). Usually the shorter the incubation period is the more acute the onset. Patients present to hospital with a history of fever, abdominal swelling and discomfort. Episodes of cough, diarrhoea and epistaxis are common complains (Rees and Kagar, 1987). Loss of weight is a marked and constant feature although the patient retains a good appetite even during febrile attacks (Woodruff, 1987). Amenorrhoea is an early symptom (Napier, 1946) but uncomplicated pregnancies continue to term resulting in healthy infants. Increased pigmentation is noted earlier in fair-skinned people. It is often first noticed on the dorsal surfaces of the hands and fingers and of the feet and toes. The face is also involved.

The fever may be continuous, remittent, and intermittent or recurs irregularly with a characteristic double or treble diurnal rise to high peaks (Maegraith, 1980). Fever presents with malaise, rigors and profuse sweating especially at night (Woodruff, 1987).

Splenomegaly is the most outstanding sign and is invariably seen in established cases (Henderson, 1937, Manson-Bahr and Heisch, 1956). It is soft and doughy to start with (Napier, 1946).
and becomes firm to hard later in the disease (Van Peenen and Reid, 1962).

Hepatomegaly is usually noted but is not as marked or constant as splenomegaly (Henderson, 1937; Manson-Bahr and Heisch, 1956). It is also slower in developing. Jaundice in rare but if present is usually late and carries a poor prognosis (Napier, 1946; Maegraith, 1980).

Lymphadenopathy is seen mainly in East African VL (Kirk and Satti, 1940b). The lymph nodes are not markedly enlarged and are firm and rubbery in consistency.

Differential diagnosis of VL includes malaria, African trypanosomiasis, hepatosplenic schistosomiasis, brucellosis, typhoid fever, bacterial endocarditis, generalized histoplasmosis, chronic myelocytic leukaemia, Hodgkin's disease and other lymphomas, sarcoidosis, hepatic cirrhosis and tuberculosis. Tropical splenomegaly is difficult to differentiate. Patients with multiple myloma and Waldenstrom macroglobulinaemia have monoclonal hyergammaglobulinaemia (Manson-Bahr and Bell, 1987).

Secondary infections complicate the course of the disease. Bacterial infections such as pneumonia, cancrum oris and
meningitis are common (Napier, 1946). Petechial and retinal haemorrhages may occur especially in terminal cases. Cirrhosis may develop as a late complication (Napier, 1946; Maegraith, 1980). Ascites is a late sign and may be associated with generalized oedema, including the face.

Post-kala-azar dermal leishmaniasis (PKDL) may occur in isolation (WHO 1990) but usually is a late complication of VL appearing after apparent cure. It presents as hypopigmented or erythematous macules on any part of the body or as a nodular eruption especially on the face.

Mortality is high in untreated VL, death occurring in 2 month to 2 years (Archibald, 1922; Maegraith, 980). Treatment is started as soon as diagnosis is established. Some clinical cases abort early not progressing to the typical presentation of febrile splenomegaly.

Pentavalent antimonials are used in various preparations and dosages in the different areas (WHO 1990). Na stibogluconate (Pentostam) is used in a daily dose of 10mg Sb /kg (0.1 ml Pentostam/kg) IM or IV. Children tolerate and need 20mg Sb/kg per day. The treatment is continued until 'cure' is achieved (no palpable spleen or 2 consecutive splenic aspirates a week apart
are negative) usually no less than 30 days. Response is rapid and patients usually request discharge 7-10 days after the start of treatment. In the minority that fail to respond diamides especially pentamidine is used. Good nursing and non-specific treatment with a high protein vitamin-rich diet and antibiotics may have a dramatic if temporary effect and are vital factors in recovery (Molyneux and Ashford, 1983) immunopathology of VL.

5. Immuno pathology of VL.

Macrophages take up the inoculated parasite which are thus sheltered from the body's immune responses and so result in a granuloma, the so called leishmanoma (Manson-Bahr, 1959). The parasite reaches the blood via the lymphatic vessels and is taken up by reticuloendothelial cells in the spleen, liver, bone marrow, lymph nodes and other organs (Manson-Bahr and Bell, 1987). Development of a granulomtous reaction and eradication of the parasite can take place and this is apparent in subclinical infection. The parasite can continue multiplying with little host resistance resulting in kala-azar (KA).

The immune response is cellular and varies across a wide spectrum from complete eradication of the parasite to unrestricted spread; thus, evidence of severe non-specific depression of T-
lymphocyte function and cell-mediated immunity through inhibition of suppressor T-cells has been shown by failure to develop a delayed hypersensitivity reaction to *Leishmania* until recovery when functions are restored (Manson-Bahr and Bell, 1987).

In contrast the humeral response is marked. Serum immunoglobulin levels, mostly the IgG and IgM classes, are raised (Maueland Behin, 1982). This increase is non-specific and results from stimulation of polyclonal B-cells. The reversal of the albumin/globulin ratio is considered to be one of the pathognomonic features of VL. High titres of anti-leishmanial antibodies can be detected by a variety of serological techniques.

6. Diagnosis of VL

The diagnosis of VL depends on the clinical, parasitological and serological findings. Clinical suspicion of VL is aroused by the presentation of the patient and the geographical epidemiology. General parasitological and immunological diagnostic methods depend on the demonstration of parasites in material by direct smear, culture and/or animal inoculation as well as serological testing or histological examination; the leishmanin skin test or Montenegro reaction is used only in the epidemiological diagnosis of VL as it only measures past infection.
6.1 Parasitological diagnosis

The definitive diagnosis of VL depends on the demonstration of *Leishmania* in material aspirated from the spleen, bone marrow, lymph node or less commonly blood. The aspirated material or blood can be examined by direct microscopy or inoculated into animals or artificial culture media.

6.1.1 Material for examination

Splenic aspirate is obtained by splenic puncture; it is the most likely to show parasites in about 90% of active cases (WHO 1984) but serious complications can occur. Bone marrow aspirate is obtained by sternal or iliac crest puncture. It is positive in up to 80% of active cases (WHO 1984); it is safer but more painful. Lymph gland aspirate is obtained from an enlarged lymph node mostly the femoral or epitrochchlear nodes. The procedure is safe and positive in 60% of cases (WHO 1984). Examination of the peripheral blood for L-D bodies is difficult and very variable. Buffy coat smears when examined long enough may show amastigotes in leucocytes (Manson-Bahr, 1987). This is only really possible in Indian KA and in East African VL. Skin scrapings or biopsies can be examined for L-D bodies. Prolonged examination of the preparations is needed and amastigotes are few.
6.1.2 Direct microscopy

Amastigotes are seen in smears prepared from the aspirated material and stained with Leishman or Giemsa stain (Cheesbrough, 1987). The L-D bodies can be seen in groups inside mononuclear cells or lying free between the cells. The cytoplasm stains palely and is often difficult to see but the nucleus and rod-shaped kinetoplast stain dark reddish-mauve.

6.1.3 Animal inoculation

The Syrian hamster has been used since the early 1930s as a means of isolating and maintaining leishmanial isolates (Schnur and Jacobson, 1987). Mice and a variety of other animal species are susceptible to leishmanial strains but mostly rodents are used. Tissue suspensions are inoculated intradermally into the skin of the nose and/or feet. The inoculated hamsters should be kept in an animal house where the temperature can be maintained at about 23-26°C (WHO 1984). The resulting infection usually becomes apparent by the development of a nodule or ulcer at the site of inoculation; the presence of amastigotes can be verified by the examination of stained smears.
6.1.4 In vitro culture of *Leishmania*

Rogers was the first to demonstrate the possibility of isolating and culturing *Leishmania* in 1904 (Schnur and Jacobson, 1987). He grew the promastigote form in a blood agar culture medium. Since then many different methods and media have been experimented with in both the Old and New Worlds.

The sample is usually bone marrow which is taken under strict aseptic technique. It is important to avoid transfer of more than small amount of whole blood or serum components along with the inoculums into the culture medium (Evans, 1987); blood has been shown to contain serum proteins highly inhibitory to growth of leishmanial promastigotes. It is safer to use only 1 - 2 drops (0.1 ml) of marrow per culture bottle than to inoculate all into one even when examination of the smear shows low parasites. Alternatively marrow taken into an anticoagulant may be centrifuged, the supernatant discarded and the pellet used as inoculums.

Many different media have been used in *in vitro* culture for the primary isolation of *Leishmania* (Evans, 1987). They include

1. Biphasic blood agar media like NNN, Difco blood agar medium and Evan's modified Tobies medium among many others.
2. Liquid media like RPMI 1640, Hank's solution and Schneider's Dorsophila medium; these are supplemented with variable concentrations of heat inactivated foetal calf serum.

3. Semi-solid media include Sloppy Evans medium and Locke-blood agar medium.

The choice of a medium is influenced by the purpose of culture (Evans, 1987). Initial isolation requires rich media and also maintenance. Semisolid media are ideal for this as well as for transportation and revival of ailing isolates. For mass cultivation liquid media are best to avoid both blood cells and agar.

Routine use of antibiotics in isolation of *Leishmania* is advocated as the chances of contamination are high during inoculation as well as later during check (Evans, 1987). Despite cleaning and sterilization of body surfaces and correct technique it is difficult to avoid using antibiotics and antimycotics in isolating media when working under field conditions. However their avoidance is important when initial integrity of primary isolates is to be maintained. There are three types of contaminants- bacteria, yeast and fungi. Bacteria are usually faster growing than promastigotes and so outgrow them but do not necessarily kill
them. Yeasts and other fungi can be controlled with the antimycotic agent 5-fluorocytosine. It is fungistatic rather than fungicidal. By frequent passaging and suppression the culture can be cleared (Schnur and Jacobson, 1987).

The inoculated media are incubated at a temperature of 22°C (Evans, 1987). A temperature of 21-23°C is better than 25-27°C because it is easier to kill promastigotes by heat than by cold. Small screw-capped universal glass bottles of ¼ fluid ounce capacity 'bijou bottles' are easier to handle than round glass tubes and take up less space in the incubator. Disposable plastic culture bottles save time and space but are expensive as they can not be autoclaved and reused.

The cultures should be examined regularly for growth (Evans, 1987). This should be done not less than once a week. Frequent inspection is important to detect contamination early and give a better chance for its control.

6.2 Immunological diagnosis

6.2.1 Leishmanin skin test

The leishmanin skin test is negative during active VL; it becomes positive only 6-8 weeks after recovery from VL and remains so for life (Manson-Bahr and Bell, 1987). It is useful in the
diagnosis of VL in two ways - retrospective diagnosis of previous illness or exposure in an endemic area and exclusion of VL a positive leishmanin test will exclude the diagnosis of active KA.

6.2.2 Serodiagnosis

Serological methods that have been applied for the diagnosis of VL include non-specific and specific tests.

(A) Non-specific tests depend on the increase in immunoglobulin seen in KA. They include:

1. Immunoglobulin levels: IgG is increased and polyclonal IgM increases to a lesser degree. Paper electrophoresis can thus differentiate VL from multiple myloma where the IgG is monoclonal.

2. Chopra’s antimony test: This test is used in India. A characteristic flocculent precipitate is produced in the positive tests. Serum is diluted ten times with double distilled water and placed in narrow-bore test-tubes; to this 4% urea stibamine solution is added and the contents are mixed (Napier, 1946). The precipitate forms almost immediately and settles as a flocculent mass in the course of about half an hour.
3. Globulin-precipitation test (Sia test): This test was described by Sia in 1925. One part of serum is mixed with three parts of distilled water. In KA a flocculent precipitate develops (Napier, 1946).

4. Formol gel test (Napier's aldehyde test): This test was described by Napier in 1922; it is simple to perform and ideal for field work in case detection (Manson-Bahr, 1987). It is performed by mixing 1ml of serum with about 2 drops of concentrated formalin in a tube which is allowed to stand for up to 20 minutes (Cheesebrough, 1987). Whitening and jellying of the serum usually within 5 minutes indicates a positive test. A milky appearance without the serum solidifying may occur in early KA. When the serum remains unchanged or whitening and jellying occur only after 20 minutes the test is read as negative.

Cheesebrough (1987) described a formol gel slide test recommending it when testing children or when venous samples of blood are difficult to obtain. In this test a large drop of serum is mixed with a small drop of formalin on a slide; a positive test is indicated by whitening and jellying of the serum usually within 5
minutes. The test becomes positive about 3 months after infection and negative about 6 months after successful treatment.

In an endemic area a definite diagnosis using the formol gel test can be made with very little risk of error in at least 70% of cases attending the outpatient department (Napier, 1946). This test has been used for field diagnosis in India especially during epidemics (Srivastiva, 1985). During the VL epidemic in Bilhar (1971-1982) the aldehyde test was positive in 612 cases out of 750 cases of VL i.e. 81.6% positively (Thakur, 1982). Cheesebrough (1987) stated a positively of 85% in VL patients. The formol gel test in its original set up was recently assessed in Bangladesh over an 18 months period (Chowdhurry et al, 1992). It picked 94% of KA patients in comparison to 96% by CFT. Although considered a non-specific test cross-reactivity that arose seldom presented diagnostic difficulty. The test was recommended because of its simplicity, negligible cost and easy performance in a rural setting.

(B) Specific tests focus mainly on antibody detection methods. Lesser effort has been devoted in the search for antigen purification methods (Voller and De Savigny, 1981); and what has
been done has improved antigen quality rather than come up with new test methodology. The specific tests include

1. Complement fixation test (CFT) has been used extensively for VL (Cheesebrough, 1987). Complement fixing antibodies appear during the first 3 months of infection and disappear within 6 months of cure. A titre of 1/10 is significant and 1/40 diagnostic. Cross-reactivity with Chagas disease and rarely tuberculosis and leprosy can occur. It is useful in diagnosis of early cases and monitoring of treatment.

2. Haemagglutination inhibition test (HIT) is more difficult to perform. It follows the same course as the CFT. Titres of 1/200 or more are significant.

3. Counter-current immunoelectrophoresis (CIEF) is positive in 80% of early VL cases and 100% in late cases. It is a rapid and less sophisticated test. Positive titres were found in other febrile conditions and in children in an endemic area suggesting previous subclinical infection (Manson-Bahr, 1987).

4. Immunofluorescent antibody test (IFAT) is a sensitive technique detecting 93% of VL patients. Titres of 1/20
or more are significant and 1/28 diagnostic of active disease. This test detects early disease and becomes negative in about 6 months after cure. Promastigote or amastigote antigens are used. Cross-reactions are noted with trypanosomal sera and rarely acute malaria, tuberculosis and leprosy. Other drawbacks of this test are the requirements for a fluorescent microscope and training in fluorescent technique as well as a high degree of subjectivity in reading the test. The test itself is time consuming however antigen can be prepared easily and stored on microscope slides for long periods. An advantage is that it permits the identification of reactions on selected sites of the parasite thus locating the antigens.

5. Enzyme-linked immunosorbent assay (ELISA) has been reported as comparable to IFAT by Hommel (1976,1978), while Edrissian and Darabian (1979) found it to be more sensitive. No cross-reactions have been noted except rarely with malaria. It is suitable for sero-epidemiological surveys as a micro-ELISA is available. Reagents are stable and no special
equipment is required. It is less expensive and large numbers of samples can be easily handled.

6. Direct agglutination test (DAT) The agglutination of living organisms has been recognized as a most sensitive technique for the detection of antibodies (Kagan, 1974). In 1971 Vattuoue and Yanovsky described a DAT of *Trypanosoma cruzi* epimastigotes for the diagnosis of Chagas disease; the same technique was then used by Allain and Kagan (1975) for the detection of leishmanial antibodies in sera of patients with VL and cutaneous leishmaniasis. They used promastigotes of *Leishmania* species. End-points of the reactions were difficult to locate and the problem was solved by E. Harith *et al* (1986) who evaluated the DAT for serodiagnostic and sero-epidemiological studies of VL. A comparison of the DAT to IFAT and ELISA showed it to be highly sensitive and specific (E.Harith *et al*, 1987). The ease and low cost of its application compared to the other two tests further recommends the DAT for mass screening and sero-epidemiological studies of VL. The DAT was improved
further by modifications in its components and procedure increasing the potential for wide scale application in diagnosis of VL both in hospitals and in the field (E. Harith et al., 1988).

PART II  VL in the Sudan

1. Introduction

The three forms of leishmaniasis - visceral, cutaneous and mucosal - are found in the Sudan with only VL posing a major health problem up to the mid-1970s when several epidemics of cutaneous leishmaniasis occurred (Satti, 1963a, 1980; Yousif, 1967). It has been estimated that KA has been responsible for 40000 deaths since 1988 (WHO 1993). A lot of cases go unreported and there are no reliable figures. In a survey of over 1000 Nuer people in Bentiu, a town in the western Upper Nile region, 52 were found to be infected. On that basis it was concluded that over 18000 of the area's 40000 people may have KA.

Up to 1930 the total cases of KA reported in the Sudan was 56 (Satti, 1963a). By 1934 it has increased to 289 cases and by 1939 to 394 cases. After a lull in the 1940s and early 1950s over
7000 cases were reported by 1956. This increase in the number of cases was due to economic development particularly in the field of agriculture which resulted in the movement of non-immune people into the endemic areas.

2. Historical background

The first case of VL was reported in May 1904 by Dr. Sheffield Neave who has been in charge of Omdurman Hospital (Neave, 1904); however VL has been known to the people of the Sudan for some time before that date. Shortly before in November 1903 M. Laveran in Tunis had described the first case of KA in Africa (Haseeb, 1959).

Two government commissions studied the disease (Bousfield, 1910; Thomson, 1911). The one headed by Dr. L. Bousfield (1906 - 7) and another headed by D.S.B. Thomson (1909-11) with Dr. W.E. Marshall as the pathologist. They investigated the prevalence and cause of KA in Eastern Sudan. In 1913 Dr. H.A. Bray moved the studies of KA to the laboratories in Khartoum and Dr. R. Archibald, pathologist at the Wellcome Tropical Research Laboratories in Khartoum, carried out thorough investigations on infectivity of the parasite and its life-cycle in the various hosts (Archibald and Hasseeb, 1937; Hasseeb, 1959).
1935 Archibald and Hasseeb revived research on KA. They did extensive epidemiological studies in 2 main centres - Singa in the Fung area and Kapoeta in Equatoria. They concluded their investigations in 1937. The same year Henderson published the results of his work in the area (Henderson, 1937). Together with Hasseeb he studied the clinical features of the disease and evaluated treatment.

From 1931 till 1956 Kirk, Satti and their co-workers investigated various aspects of the disease including treatment and problems of transmission and released their findings in a series of publications (Kirk and Satti, 1940a,b; Kirk and Lewis, 1955; Satti, 1963a,b). Until then the disease was typically sporadic but a distinctive feature was the occurrence of sharp outbreaks in military troops. Four such outbreaks took place between 1913 and 1931; two of them in practically uninhabited country. This pointed to the presence of an animal reservoir which was then extensively sought (Hasseeb, 1959).

During the 1940s and early 1950s there was no appreciable increase in cases (Satti, 1963a). However, economic development especially in agriculture started in the endemic areas resulting in the movement of hundreds of non-immunes. This led to a marked
increase in cases reaching epidemic proportions in 1956-59. Recognizing the scope and urgency of the problem the Sudan Government invited in 1958 the United States Naval Medical Research Unit Number 3 (NAMRU-3) to assist in providing epidemiological data and clues for control and prevention of KA (Hoogstral and Heyneman, 1969). From 1960-64 a team from NAMRU-3 studied all aspects of the disease in the Melut-Paloich area in Upper Nile province. They established two research laboratories one in Malakal in 1960 and another in Paloich near Melut in 1962 (Zeese and Frank, 1987). Their findings were published in a series of papers entitled 'Leishmaniasis in the Sudan Republic' 1961–69 (Hoogstraal, and Heyneman, 1969).

In late 1979 an Austrian missionary doctor observed an increasing number of VL cases in the Melut area (Zeese and Frank, 1987). Accordingly Zeese and Frank travelled widely in the area from 1981-84. They reported an average of 1300 patients treated for KA per year in the whole of Sudan with approximately 1000 cases (75% of total) treated in 2 hospitals - Gadarif and Hawata. 80% of these patients came from an area south of Gadarif between the Blue Nile in the west and the Ethiopian border in the east.
Late in 1988 a Medecins san Frontiere (MSF-Holland) team based in Khartoum to assist in the health care of displaced people diagnosed several hundred cases of VL among displaced southerners from the Nuer tribe originating from Bentiu area in western Upper Nile (de Beer, 1991; Goris, 1993). According to local history the epidemic had started in Pak, a town in Jikany area. Between 1985 and 1989 the disease was reported from Jagei, Leek, Jikany and Panarou; thus involving the whole of Western Upper Nile province. The UNICEF mission (February 1993) reported 30000 killed in one area while 50000 further deaths were reported as the disease spread northwards to Panarou area. More than half the population has been estimated to have perished.

3. Epidemiology

3.1 Geographical distribution

The disease is endemic in Kassala and the Blue Nile provinces bordering the Ethiopian frontier (Kirk, 1939) (see fig.3). The distribution of the disease is wide, erratic and variable from time to time. It clings to the banks of the Blue Nile and its tributaries and the upper reaches of the River Atbara. As it approaches Medani its incidence decreases. The disease is also
endemic in the Upper Nile province (Stepfenson, 1940; Hoogstraal and Heyneman, 1969) in the Melut-Paloich area and Doleib Hill; according to NAMRU-3 VL extends beyond the inter-river region from Kassala in the north, south to Malakal and Nasir in Upper Nile province. Another focus in the south occurs in south Kapoeta in Equatoria province (Archibald and Hasseeb, 1937). El Fasher in Darfur province is a focus in the west of Sudan (Kirk, 1939; Hoogstraal and Heyneman, 1969). Sporadic cases have been reported from Kordofan (Kirk, 1939) and the west. A case has been reported in Al-Alafoon in Khartoum province (Shamsidin, 1962) and an outbreak in El Khogalab in 22 school children (Hamza et al, 1976). VL cases have also been reported from Gebal Awlia in 1987 in the White Nile province and Umeish village in 1982 (El Safi, 1989)
Fig. 3 Distribution of visceral leishmaniasis in the Sudan

The disease is found mainly in small villages and rural populations (Yousif, 1967). Human infection is associated with certain areas and occurs in outbreaks sometimes associated with amine or epidemics and usually associated with people movements. Males are affected more than females (Kirk, 1939; Archibald and Hasseeb, 1937). No age is exempt but the disease
is more common in adults, especially young adults, than children. New-comers to an endemic area are affected more easily (Yousif, 1967). The disease can affect any sector of the population but those whose work requires their being out in the country are affected more.

The incidence of the disease is related to adequate rainfall and humidity (Archibald and Hasseeb, 1937). Seasonal incidence of the disease corresponds to the months July to October. Most of the cases occur in October towards the end of the rainy season (Yousif, 1967). Great variations in incidence amounting to epidemics have occurred but have not been thoroughly documented.

Several epidemics of VL have been reported in the Sudan. A severe outbreak was reported by Stephenson (1940) in Melut and Kaka in the Upper Nile province. In 1956 a violent epidemic took place in Wadega and Kurmuk in the southern Fung district of Blue Nile province (Satti, 1962). An outbreak of some 204 cases also occurred in 1961-62 at Khor Falus, south of Malakal in the Upper Nile Province (Van Peenen and Reid, 1963). A small outbreak involving 22 school children in the village of El Khogalab in Khartoum province was reported (Hamza et al, 1976). Another
took place in Edumish village in the White Nile province (El Safi, 1989). An outbreak occurred in El Dinder in the Blue Nile province in 1985. Late in 1988 the Nuer tribe in Bentiu area near Bahr el Gazal in Upper Nile province was hit by a severe epidemic (De Beer et al, 1991; El Hassan et al, 1993). They moved northwards to Kadugli, Kosti, El Obeid and Khartoum seeking treatment. This epidemic spread in 1991 to a nomadic Misairiya tribe in Babanosa in the southern part of Kordofan province. They are cattle owners who regularly move southward during the dry season in search of pasture and water. Parasite studies showed the responsible Leishmania to be of the same zymodeme as that isolated from the Nuer in Upper Nile (Ashford et al, 1992).

### 3.2 Parasite

The identity of the causative Leishmania species in the Sudan is not easy to determine as all three forms of the disease co-exist in known endemic areas of VL (Kirk, 1942). Also VL may be preceded, accompanied or followed by cutaneous or mucocutaneous lesions (Kirk and Satti, 1940a, b; Satti, 1963a; Hoogstraal and Heynenman, 1969).

Electron microscopic morphometric studies showed that all three forms were caused by one species and this was *L. donovani*
Studies using monoclonal antibodies were carried out and revealed their importance in serodiagnosis of VL cases and identification of its causative parasite (El Amin et al., 1985).

*Leishmania donovani* from a Sudanese VL patient was shown by isoenzyme characterization to be zymodeme LON-46 (El Safi et al., 1991). Ashford et al. (1992) showed that 25 strains of *Leishmania donovani* isolated from humans and *Phlebotomus orientalis* in the endemic area of Bentiu in southern Sudan belonged to 3 similar zymodemes (MON 18, MON 30 and MON 82). The parasite isolated from Babanosa cases was found to belong to these same 3 zymodemes (El Hassan et al., 1993).

### 3.3 Reservoir

Search was started by early workers but has still not reached definite results. L-D bodies have been discovered in a dog in Singa (Bousfield, 1910), a fox (Kirk, 1956) and a monkey. Rodents and small animals that live in burrows in the ground are the most likely suspects. In 1963 L-D bodies were detected in the spleen of 4 wild Nile grass rats (*Arvicanthus nilotica*) and in 1966 Adler demonstrated that the strains from human cases of KA, from *P.orientalis* and from the Nile rats of Southern Sudan were
immunologically identical species (Cahill, 1968). NAMRU-3 found two rodent species infected in the Malut-Paloich area - the Nile grass rat *Arvicanthis niloticus luctuosus* and the spiny mouse *Acomys albigena* (Hoogstraal and Heyneman, 1969). Two carnivorous were also infected - the Senegal genet *Genetta g.senegalensis* and the Sudanese serval *Felis serval phillipsi*. In Malakal city NAMRU-3 found *Acomys albigena* and *Rattus rattus* to be infected. Variations in the reservoir population and infection were demonstrated by other studies (Sixl *et al*, 1987). Sixl *et al* (1987) could only find *Leishmania* in the spleen of one jackal after examining 628 wild animals belonging to 17 different species in the Melut-Polaich area.

### 3.4 Vector and transmission

The distribution of KA in the Sudan is coincident with that of the vector *Phlebotomus orientalis* which is found from Lake Chad in the west to the southwest corner of the Arabian Peninsula (Manson-Bahr and Bell, 1987). Nomads and semi-nomads who set up temporary villages on the Nile flood plains in the dry season near patches of acacia-balanites scrub which harbour *P. orientalis* are affected.
*Phlebotomus orientalis* was found suitable to act as vector of KA to man in the southern Sudan (Hoogstraal and Heyneman, 1969). It showed a remarkably consistent infection rate ranging from 1.9-5.0%. Its activity was found to peak between April and June. It is restricted to *Acacia Balanities* forest and nearby villages. It bites from dusk to shortly after dawn and its flight range is at least 730m. *Phlebotomus papatasi* as suspected in the Gadarif area as being the vector of VL (Zeese and Frank, 1987). Other modes of transmission have been reported in the Sudan. Direct contact was reported by Symmers (1960). Transmission through nasal secretions was suggested by Satti (1963a); a monkey was infected experimentally by breathing a spray containing a saline emulsion of a patient's nasal secretions in which *Leishmania* had been demonstrated (Kirk, 1942).

4. Clinical features and treatment

In general the clinical findings are similar in the different endemic areas (Van Peenen and Reid, 1962; El Safi *et al*, 1991; Zijlstra *et al*, 1992). The average incubation period is 2 months with a range of 3-6 months depending on the physical condition of the patient at the time of infection (Archibald, 1923; Kirk, (1939). It may be as short as 14 days (Yousif, 1967). Henderson (1939)
found the disease to be mostly chronic with periodic exacerbations, 40% giving history of 1-2 months. The disease presents mainly with fever which occurs late in the day subsiding after a few hours with profuse sweating. It is usually remittent with a double rise in nearly 80% of cases. The patient usually has a good appetite and does not look acutely ill during the febrile attacks. All patients have splenomegaly of varying degree - and nearly all hepatomegaly. Emaciation and weakness are common and occur early during the course of the disease. Diarrhoea is a troublesome symptom in about a quarter of the patients. Oedema is marked in the face and ankles. Lymphadenopathy is common and may be the only manifestation together with fever (Satti, 1963a; Kirk and Satti, 1940b).

PKDL has been reported by several workers in the Sudan (Kirk and Satti, 1940a, b; Kirk, 1942). The skin eruption may occur in KA patients either in untreated cases or following treatment. Mostly it disappears in 6-9 months with return of the skin to normal. Kirk (1944) suggested three stages in the evolution of VL in the human subject. These include a primary stage in which a cutaneous lesion develops at the site of inoculation, a secondary stage which corresponds to the generalized infection i.e. KA and a
tertiary stage in which cutaneous and mucocutaneous lesions may develop. According to the virulence of the strain and defence mechanism of the host the infection may terminate at any stage. Locally prevalent conditions that simulate KA include chronic malaria, typhoid fever, brucellosis, schistosomiasis and tuberculosis (Henderson, 1937).

Pentavalent antimony drugs are regarded as the best medicine for treating VL; but it is expensive, costing the equivalent of US$ 100 in Khartoum (the monthly salary of a senior government official) and more than twice that amount in the south (WHO 1993). Sodium stibogluconate (Pentostam) is given in a dose of 10mg/Kg IV for 30 days. The minimum daily dose for children is 200mg. Doses in the Sudan are higher than in India (Molyneux and Ashford, 1983). No evidence of toxicity is usually seen and response is rapid with clinical improvement and disappearance of fever in the first week (Zijlstra et al, 1992). The drug performed well in children and adults with respect to relapse rate and death rate.

5. Diagnosis

5.1 Parasitological diagnosis
The parasite has been demonstrated in smears from the spleen (Henderson, 1937), bone marrow (Van Peenen and Reid, 1962) and lymph node aspirate (Kirk and Satti, 1940b). It has also been detected in peripheral blood phagocytosed within leucocytes, nasal and tonsil swaps (Archibald and Hasseeb, 1937). It was observed that while it was rare to find the parasite in the peripheral blood in patients in the Blue Nile province it was invariably present in those from Kapoeta area.

In vitro cultivation has also been used to demonstrate promastigotes (Marshall, 1911a; Archibald, 1913, 1914; Van Peenen and Reid, 1962). Animal inoculation has been used both in monkeys and hamsters (Marshall, 1911a, b, 1912; Archibald, 1913, 1914; Hoogstraal and Heyneman, 1969). Satti (1963b) reported that the primate Galago senegalensis senegalensis was more susceptible than the hamster to infection with L. donovani in the Sudan.

5.2 Leishmanin test

The leishmanin test has been used in several surveys in the different endemic areas (Cahill, 1965, 1968; Van Peenen and Deitlein, 1963), where its value as an epidemiological tool has been demonstrated. This test has also been used in active disease
(Van Peenen and Reid, 1962). All 20 patients with VL that have been studied in Upper Nile province gave negative tests with leishmanin antigen except one with a past history of treated KA.

5.3 Serological diagnosis

IgG and IgM are elevated in VL; IgG1 subclass is largely responsible for the increase in IgG. IgG2 and IgG4 are not elevated (El Amin et al, 1986). Parasite specific antibodies were mainly of the IgG1 and IgG3 subclasses and both were significantly reduced following treatment (Elassad et al, 1994).

The direct agglutination test was first used by Archibald (1914) who could not detect specific agglutinins in the serum of VL patients. Immunodiffusion, counter-immunoelectrophoresis and immunofluorescence have all been used for the serodiagnosis of VL in the Sudan (Abdalla, 1980). A high sensitivity with the IFAT was observed. ELISA was reported as more sensitive and specific than both IFAT and indirect HA in serodiagnosis of VL (El Amin et al, 1986). However, these serum tests can not be applied for routine diagnosis of VL in the Sudan as most of them require technical facilities which are often not available in developing countries particularly in endemic areas.
A simple and economic test that has been developed by E. Harith et al (1986) was evaluated by El Safi and Evans (1989) for the diagnosis of VL in the Sudan. They found all sera from parasitological confirmed patients positive in both ELISA and DAT. On the grounds of simplicity and low cost the DAT was preferred. The DAT was tested in an endemic area in the Sudan, Hawata district in Kassala province, by Abdil Hamid et al (1989). They showed 100% concordance between DAT and parasitological diagnosis in 40 confirmed cases out of 49 patients chosen on the basis of previous treatment for VL. In the remaining 9 unconfirmed parasitological but highly suspected clinically, the DAT was indicative of VL. All other diseases gave negative results. They concluded that considering the low costs involved, easiness in performance and stability of the antigen the DAT appears to possess a high potential for routine application in the Sudan.

The aldehyde test of Napier was used for the diagnosis of KA (Archibald and Hasseeb, 1937; Henderson, 1937; Kirk and Satti, 1940). Henderson thought it useful as a screening test. However, Van Peenen and Miale (1962) found that it was positive in less than half the cases and so was not very sensitive but it was relatively specific as no positives were observed in control serum.
These workers carried out the test in capillary tubes to conserve serum. They interpreted the test according to Napier (1946) and only sera giving a positive reaction within 10 minutes were read as positive. The best correlation between positive tests and elevation of gamma globulins was noted in the formol gel reaction.

AIMS AND OBJECTIVES OF THE STUDY
1. Comparison of bone marrow smear and \textit{in vitro} culture for the parasitological diagnosis of VL.

2. Comparison of six different media used in \textit{in vitro} cultivation of \textit{Leishmania} to determine the best medium for primary isolation in VL cases.

3. Comparison of the formol gel slide test with the DAT for the diagnosis of VL in Sudanese cases; and determination of cross-reactivity with other diseases.
MATERIAL AND METHODS

1. Patients

Patients with clinical features suggestive of VL presenting to the KA ward of Soba University Hospital and to the Tropical Diseases Hospital in Omdurman from early September 1993 till late December 1993 were studied. Some cases were also recruited from the medical wards of Khartoum Teaching Hospital.

A questionnaire was filled regarding personal and clinical history of the disease as well as physical examination and the preliminary investigations done (Appendix).

A venous blood sample (4-5 ml) was then drawn. 4 drops of blood were directly placed on a piece of filter paper (Whatman Chromatography paper No 3) for the DAT and the rest was allowed to clot and separate. Serum was then preserved for the formol gel slide test frozen at -20°C.

The bone marrow samples were obtained from the posterior superior iliac spine using a bone marrow aspirate needle (Klima) and 5ml disposable syringes. The puncture site was determined by dropping a perpendicular line from the spine to the highest point of the iliac crest. The skin was cleaned and wiped with 70% alcohol.
The skin, subcutaneous tissues and periostium were infiltrated with 1 - 1.5ml of 2% xylocaine. After 5-10 minutes the aspirate needle was pushed into the bone (indicated by a give in to the pressure). The stilet was removed and 1-2ml of bone marrow was aspirated into each of 2 syringes one containing a drop of heparin and the other did not. Smears were made from the nonheparinized sample, stained by Leishman and examined by light microscope for L-D bodies. The two syringes were then closed with their covered needles and taken to the central laboratory for inoculation into the culture media.

2. Parasitological studies

2.1 Smear

Two or more smears were made from the nonheparinized bone marrow sample of each VL suspect. A drop was spread on each slide and allowed to air dry. The smears were then stained with Leishman stain and examined systematically for L-D bodies using the X100 objective and oil immersion. Amastigotes were identified by clearly visible nuclei and kinetoplasts.
2.2 In vitro cultivation

2.2.1 Culture media

(a) Blood Agar Base No 2 (El Safi, 1989) This is a biphasic medium made up of Oxoid Blood Agar Base No 2 and supplemented with 10% defibrinated rabbit heart blood. 40 gm of this blood agar were dissolved in 100 ml of distilled water in a water bath at 56°C and then autoclaved. After cooling the blood was added as well as penicillin and streptomycin (p/s in a concentration of 200U/ml and 200µg/ml respectively). After good mixing the medium was dispensed into small glass screw-capped containers and allowed to set as a slope. Hank's balanced salt solution was used as overlay.

(b) Novy-Nicolle-Mac-Neal medium (NNN medium) (Evans 1989). This is a biphasic blood agar based medium. Plain non-nutrient agar (1.4 gm), NaCl (0.6 gm) and distilled water (90 ml) are heated in a flask. The agar is allowed to melt and the contents of the flask are well mixed before autoclaving the mixture at 21°C for 15 minutes. The mixture is then allowed to cool to about 50°C before adding aseptically collected defibrinated rabbit heart blood to a final concentration of approximately 15% p/s is added and all
is mixed by rolling before dividing the medium into small glass screw-capped containers. These are placed in a sloped position until the agar sets and can then be stored in a refrigerator. Before use a few drops of sterile distilled water are added if the water of condensation is not enough.

(c) RPMI 1640 medium (Flow Lab.) This liquid medium is commercially available. It is used supplemented with 20% heat-inactivated fetal calf serum (FCS) p/s is added. 1 - 2 ml are used per container.

(d) Hanks's balanced salt solution (El Safi, 1989) This liquid medium is prepared by mixing two solutions. These are prepared as follows:

Solution 1

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>80 g</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>4 g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>1.4 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>2 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>500 ml</td>
</tr>
</tbody>
</table>

Solution 2

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>1.25g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.60g</td>
</tr>
</tbody>
</table>
Glucose 10 g
1% Phenol red 16 g
Distilled water 500 ml

Solution 2 is slowly added into solution 1 and shaken well. 9 litres of distilled water are added to give a final volume of 10 litres and distributed in 500ml bottles to be autoclaved (10min. at 10lbs.). Before use 10ml of 2.8% sodium bicarbonate is added to each 500ml as well as 10ml of a mixture of penicillin and streptomycin (1/100 IU Penicillin and 1g streptomycin per 100ml distilled water).

(e) Schneider’s Drosophila medium (Evans, 1989) This commercially available liquid insect tissue culture medium is supplemented with 20% (FCS). 1-2 ml is used per container.

(f) ‘Sloppy Evans’ (Evans, 1989). This is a nutritionally rich semi-solid medium containing rabbit blood and FCS. Its preparation involves two steps. Step (1) is the preparation of proline balanced salt solution (PBSS). This is prepared one litre at a time as it has good keeping properties. The following ingredients are used.

KCl 0.4g
Na$_2$HPO$_4$ .12H$_2$O     0.06g  
KH$_2$ PO$_4$                0.06g  
CaCl . 2H$_2$O           0.185g  
MgSO$_4$ . 7H$_2$O        0.1g  
MgCl . 6H$_2$O           0.1g  
NaCl                       8.0g  
L-Proline                1.0g  
Phenol red             (q.s.)

These ingredients are dissolved one at a time in about 750ml of distilled water. The pH is adjusted to 7.2 with solid Tris (tris(hydroxymethyl) amino-methane). The volume is made up to1000ml, dispensed into screw-capped containers and autoclaved (121°C, 15 min). Preferably it should be stored at 4°C but will not spoil for several months at room temperature.

Step (2) The following materials are weighed.

PBSS             80 ml  
Bacteriological peptone (Oxid)     0.1g  
Beef Extract (Oxid, Lablemco)     0.03g  
Rabbit heart blood (defibrinated)  10 ml  
FCS (inactivitated)             20 ml
Agar (plain, non-nutrient) 0.3g

The ingredients 1-4 are mixed in a screw-capped container and autoclaved (121°C, 15 min). After cooling to about 50°C the blood and FCS are added as well as p/s. The total is mixed by rolling and then dispensed into small culture containers while still molten. After cooling the medium remains semi-solid and is refrigerated until needed.

2.2.2 Primary isolation of the parasite

In the central laboratory the heparinized and nonheparinized samples were inoculated into the 6 culture media. This was mostly done within 12 hours and not later than 24 hours; during which time the samples were kept at room temperature or in the door of the fridge. The separated serum was removed and only 1-2 drops of the marrow was used to inoculate each culture bottle. A locally made cabinet without UV-light and a Bunsen flame were used. The inoculated media were incubated at 22°C.

Routine check was done every 7-10 days with earlier check on the liquid media. In our case check was kept to a minimum to avoid contamination. Samples were discarded only after the 3rd check or if contaminated. The containers were opened near the flame in the cabinet. A drop was drawn using a sterile Pasteur
pipette, placed on a clean glass slide and covered with a cover-glass. The wet preparation was then systematically examined using the X40 objective for the presence of promastigotes (see plate 1 page 53).

Results were noted as negative growth or positive growth, intensity being shown by the number of crosses with the addition of R for rosette formation. Contamination was recorded as CB for bacterial and CF for fungal contamination. Positive cultures were send for isoenzymes analysis to Dr. D. Evans at the Department of Medical Parasitology, London School of Hygiene and Tropical Medicine with the collaboration of Sudan Airways Crew.

3. Serological Studies

3.1. Direct agglutination test (DAT)

The DAT was performed on sera from all patients involved in the other two studies, in vitro culture and formol gel slide test.

Sera were eluted from blood spots on filter paper. Each spot, approximately in 1 cm in diameter, was cut and covered with 1ml of buffered saline overnight. The elute was used for the test.

The antigen was kindly supplied by Dr.A.El Harith, Department of Medical Microbiology, University of Amsterdam.
The antigen (1S strain) is a stabilized suspension of whole promastigotes of *L. donovani* that have been enzyme-treated, formalin-fixed and Coomassie blue-stained (El Harith *et al*, 1986).

The test was performed according to E Harith *et al* (1988) using microtitre plates with 96 V-shaped wells. The diluent does not keep and must be prepared fresh. It was prepared by dissolving 0.2g of gelatine in 100ml of normal saline. This was brought to boiling in a water bath and allowed to cool before adding 0.8µl 2-mercaptoethanol. 50µl of diluent were added to all wells. 2-fold serial dilutions of serum were made starting from well 2 with 50µl of elute (serum diluted 1:100). Well 1 was used as a negative control. The antigen suspension was allowed to attain room temperature and shaken gently before use. 50µl were added to each well. The plate was carefully shaken on a level surface for a few minutes. It was then covered and placed at 22°C for 18 hours (overnight). The test was read against a white background. The first well to show a clear sharp-edged blue spot comparable to the negative control is the end point; the titre corresponding to the reciprocal of the dilution in the well just before this (see plate 2 page 54). The principal of the test is based on the fact that the blue promastigote sediment in the absence of agglutinating antibodies
gives rise to the well-demarcated blue spot. In the presence of antibody sedimentation is sufficiently prevented to result in a less clearly demarcated blue area or a blue suspension.

**3.2 The formol gel slide test**

The test was carried out on 64 serum samples of bone marrow smear positive and DAT positive patients of VL. Sera were obtained by centrifugation of venous blood samples collected from VL suspects and allowed to clot overnight; they were stored at 20°C until used for the test. In addition 10 DAT negative serum samples from healthy individuals from a non-endemic area were used as control.

To test cross reactivity with other leishmanial infections sera from 8 parasitologically confirmed cutaneous leishmaniasis cases were tested. Serum samples from patients with other diseases were also tested. These included malaria (5 samples), typhoid (5), schistosomiasis (5), tuberculosis (5), onchocerciasis (5) and viral hepatitis (5) as well as sera positive for ASO (4), rheumatoid factor (5) and VDRL (5); all these sera were DAT negative.

The test was performed by mixing a large drop of serum (50 µl) and a small drop of concentrated formalin solution 40% w/v (10 µl) on a slide (Cheesbrough, 1987; Goris, 1993). The test was
read against a dark background within 15 minutes as usually the sample was drying out by then.

Results were read as (see plate 3 page 55)
- (negative) when no changes occurred within 15 minutes
+/- (weakly positive) when whitening but no jellying occurred within 15 minutes
+ (positive) when whitening and jellying occurred within 15 minutes
+ Q (highly positive) Q for quick when whitening and jellying occurred within the first 3 minutes.

CHAPTER 3

RESULTS

1. Clinical studies
Table 1 shows the clinical features of 68 parasitological confirmed VL patients included in the study. The male to female ratio was 8:1. The age ranged from 8 - 75 years with 75% occurring in the age group 10 - 29, i.e. children and young adults. Most of the cases came from Gadarif and Babanosa in the Blue Nile province. A few came from El Dinder, El Nuhud, Managil, Damazin and Kadugli. The cases from the South came from Malakal and Bentiu as well as a case from Equatoria and another from Bahr el Gazal. Duration of the illness before presentation was 3-6 weeks in 50% of the patients with a range in the total series of 2 weeks to over one year.

All patients complained of fever at presentation. Loss of weight was a common complain (88%) as well as cough (75%). Epistaxis was a main complain in 45% of the cases. Abdominal pain occurred in 40% and diarrhoea in 14%. Splenomegaly was found in 96% of the cases. Hepatomegaly was less pronounced and occurred in 65%. Lymphadenopathy was common occurring in 63%.

2. Parasitological studies
The results of *in vitro* culture of 44 bone marrow samples from VL suspect are summarized in Table 2. The results of heparinized and nonheparinized bone marrow samples obtained from each patient and inoculated in the 6 media are shown.

14 smears of the total 44 cases were positive for LD bodies and 19 samples were isolated by culture in the different media. Contamination both bacterial (cb) and fungal (cf) was common. Most of the growth occurred in Sloppy Evans medium (SLO) and was remarkably intense. Blood Agar base No 2 (BA No2) showed growth but was affected by contamination of the overlay. NNN did not show good growth in this study and was disappointing. RPMI and Hank's solution (HAN) also did not show remarkable growth. Schneider's medium (SCH) was unfortunately lost to contamination early in the study. However it gave an intense growth with rosette formation in sample 16. The DAT results of 27 of the total 44 cases ranges from 100 >102400. Samples 9, 17, 25 and 26 proved to be non-VL cases.

Table 3 gives a comparison of the smear and *in vitro* culture results of the 44 VL suspects. Growth was obtained in 10 (71%) of the 14 smear positive samples, while 9 (30%) samples of the 30 smear negative samples showed growth. 9 (20%) of the total gave
positive cultures but were smear negative as compared to only 4 smear positive samples in which growth was not detected. Statistical analysis by the McNemar test (binomial 2-tailed \( p=0.2668 \)) showed the difference between the smear and in vitro culture results to be insignificant. Analysis was repeated without the last 17 samples in which contamination was marked.

Table 4 shows a comparison between the smear and in vitro culture results of the first 27 VL suspects. Primary isolation was obtained in 9 (90%) of the 10 smear positive samples. Out of 17 smear negative samples culture was positive in 8 (47%). 8 (30%) of the 27 studied samples were culture positive but smear negative as compared to 1 (4%) sample which was positive by smear but did not show any growth. Statistical analysis by McNemar test (binomial 2-tailed \( p=0.0391 \)) showed the difference between the smear and in vitro culture results to be significant at \( p<0.05 \).

Table 5 shows the overall growth of 19 Leishmania primary isolates from bone marrow samples in different culture media. The overall number of isolates in Sloppy Evans medium was 17(89%) of the 19 positive cultures. In the other media 11 (58%) were in Blood Agar base No. 2, 7 (37%) in NNN, 5(26%) in RPMI + 20%
FCS, 5 (26%) in Hank's solution and 2 (10%) in Schneider's medium.

Table 6 shows the rate of *in vitro* growth of *Leishmania* in the 14 smear positive bone marrow samples in the different culture media. Evans' sloppy medium was positive in 10 (71%) of the smear positive samples, Blood Agar base No. 2 was positive in 7 (50%), Hank's in 2 (14%) and Schneider in one (7%).

### 3. Serological studies

Table 7 records the results of the formol gel slide test and the DAT for sera of 64 parasitological confirmed VL cases. All are DAT positive. Of the total VL samples tested 59 (92%) gave a positive result. Of these 23 (39%) were weakly positive (+/-) and 22 (37%) highly positive. The 10 control samples from a non-endemic area all gave negative results. The samples tested for cross reactivity gave negative results except in the case of onchocerciasis where incomplete whitening occurred.
Plate (1): Promastigotes of *Leishmania* in culture
Plate (3): Formol gel slide test.

A. Negative and positive control.
B. 2 positive samples.
C. 2 negative samples.
D. 2 positive samples, one showing jellified serum.
### TABLE (1): CLINICAL FEATURES OF 17 PARASITOLOGICALLY CONFIRMED VL PATIENTS.

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<td>Lymphadenopathy</td>
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TABLE (2): *IN VITRO CULTURE OF BONE MARROW SAMPLES FROM VL SUSPECTS*.

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*cb = contamination (bacterial)*

*cf = contamination (fungal)*
Table (3): Comparison between smear and *in vitro* culture results of 44 bone marrow samples from VL suspects

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Table (4): Comparison between smear and *in vitro* culture results of 27 bone marrow samples from VL suspects

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TABLE (5) : The overall growth of 19 *Leishmania* primary isolates from VL suspect bone marrow samples in different culture media

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<th>Culture medium</th>
<th>Sample with heparin</th>
<th>Sample without heparin</th>
<th>Overall No</th>
<th>%</th>
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<td>58</td>
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Table (6): the rate of *in vitro* growth of *Leishmania* in 14 smear positive bone marrow samples in different culture media.

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<th>Culture medium</th>
<th>Sample with heparin</th>
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Table (7): The slide formol gel and DAT Results of sera from parasitologically confirmed VL patients.

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W * : Whitening.
G ** : Jellying.
Y *** : Seen.
O **** : Not seen.
Q ***** : W & G seen in first 3 minutes.
1. Clinical studies


Children and young adults were affected most with a higher incidence among males. El Safi et al (1991) working on VL cases from Kassala and the Blue Nile provinces found the disease to affect young male adults most. Archibald and Haseeb (1937) observed that in the Kapoeta focus the disease affects mainly young children. In the epidemic that hit the Nuer tribe in Bentiu in Upper Nile province de Beer et al (1991) found children less than 15 years forming 40% of their patients and another 40% falling in the age group 15-34 years.

Fever was a presenting feature in all cases in this study and weight loss was very common. Epistaxis was a complaint in 45% of patients. Ali et al (1987) reported epistaxis in 2/3 of 120 VL
cases mainly from Gadarif in the Blue Nile province. Zijlstra et al (1992), who studied 43 children and 45 adults from the Bentiu epidemic, reported epistaxis in 55% of adults and 44% of children. Diarrhoea was not common but when present was very weakening and difficult to treat. Abdominal pain was common and was mostly related to hepatosplenomegaly. 96% of the patients had splenomegaly; the fact that a palpable spleen may not be found in KA has earlier been observed by Cole (1944). Only 65% had hepatomegaly in this series.


2. **In vitro cultivation**

The choice of the isolation method depends on the immediate circumstances and to some extent on the technical capability and experience of the staff. *In vitro* isolation offers certain advantages over *in vivo* methods (Evans, 1989). Cultures become positive more rapidly. Materials required are less expensive. The cultured organism can be cryopreserved thus reducing time and personnel required for maintenance. The disadvantages of *in vitro* isolation
are that some *Leishmania* species are extremely difficult to culture and sterile conditions may be difficult to attain in the field.

*In vitro* cultivation of the causative organism probably plays a more important role in the study and treatment of the leishmaniases than in any other group of diseases caused by protozoa (Evans, 1987). Parasitological diagnosis, identification and characterization of the organism, biochemical, biological and immunological as well as chemotherapeutic studies usually involve *in vitro* cultivation of the parasite in one form or another.

Most of the trouble and loss of organism occurs at the stage of isolation and the initial establishment of the organism in the culture medium. The artificial medium must permit efficient transformation from amastigote to promastigote form and then support division and nutrition. Important is as well the methods used to obtain the sample containing the amastigotes and its inoculation into the medium. In the present study bone marrow samples have been used for the cultivation of *Leishmania*.

Although bone marrow aspiration may be painful it is definitely safer than splenic puncture which has limitations such as an abnormal prothrombin, clotting or bleeding time; in addition
splenic aspiration can result in serious complications which may be fatal if adequate facilities are not available.

The results of the present studies (Table 4) show that 8 (47%) of the 17 smear negative samples from VL suspect gave positive cultures. When 4 patients of these 17 that were finally diagnosed as non-VL are excluded the percentage increases to 61.5% (that is 8 out of 13). In vitro culture is statistically more sensitive than smear examination for the demonstration of *Leishmania* (*p*<0.5); it is easy to perform and should be included in the routine parasitological diagnosis of VL.

The desirable qualities of a medium for the diagnosis and primary isolation of *Leishmania* include a high sensitivity for a small number of amastigotes and a liquid formulation, preferably lyophilizable, that will not lose its efficacy upon reconstitution (Miles, 1982). Low cost and a long shelf-life would be ideal. It should be possible to protect the growth against bacterial and fungal contamination by antibiotics and antimycotics. The capacity to reconstitute the medium and seed it 'through-the-cap' would be an advantage in the field.

A 'universal' culture medium that is suitable for the growth of the different *Leishmania* is still lacking (Evans, 1987). Work to
establish which medium is best suited for the growth of a particular isolate is tedious and almost impossible. Initial isolation has its problems. Mass cultivation for the collection of promastigotes is easier. Cloning, cultures derived from a single promastigote, has become important especially for immunologists and molecular biologists. Several methods have been devised for this.

Ideally different media should be inoculated hoping that at least one will prove suitable for isolation (Evans, 1987). The traditional biphasic blood agar medium should be used in preference to a tissue culture medium supplemented with foetal calf serum when faced with an unknown organism. Results of this study have shown that Evans' sloppy medium is the best of the 6 studied; it produces and supports the greatest intensity of growth and if growth took place at all it was always (except in one case where it occurred in Blood Agar base No.2) in this medium. Evans (1987) recommended it and Rassam and Al-Mudhaffar (1979) obtained 80% recovery culturing bone marrow samples. It also showed relatively less contamination than the biphasic media. The effect of contamination was less damaging in this medium and a number of the contaminated samples send to England have been cleared and are now thriving.
A great intensity of growth was observed in two cases from the south who had smears full of amastigotes. However, in another case from Gadarif there was an intense growth although the smear showed scanty parasites. This difference could be due to the intrinsic differences of the parasite strains that are found in the different regions and can be clarified by isoenzyme characterization. Further work is required to investigate the correlation between smear positivity and intensity of growth.

The shortest time for growth to be detected in Evans sloppy medium was 6 days after inoculation. Most positive cultures were so by the 2nd check (14 days after inoculation). Promastigotes were still alive in 3 samples 8 months after inoculation. These were sub cultured into fresh medium where active growth was noted 7 days later. This confirms the findings of previous workers (Evans, 1987) that Evans' sloppy medium is a valuable transport and storage facility.

As shown in Table 6, 10 (71%) of the 14 smear positive bone marrow samples inoculated in Evans sloppy medium, showed growth of the parasite. This finding refers to the fact that *Leishmania* responsible for VL in the Sudan is not intrinsically
difficult to grow as previously thought. El Safi (1989) had only 9% growth in bone marrow samples of VL cases.

The second best medium was Blood Agar base No.2. Control of contamination can elevate this medium to the rank of Evans sloppy medium with the added advantages of ease of preparation and lower cost. Schneider's medium was not properly evaluated due to early contamination in the main bottle. However further evaluation is warranted as it showed good growth in the few cases before contamination had occurred. Another advantage of Schneider's medium is its being a liquid medium; this saves time and effort required to obtain clean promastigote suspensions that are needed before it can be used. Miles (1982) was of this opinion and like other workers recommended its use; however its cost may be a limiting factor in developing countries.

NNN medium was first used without overlay and this resulted in drying out and contamination. Hank's was then used as overlay but growth was not remarkable in this much used medium in the present study. Abdalla (1980) used NNN to culture promastigotes to prepare antigen for his studies and Evans (1989) recommends primary isolation in it in preference to a richer medium as the isolate may die on subculture in such a medium. Rassam and Al-
Mudhaffar (1979) found NNN with Locke's overlay superior to blood agar base No 2 with glucose and Lock's overlay. Saran et al (1986) found NNN with Hank's solution as overlay inferior to Grace's insect medium and taking longer to show peak growth.

Hank's solution was used without FCS and growth occurred although it was not intense; the fact that this simple medium could yield growth is interesting. It showed intense contamination however spoiling many samples inoculated in NNN and Blood Agar base No. 2 media where it was used as overlay. RPMI + 20% FCS did not show good growth in this study. It also does not maintain growth for more than 10 days.

Although the present study has clearly shown the value of Evans sloppy medium in the primary isolation of *Leishmania donovani* further study in the area is required. This includes work to determine the best medium in terms of shortest time to show growth. This would require daily check and use should be made of culture bottles that can be checked for by an inverted microscope to avoid loss of samples by contamination. It would be of interest to find out the influence of the size and amastigote content of inoculums on the occurrence of growth and its rate. This involves the meticulous grading of parasites in the smears and is influenced
by the intrinsic properties of the parasite strain regarding ease of growth in *in vitro* culture. To determine the effect of serum the samples need to be centrifuged to remove all serum before inoculating the pellet resuspended in saline. It has been reported that human serum has an inhibitory effect on promastigotes (Evans, 1989).

Contamination is a major problem and further measures should be taken for its control as it has serious effects on the growth of the parasite. The diagnostic value of *in vitro* culture requires more evaluation under optimum aseptic conditions; these include the use of a proper cabinet with UV-light, the utilization of disposable culture bottles that can be checked for growth without opening by an inverted microscope as well as evaluation of the use of the flame. However the cost of the procedure must be carefully balanced to be within reach of those who need it most.

Schneider's medium and Grace's insect medium are two liquid media that deserve further study and evaluation. They would be of great use when promastigotes are needed for mass cultivation to prepare antigen for serology or biochemical studies. Research to study and evaluate media for mass cultivation is the
next priority. The production of a vaccine can also benefit from such liquid media that support profuse growth.

3. Formol gel slide test

Most of the parasitic diseases occur in the developing regions of the world where the climate is usually hot (either arid or humid) and basic services such as transport, electricity and water are frequently inadequate or unreliable. The financial conditions in these areas impose marked limitations on the availability of laboratory facilities and equipment as well as a shortage of trained personnel. Ease of commercial and recreational travel to the tropics and movement of refugees has spread the disease more and exposed a greater number of people to it.

The immense development seen during the last 2 decades in parasitic serology has resulted because of a great need for effective immunodiagnostic methods for tropical diseases; the usual method of direct parasitological demonstration of parasite involves a laboratory effort costly both in material and human resources (Voller and De Savigny, 1981). In both fields of diagnosis and epidemiology immunodiagnostic techniques carry the advantages of early detection of infection, detection of infections of low intensity, cost-effectiveness when compared with
conventional parasitological techniques and ease of automation and batch processing. However the developed techniques need to be specific to be used for diagnosis. Cross-reactivity between different parasite antigens and multiparasitism of individual hosts need to be considered. The techniques need to be sensitive to both early and light infections to be of use in epidemiological monitoring procedures as well as practical and economical in field conditions.

In this study the formol gel test has shown a sensitivity of 92%. Comparable results were earlier obtained in Bangladesh where the sensitivity was found to be 94% as compared to IFAT which had a sensitivity of 96% (Chowdhurry et al, 1992). However contradictory results have been obtained by earlier workers for Sudanese KA. Van Peenen and Miale (1962) reported a sensitivity of less than 50% sensitive in VL cases from Upper Nile province. However they noted a closer correlation of positive formol gel tests with elevation of gammaglobulins than thymol turbidity and flocculation tests. Henderson (1937) thought it useful as a screening test. Cole (1944) in his extensive review of KA in East Africa (including a series from an area near Kapoeta in southern
Sudan) found that the test was negative in early cases however it became positive in patients who have had fever for 3 or 4 months.

The formol gel test has been thought to be non-specific (Cheesebrough, 1987). However cross-reactivity testing of the formol gel test in the present study showed it to be quite specific. The test gave negative results when tested on sera from patients with malaria, typhoid, schistosomiasis, tuberculosis and viral hepatitis as well as sera positive for ASO, rheumatoid factor and VDRL. In the case of the onchocerciasis samples whitening developed in the periphery of the serum drop.

The present study has demonstrated the value of the formol gel test in the serodiagnosis of VL. The slide test is a valuable test in circumstances where demanding serological tests can not be done and where the performance of parasitological diagnosis is difficult. The test requires a small amount of serum which is ideal for children and as well under field conditions. It is simple to perform, involves negligible cost and can be done in any rural setting even by the clinician.

It has been found that the formol gel test becomes positive about 3 months after infection (Cheesebrough, 1987). Thus it may be of value as a complimentary test to the DAT which, due to its
higher sensitivity, does not differentiate between active disease, and subclinical infection in an endemic area (Zijlstra et al, 1991). A broad evaluation of the test is required under field conditions to test its value in the detection of subclinical infection and past disease. It would also be of interest to determine its potentials as a therapeutic test. This entails study of its variation with treatment and further correlation with globulin levels.
CHAPTER 5

CONCLUSION and RECOMMENDATIONS

The clinical features of the VL cases included in the present study were similar to those found by earlier workers. In addition to Gadarif area in Eastern Sudan which has been known to be endemic for VL for many years, many patients in the present study came from Babanosa in Western Sudan.

In vitro cultivation of bone marrow samples from VL suspects was found to be significantly more sensitive than stained smear examination in VL (p<0.5). Leishmania donovani responsible for VL in the Sudan is not difficult to grow as primary isolation of the parasite was successful in 90% of smear positive samples. For initial parasitological diagnosis of VL, however, both in vitro cultivation and direct microscopy of aspirates should be employed.
Contamination was a major problem as it has adverse effects on the in vitro growth of the parasite; further measures should be taken for its control.

The best of the six studied culture media used for the primary isolation of Leishmania was found to be Evans sloppy medium which showed a detection rate of 89%; growth was mostly intense with profuse rosette formation and contamination was relatively less damaging than in the other media tested. In addition this medium is ideal for storage and transportation as it can maintain growth of the parasite for more than 8 months.

Blood agar Base No.2 was the second best medium and would be a cheaper substitute.

The formol gel slide test was sensitive, specific, and simple to perform, of negligible cost and requires a small amount of serum. It is still of value in the serodiagnosis of VL and can be integrated in the most peripheral levels of primary health care.
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