بعض الأوجه المناعية والبيوكيمياوية لمرضى اللشمانية الأحشائية

أطروحة مقدمة من قبل جاسم حميد طاهر

إلى مجلس كلية العلوم - جامعة بابل

وهي جزء من متطلبات نيل درجة الدكتوراه فلسفة

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SOME BIOCHEMICAL & IMMUNOLOGICAL ASPECTS OF PATIENTS INFECTED WITH VISCERAL LEISHMANIASIS

A thesis

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to the Council of the College of Science

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in

Biology/Zoology

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الخلاصة

تضمنت هذه الدراسة قياس بعض الاختبارات المناعية والبايوكيمياوية لعينات دم أطفال أصحاء ومرضى ، شملت ٨٦ عينة لأطفال مخمجين باللشمانية الاحشائية *Leishmania donovani* (أُكدت اخماجهم من خلال وجود الطفيلي في خزعة نخاع العظم) ، ٣٦ عينة لأطفال أصحاء و ٢٠ عينة لأطفال مخمجين بأمراض غير اللشمانية الأحشائية وهي التدرن الرئوي ، حمى مالطة ، الحمى التيفوئيدية وداء المقوسات.

أُستخدمت عدتان لتشخيص خمج اللشمانية الاحشائية ، احدهما تجارية تتضمن قياس المناعة المُمتزه عن طريق الخميرة المرتبطة بها (أليزا ، ELISA) والثانية صنعيّة تم تهيئة مستلزماتها في المختبر من خلال تحضير المستضد النوعي لها (طور اللشمانية الاحشائية أمامي السوط) وتدعى الاليزا النقطية (Dot-ELISA). أعطت الاليزا التجارية خصوصية ٩٩٪ وحساسية ٩٧٪ أعلى قليلاً مما هو عليه في الأليزا النقطية والتي كانت ٩٢٪ و ٩٦.٥٪ على التوالي.

ارتفعت معدلات قيم الكلوبيولينات المناعية IgG و IgM أثناء الخمج بشكل ملحوظ مقارنة بمجموعة السيطرة ، ثم انخفضت تدريجيا بعد العلاج فيما لم تظهر فروق معنوية في معدل قيم IgA في مجموعة المخمجين عما هو عليه في مجموعة السيطرة. ازدادت معدلات تركيز المكون الثالث والرابع للمتمم (C_3 & C_4) في أثناء الخمج بالمقارنة مع مجموعة السيطرة ثم انخفضت تدريجيا بعد العلاج.

تفاعلت مصول المرضى مع ١٢ موقعاً ببتايدياً تراوحت أوزانه الجزيئية بين ١٣ - ٧٤ كيلودالتون عند استخدام هلام البولي اكريلمايد و الوصمة المناعية. إن تشخيص الوحدة البروتينية ١٨ كيلودالتون من قبل الأضداد في مصول المرضى يعد فحصاً تأكيديا لتشخيص خمج اللشمانية الأحشائية ، وقد تفاوتت شدة التفاعل للأضداد النوعية في مصول المرضى مع هذه الوحدة باختلاف نوع المستضد .

ظهر انخفاض واضح في قيم الخلايا اللمفاوية التائية والخلايا اللمفاوية المساعدة وكذلك في النسبة المئوية ما بين الخلايا اللمفاوية المساعدة والخلايا اللمفاوية السّمية ، كما ازدادت نسب الخلايا اللمفاوية السّمية زيادة معنوية إضافة إلى زيادة الخلايا اللمفاوية البائية زيادة غير معنوية مقارنة بمجاميع السيطرة. لم تُظهر النتائج بصورة عامة قبل العلاج أية فروقات معنوية بين بعض الدلائل الطبية وهي العمر والجنس ، وعند متابعة نسب هذه الخلايا بعد العلاج لوحظ إن هناك ارتفاعاً في النسبة المئوية مابين الخلايا اللمفاوية المساعدة والخلايا اللمفاوية السمّية مع بقاء

هذه القيم اقل من معدلاتها في مجموعة السيطرة ، في حين انخفضت نسبة الخلايا اللمفاوية البائية إلى نسبة اقل إلا إنها بقيت أعلى من معدل مجموعة السيطرة.

أظهرت قيم المحركات الخلوية زيادة معنوية في الانترلوكين ـ العاشر (IL-١٠) ، الانترفيرون كاما (IFN-γ) وعامل نخر الورم ـ الفا (TNF-α) لمرضى اللشمانية الاحشائية قبل العلاج . أما بعد العلاج فكان هناك انخفاضاً معنوياً ولكنه بقي أعلى من قيمة مجموعة السيطرة.حصلت زيادة في قيمة البروتين الموجب للحمضات (ECP) في مصول الأطفال المخمجين باللشمانية الاحشائية قبل العلاج ، أما بعد العلاج فان هذه القيمة انخفضت بصورة معنوية عما هو عليه قبل العلاج.

كانت هناك زيادة معنوية في مستويات الإنزيمات ادينوسين دي أمنيز (ADA) ، كلوتاثايون ردكتيز (GHR) و سوبرأوكساید دسميوتيز (SOD) في مصول الأطفال المخمجين باللشمانية الاحشائية مقارنة بمعدلاتها السوية في الأصحاء ولم تكن هناك فروق معنوية بدلالة العمر و الجنس.

أظهرت معدلات الإنزيمات ألكلاين فوسفاتيز (ALP) ، لاكتيت ديهايدروجينيز (LD) ، آلانين امانوترانسفيريز (ALT) و اسبارتيت امانوترانسفيريز (AST) ارتفاعاً معنوياً في مصول الأطفال المخمجين باللشمانية الاحشائية مقارنة بمعدلات السيطرة ولا توجد فروقات معنوية بدلالة العمر والجنس.

ظهرت ثلاثة أنماط إنزيمية لمتناظرات الإنزيم ALP و خمسة أنماط إنزيمية لمتناظرات الإنزيم LD باستخدام هلام البولي اكريلمايد ، اختلفت حزم هذه المتناظرات من الناحية الكمية عما هو عليه في مجموعة السيطرة عند اختبار فعالية الإنزيمين قبل وبعد المعالجة.

كانت هناك زيادة معنوية في عنصر النحاس وانخفاضاً معنوياً في عنصري الخارصين والمغنيسيوم مقارنةً بمجموعة السيطرة. تميزت فئة سنة واحدة من العمر بزيادة في قيمة عنصر النحاس (وليس في عنصري الخارصين والمغنيسيوم) عما هو عليه في الفئتين الاخريتين (اقل واكثر من سنة) من العمر.

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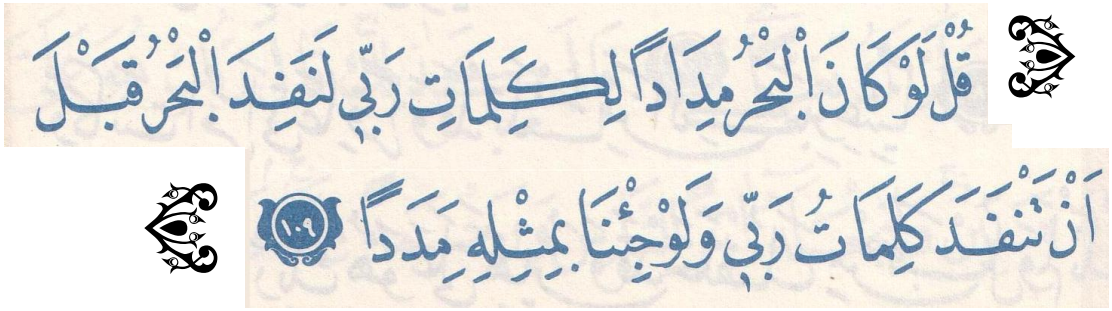
بسم الله الرحمن الرحيم


قُلْ لَوْ كَانَ الْبَحْرُ مِدَادًا لِكَلِمَاتِ رَبِّي لَنَفِدَ الْبَحْرُ قَبْلَ
أَنْ تَنْفَدَ كَلِمَاتُ رَبِّي وَلَوْ جِئْنَا بِمِثْلِهِ مَدَدًا ١٠٩

صدق الله العظيم

سورة الكهف

الآية ١٠٩

Certification

We certify that this thesis was prepared under our supervision at the Department of Biology, College of Science, University of Babylon as a partial of fulfillment of the requirements for the degree of Doctor Philosophy in Biology (zoology) and this work has never been published any where.

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Jasim

Summary

This work was carried out to study some immunological and biochemical aspects which were performed on blood samples of visceral leishmaniasis and control groups. Eighty six blood samples were confirmed parasitologically by bone marrow smears, thirty six samples from apparently healthy children and twenty children samples with infectious diseases other than VL including tuberculosis (٥) , typhoid fever (٥) , brucellosis (٥) and toxoplasmosis (٥).

Two types of ELISA kits were used for the diagnosis of VL disease, the commercial ELISA kit and the Dot – ELISA kit, which were prepared from *Leishmania donovani* promastigote antigen. ELISA kit gave slightly higher specificity (٩٩٪) and sensitivity (٩٧ %) than Dot – ELISA (٩٢ %) and (٩٦.٥ %) respectively. The two methods failed to detect four parasitologically proven cases. The cross– reactivity was ١٠ % for results of the two methods.

The results of immunoglobulins revealed that there was a significant increase in levels of IgG and IgM measured by single radial immunodiffusion assay in comparison to control, and then reduced after treatment. No significant differences in the mean level of IgA in infected children in comparison to that of healthy control. The mean level of the third and fourth complement components ($C^{٣}$ & $C^{٤}$) was significantly higher than that of control group , then reduced after treatment.

By using SDS – PAGE and Western blot assay, the patients' sera reacted with ١٢ proteins bands ranging from ١٣ – ٧٤ k Da. The recognition of ١٨ k Da VL antigens by sera of patients considered as specific test confirming a clinical diagnosis of VL. Variable reaction strength showed with this specific band according to the type of antigen.

A significance decrease was found in number of $CD_{٣}$ and $CD_{٤}$ cells in VL patients compared to the healthy control group, while the results showed that there was a significant increase in $CD_{٨}$ lymphocytes with VL patients in comparison to the control group. The $CD_{٤}/CD_{٨}$ ratio was lower in VL patients than that of the control group. There

was a relative increase in $CD_{٢٢}$ of VL patients compared to the control group but was not significant. There was no significant difference in all types of CD cells percentage of VL in relation to some clinical parameters such as sex and age.

Follow up of the patients showed there was a significant increase in the percentage of $CD_{٣}$, $CD_{٤}$ and $CD_{٤}/CD_{٨}$ ratio at the end of therapy. A relative decrease in $CD_{٢٢}$ but still more than control group.

The result of cytokines showed that there was a significant increase in IL – ١٠ , IFN – γ and TNF – α levels in sera of patients with VL before treatment in comparison to control group. Although the levels in patients after treatment still slightly higher than the control group, there was no significant differences in comparison to control.

There was an increase of eosinophil cationic protein level in the sera of VL patients when compared to that of normal control group. In the post treated VL group, the level was significantly lower than pretreated VL group. There was no important differences between the post treated and control group.

There was a significant increase in the levels of adenosine deaminase, glutathione reductase and superoxide dismutase enzymes in VL patients compared to the healthy control group. There was no significant change in relation to the sex and age.

The mean levels of alkaline phosphatase, lactate dehydrogenase , alanine aminotransferase and aspartate aminotransferase enzymes exhibited a significant elevation in VL patients compared to the control. There was no significant change in relation to the sex and age.

ALP isoenzymes revealed three banding patterns which differ from the three zymodems which were obtained from control group. These differences may be due to isoenzymes activity of patients with VL before and after therapy. LD isoenzymes revealed five banding patterns differ from the five normal zymodems. These differences

mainly occurred due to LD isoenzymes activity in patients with VL before and after therapy.

The study also showed that there was an increase in the level of copper and a decreased in the levels of zinc and magnesium in sera of VL patient and it was significant as compared with the healthy control. There is an age dependence of serum copper (but not zinc or magnesium) in one age patients, and had a higher serum copper than the patients of more and less than one year old.

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List of abbreviations

Adenosine deaminase

Atomic absorption spectrophotometry

Antibody

Enzyme linked immunosorbent assay	Antibody – dependent cellular cytotoxicity
Glutathione reductase	Alkaline phosphatase
Granulocyte macrophage colony stimulating factor	Antigen presenting cell
٦٣ k Da glycoprotein	Aspartate aminotransferase
Reduced glutathione	٥-bromo-٤-chloro-٣-indyol phosphate
AAS	Bovine serum albumin
Ab	Cluster of differentiation
ADA	Circulating immune complex
ADCC	Cutaneous leishmaniasis
ALP	Cell mediated immunity
APC	Cytotoxic T-Lymphocyte Associated Antigen
AST	٣,٣`- Diamino benzidine tetrahydrochloride
BCIP	deoxy Adenosine Monophosphate
BSA	Deionized distilled water
	Dot-blot immunobinding assay
	Deoxy ribonucleic acid
	deoxy Nucleotide Triphosphate
	Delayed type hypersensitivity
	Distilled water
	Eosinophil cationic protein
	Ethylene diamine tetra acetic acid

Glutathione peroxidase	**CD**
Oxidized glutathione	**CIC**
Horseradish peroxidase	**CL**
Immunoblotting	**CMI**
Immunoelectrophoresis	**CTLA**
Indirect fluorescent antibody test	**DAB**
Interferon - gamma	**dAMP**
Immunoglobulin	**DDW**
Indirect hemagglutination	**DIBA**
Interleukin	**DNA**
Kala - azar	**dNTP**
kilo Dalton	**DTH**
***Leishmania* – Homologue of**	**DW**
	ECP
	EDTA
	ELISA
	GHR
	GM-CSF
	$GP_{۱۳}$
	GSH

GSSG	**receptor for activated C-Kinase**
HRP	**Lactate dehydrogenase**
IB	**Lipophosphoglycan**
IEP	**Monoclonal antibody**
IFAT	**Major histocompatibility complex**
IFN-γ	**Molecular weight**
Ig	**Nicotineamide adenine dinucleotide (oxidized)**
IHA	**Nicotineamide adenine dinucleotide (reduced)**
IL	**Nicotineamide adenine dinucleotide phosphate (oxidized)**
KA	**Nicotineamide adenine dinucleotide phosphate (reduced)**
k Da	**Nitroblue tetrazolium**
LACK	**Nanogram ($١٠^{-٩}$ gram)**
LD	**Natural killer cell**
LPG	**Nitric oxide**
Mc Ab	**Optical density**
MHC	**O – Phenylene diamine**
MW	**Peripheral blood lymphocytes**
NAD	**Phosphate buffer saline**
NADH	
NADP	
NADPH	**GSH-Px**
NBT	

T – cell receptor	**Ng**
Tetramethyl ethylene diamine	**NK**
Transforming growth factor	**No**
Naïve T – helper cell	**OD**
T – helper cell type ١	**OPD**
T – helper cell type ٢	**PBL**
Tumor necrosis factor – alpha	**PBS**
Visceral leishmaniasis	**Programmed cells death**
Western blot	**Polymerase chain reaction**
World Health Organization	**Picogram ($١٠^{-١٢}$ gram)**
	Post kala – azar dermal leishmaniasis
	Phenazine methosulfate
	Protein surface antigen
	Relative mobility
	Sever combined immunodeficiency
	Standard deviation
	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
	Standard error
	Superoxide dismutase
	Tris buffer saline

Th_0

Th_1

Th_2

TNF – α

VL

WB

WHO

PCD

PCR

Pg

PKDL

PMS

PSA

RM

SCID

SD

SDS-PAGE

SE

SOD

TBS

TCR

TEMED

TGF

Chapter One

Introduction

Introduction

Leishmaniasis is one of the major parasitic diseases targeted by the world health organization (WHO, ٢٠٠٠). Two million new cases occur each year and ٣٥٠ million people at risk of infection (Handman & Hocking, ١٩٨٢).The control of Leishmaniasis in several (sub) tropical areas is complicated by the variety of different *Leishmania* species and their diverse clinical manifestation and by the fact that each parasite species has a unique epidemiological pattern (Dye, ١٩٩٦).

In Iraq, as in most of the countries of the Mediterranean Littoral and the Arabian Peninsula, there has been an increase in the number of reported cases during the last few years. A six-fold rise in the number of visceral leishmaniasis cases in ١٩٩١ compared to ١٩٩٠ was observed namely ٥٧٦ cases in ١٩٩٠ and ٣٧١٣ cases in ١٩٩١. This is possibly due to many factors, such as population movement ; the debility of health and vector control facilities during the war (Neouimine, ١٩٩٦). Visceral Leishmaniasis is usually detected in infants and children ; the vast majority of

cases recorded (٩٩٪) occurred in the first seven years of life (AL-Nouri & AL-Jeboori, ١٩٧٣; Sukker, ١٩٧٦). Six isolates were found to be similar to Mediterranean references stock while the seventh was similar to the Ethiopian; thus confirming the unique heterogeneous make up of *Leishmania donovani* stocks isolated from Iraqi children with VL (Rassam & AL-Muddaffar, ١٩٧٩).

The immunology of *L. donovani* has not been studied to quite extent of *L. major* at least in mice and many researchers have shown that the experimental animals respond differently to visceral parasites than they do to cutaneous species. *L. donovani* posses membrane lipophosphoglycans that may inhibit gene expression in macrophages. The inhibition is of protein kinase-dependent expression, such as that involved in macrophage activation by TNF-α and IFN- γ (Locksley & Louis, ١٩٩٢).

Patients with symptomatic kala-azar do not develop $Th_١$ responses against *L.donovani* and their macrophage do not secretes IFN-γ or IL-٢ in the presence of leishmanial antigens. However, those patients regularly had high titer of antileishmanial antibodies that is their $Th_٢$ arm is activated and the $Th_١$ arm is down regulated (Pearson *et al.,* ١٩٨٦).

Children are at greater risk than adult in endemic areas. Malnutrition contributes to the development of disease, and incomplete therapy of initial disease is a risk factor for recurrence of leishmaniasis. Children usually present with intermittent fever , paleness, refusal to feed or anorexia , weight loss, and abdominal distention. Splenomegaly, hepatomegaly , lymph node enlargement , thrombocytopaenia , anemia , leukopaenia and hypergammaglobulinemia are the most common finding in pediatric leishmaniasis (Kafetzis, ٢٠٠٣).

Stibogluconate is the chemotherapy of leishmaniasis. The exact mechanism of action has been determined. Evidence for inhibition of glycolysis in the parasite at the phosphofructokinase reaction has been found. Metabolism is minimal, and the drug is excreted in the urine. Adverse effects include pain at the injection site , gastrointestinal upsets, and cardiac arrhythmias. Renal and hepatic function should be monitored periodically (Richard *et al*., ٢٠٠٦).

Visceral leishmaniasis could be considered as an important cause of liver enlargement with an increase in enzyme activity (Rees & Kager, ١٩٨٧). So many research works have been done on different aspects of leishmaniasis. In Iraq very little information is available about biochemical changes associated with this disease, and to provide further immunological and biochemical evidence on the involvement of *L. donovani* in evasion strategies and to clarify some of the factors that may eventually lead to establishment of visceral leishmaniasis.

Studies on the murine model of visceral leishmaniasis will provide a means to characterize the operative protective immune mechanisms as well as identify parasite antigens with some adjuvants which have potential use for human vaccination. Characterization of the protective immune mechanism in experimental

visceral leishmaniasis will be accomplished by studies of the in situ cytokines response to primary infection in susceptible mice, as well as identification of responses associated with the acquisition of immunity. Once the mechanism associated with protective immunity have been defined the parasite antigens relevant to immunity can be identified. Purified *L. donovani* antigens is elicited as in *vitro* cytokine response which correlates with in *vivo* immunity will be identified and characterized (Rachman & Jaffe, ١٩٩٣).

Therefore this study aims at:

١. Trying to develop Dot-blot immunobinding assay in the laboratory and assess leishmanial promastigote antigen to study its diagnostic value for kala – azar. The results compared with commercial ELISA kit to test their sensitivity and specificity for visceral leishmaniasis in Iraq.

٢. Assessing the role of immunoblot as a confirmatory test by detection of some subunits this might be specific for leishmaniasis.

٣. Recognition numerous antigens of leishmanial promastigote and identifying their molecular weights.

٤. Determining the lymphocyte subsets involvement in the host response (CD٣, CD٤, CD٨ and CD٢٢) to infection by the use of CD-markers.

٥. Assessing in vitro, lymphocyte function by cytokines production to further assess the involvement of Th_1 and Th_2 like cytokines in regulation of immune response.

٦. Assessing the eosinophil cationic protein (ECP) to investigate the role of eosinophil in the evolution of visceral leishmaniasis.

٧. Carrying a biochemical study to monitor the level and the prognostic value of some enzymes in visceral leishmaniasis as follow :

   a. Hepatic enzymes (ALP, LD, ALT and AST).

b. The possible changes in the isoenzymes of some patients' sera.
c. Adenosine deaminase (ADA) which may reflect changes in the immune response.
d. Glutathione reductase (GHR) which is important in antioxidant defense.
e. Superoxide dismustase (SOD), this enzyme is important in the respiratory burst that occurs in host macrophage during phagocytosis .

٨. Assessing the level of some trace elements and their relation to enzymes and the immune response of the disease.

CHAPTER TWO

٣

Literature Review

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٢.١. Historical background

Leishmaniasis, also know as “Kala – azar” , “ black fever” or “black sickness” is a wide – spreading infectious disease in tropical or subtropical region (WHO,٢٠٠٠). William Leishman and Charles Donovan first demonstrated the protozoan parasite in the spleen of patients suffering from a malaria – like illness, which became known as visceral leishmaniasis (VL), separately but simultaneously in

١٩٠٣. The causative agent of VL was named *L. donovani* after its co-discovers. Human leishmaniasis with a prevalence of ١٢ million cases and an approximate incidence of ٠.٥ million cases of VL and ١.٥ million cases of cutaneous leishmaniasis. The Indian kala-azar commission (١٩٣١ – ١٩٣٤) demonstrated the transmission of *L. donovani* by *Phlebotomus* spp. (Amit *et al*., ٢٠٠٤).

٢.٢. Human leishmaniasis

The leishmaniasis had been classified into different classes on the basis of the basic syndromes of the disease. Different species of *Leishmania* appeared identical and were generally distinguished by clinical and geographic characteristic. Modern speciation by isozyme pattern, monoclonal antibodies, DNA hybridization, DNA restriction endonuclease fragment analysis, and chromosomal karyotyping was continuous to delineate new species, particularly in the new world, and to demonstrate the capacity of different species to cause similar clinical syndromes. There are four major syndromes – visceral leishmanasis (kala-azar) , cutaneous leishmaniasis, mucocutaneous leishmaniasis (espundia) and diffuse cutaneous leishmaniasis (Asilian *et al.,* ١٩٩٨).

- Visceral leishmaniasis: a disease, that may be endemic, epidemic or may sporadic. African kala azar is found in the eastern half of Africa. Indian kala azar has an age and sex distribution similar to African kala – azar . The manifestation of clinical aspects appeared generally in three months. Fever, typically nocturnal and occasionally double – quotidian, is almost universal and is accompanied by tachycardia without sign of toxaemia. Diarrhea and cough are frequent . Non tender splenomegaly becomes dramatic by the third month, the liver enlarges conspicuously, hypoalbuminaemia and polyclonal - hypergammaglobulinaemia (IgG and IgM) are constant feature , circulating immune complex glomerulonephritis and interstitial nephritis had been described. Edema cachexia, and hyperpigmentation (

kala – azar means " black fever") are late manifestations . After successful treatment, ٣ to ١٠ percent of cases developed post kala azar leishmaniasis (PKDL) wart like nodules over the face and extensor surface of the limbs. In the Indian disease, PKDL appeared after a latent period of ١ to ٢ year and may last for years. (Carvalho *et al.*, ١٩٩٢) .

• Cutaneous and mucocutaneous leishmaniasis : this form of leishmaniasis was caused by a number of species in both the old and the new world. The disease was characterized by single or multiple localized lesions on exposed areas of skin that typically ulcerate. *L. tropica* and *L. major* caused old world whereas *L. mexicana* and *L. brasiliensis* caused new world cutaneous leishmaniasis. The incubation period ranges from ٢ to ٦ weeks. The initial lesions are often multiple and located to lower extremities. Regional lymphodenopathy and satellite lesions are common. Mucocutaneous leishmaniasis and/or espundia, was caused primarily by *L. brasiliensis* which typically produced several lesions on the lower extremities that undergo extensive ulcerations. After months to year, metastatic lesions appeared in the nasopharynx, nasal obstruction epistaxis are frequent presenting symptoms (Herwaldt, ١٩٩٩).

٢.٣. Leishmania life cycle and metacyclogenesis

The sand fly vector of genus *Phlebotomus* (old world) or *Lutzomyia* (new world) became infected when feeding on the blood of an infected individual or an animal reservoir. The *Leishmania* parasites live in the macrophages as round , non – motile amastigote (٣-٧ µm in diameter). The fly ingested the macrophage during the blood meal and the amastigotes were released into the gut of insect (Killick – Kendrick, ١٩٩٠). Almost immediately the amastigotes transform into the motile, elongated (١٠ – ٢٠ µm) flagellate promastigote form. The promastigotes then

migrate to the alimentary tract of the fly, where they live extracellularly and multiply by binary fission (Guevara *et al.*, ٢٠٠١).

Sand fly saliva selectively inhibited parasite killing by macrophages and nitric oxide production (Hall & Titus, ١٩٩٥). The major surface glycoconjugate lipophosphoglycan (LPG) constitutes a dense glycocalyx that covers the entire surface of the parasite including the flagellum. Immature organisms, termed procyclic, express shorter LPG molecules but mature metacyclic bear the capping at the terminal galactose residuces with α-arabinose and elongation by increasing the numbers of repeating disaccharide unit by ٢ to ٣ fold. This mature metacyclic form of the organisms is released from the mid gut and migrates to the proboscis. Whereas procyclic organisms from log phase cultures are extremely sensitive to complement – mediated lysis through the alternative pathway, metacyclic organism activities the classical pathway but are not lysed. When the sand fly next feeds on a mammalian hosts, it transferred the metacyclic *Leishmania* promastigotes to the host along with saliva (Sacks and Kamhawi, ٢٠٠١). Once in the host , the promastigote are taken up by the macrophages where the rapidly revert to the amastigote form, survive and multiply inside the macrophages eventually leading to the lysis of the macrophages are taken up by additional macrophages and so the cycle continued. Ultimately all the organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow (Pulverta & Hoyle, ١٩٦٠).

٢.٤. Clinical feature

Leishmaniasis, a parasitic disease transmitted by the bite of some species of sand fly affected various age groups depending on the infecting *Leishmania* species, geographic location, disease reservoir, and host immunocompetence. Visceral leishmaniasis was the most sever form of the disease affecting children. The extent and presentation of the disease depended on several factors, including the humoral and cell – mediated immune response of the host, the virulence of the

infected species , and the parasites burden. Children are at greater risk than adults in endemic area. Malnutrition contributes to the development of disease, and in complete therapy of initial disease was a risk factor for recurrence of leishmaniasis (Kafetzis, ٢٠٠٣).

Children usually present with intermittent fever, paleness , refusal to feed or anorexia, weight loss , and abdominal distension. Splenomegaly, hepatomegaly, lymph node enlargement, thrombocytopenia, anemia, leukopaenia and hypergammaglobulinemia are the most common finding in pediatric leishmaniasis (Murray and Delph, ٢٠٠٠). Clinically *L. donovani* infections may range from asymptomatic to progressive, full developed kala – azar. The incubation period in humans may be as short as ١٠ days or as long as a year but usually is to ٤ months. The disease usually begins slowly with low grade fever and malaise and is followed by progressive wasting and anemia, protrusion of the abdomen from enlarged liver and spleen, and finally death (in untreated cases) in two to three years. In some cases symptoms may be more acute in onset, with chills, fever up to ٤٠ ^{o}C (١٠٤ ^{o}F) , and vomiting, death may occur within ٦ to ١٢ months. Accompanying symptoms are edema, especially of the face, bleeding of mucous membranes, breathing difficulty, and diarrhea. The immediate cause of death often is invasion of secondary pathogens that the body is unable to combat. A certain proportion of cases, especially in India, recover spontaneously (Bryceson & Hays, ١٩٩٤).

Visceral leishmaniasis may be viewed essentially as a disease of reticulo-endothelial system. The phagocytic cells, which are so important in defending the host against invasion, are themselves the habit of the parasites. Blood – forming organs, such as spleen and bone marrow, undergo compensatory production of macrophages and other phagocytes (hyperplasia) to the determinant of red cell production. The spleen and liver become greatly enlarged (hepatosplenomegaly), while the patient became severely anemic and emaciated (Ashford *et al.*, ١٩٩٢) . A skin condition known as post kala – azar dermal leishmanoid develops in the same

cases. It is rare in the Mediterranean and Latin American areas but develops in ٥٪ to ١٠٪ of cases in India. The condition usually becomes apparent about one to two years after in adequate treatment for kala – azar. It is marked by reddish depigmented nodules that sometimes become quite disfiguring (Morgan *et al.*, ١٩٦٢).

٢.٥. Diagnosis

In children, however, a definite diagnosis of VL relies on the demonstration of *Leishmania* in tissue specimens or tissue culture. The parasite can be demonstrated through direct evidence from peripheral blood, bone marrow , or splenic aspirates. Microscopy of bone marrow aspirates is the safest diagnostic approach for pediatric patients, with amastigote seen in more than ٩٠٪ of the cases by an experienced observer (Maltezou *et al.*, ٢٠٠٠). Repeated sampling, however may be required. In adult patients, bone marrow microscopy, is less sensitive (~ ٧٠٪). The higher diagnostic efficacy of the bone marrow examination in children is probably related to heavier parasitisation encountered in children. Microscopic examination of splenic aspirates offer the highest sensitivity (up to ٩٨٪), but is associated with risk of life – threating haemorrhage in cases with profound thrombocytopenia (Herwaldt, ١٩٩٩). In immunocompetent individuals, serological assays (direct agglutination, enzyme – linked immunosorbent assay and indirect immuno-fluorescence) are considered to be sensitive for the diagnosis of VL. In a study conducted in Brazil , the indirect immunofluorescence assay achieved sensitivity and specificity rates close to ١٠٠٪ for the diagnosis of VL, with immunoglobulin G antibodies significantly reduced after treatment (Da Matta *et al.*, ٢٠٠٠). Recently , a strip test employing the recombinant $K_{٣٩}$ antigen of *L. chagasi* was develop. This strip test had a sensitivity of ١٠٠٪ and a specificity of ٩٨٪ among

patients in India, however , in a study from Sudan , which compared the use of the recombinant $K_{٣٩}$ strip test with that of the enzyme – linked immunosorbent assay and direct agglutination test, the former gave limited sensitivity [٦٧٪ versus ١٠٠٪ and ٩١٪ ٩ at ≥ ١:١٦٠٠ cut off), respectively] .The interpretation of serological results was complicated by high titers observed for months or years after successful treatment and in cases of symptomatic infection (Zijlstra *et al.*, ٢٠٠١). Moreover, serological tests are positive in only half of HIV infected patients with leishmaniasis (Printado *et al.*, ٢٠٠١).

Over the past ٥ years, the value of Polymerase Chain Reaction (PCR) for the diagnosis of VL had been assessed using different clinical specimens (peripheral blood, bone marrow and spleen) (Spanakose *et al.*, ٢٠٠٢). The detection of persistent parasite DNA in infected tissues by means of PCR may also be used as a

marker for the risk of relapse after initial cure. Currently , PCR should be regarded as a promising tool, with potential advantage of using blood specimens rather than the conventional invasive producers such as splenic aspirate, bone marrow aspiration and liver biopsy.

Leishmanin skin test (Montenegro test) is of value for epidemiological studies. The test involves an intra-dermal injection of ٠.١ ml of promastigote antigen. The reaction is interpreted after ٧٢ hours later. It is negative during active VL, but usually becomes positive after successful treatment. This test is also positive in patients with previous symptomatic infection (Herwaldt, ١٩٩٩) and in cases with dermal leishmaniasis (Barral *et al.*, ٢٠٠٠) .

Differential diagnosis of VL should include other conditions associated with massive splenomegaly, such as malaria, tropical splenomegaly syndrome, typhoid , military tuberculosis, portal hypertension, leukemias, lymphomas and haemolytic anemia. Post kala – azar dermal leishmaniasis should be differentiated from yaws, syphilis and leprosy. Sarcoidosis, midline granuloma and histoplasmosis should be differentiated from mucocutaneous leishmaniasis (Kafetzis, ٢٠٠٣).

ELISA technique had been applied successfully for diagnosis, follow up of treatment and epidemiologic studies (Mohammad *et al*., ١٩٨٥). Some procedure use soluble antigens, other use total promastigote, however, antigen prepared with the isolate from the same area where the sera originated yield higher mean absorbance than the others (Badaro *et al.,* ١٩٨٦). Sensitivity and specificity were excellent through variable ranging from ٩٢ – ٩٨.٤ % (HO *et al.,* ١٩٨٣ ; Srivastava , ١٩٨٩) and from ٧٥ – ١٠٠٪ (Sinha & Sehgal, ١٩٩٤).

Since its introduction by Hommel (١٩٧٦) as a preliminary assay for diagnosis of kala - azar using a soluble parasite antigen, a large number of modifications was applied to improved of facilitate its application.The first modification was made so that ELISA can be read as visual end point titer to facilitate field application (Jahn & Diesfeld, ١٩٨٣). Intact cultured promastigote suspension have been used to develop an ELISA which is

specific and sensitive while avoiding the extraordinary and inherited difficulty in antigen preparation which is a source of variability and lack of reproducibility (Mohammad *et al.*, ١٩٨٥) .Dot – Enzyme linked immunosorbent assay, which utilizes an antigen dotted onto nitrocellulose filter disk, was developed and proved to be an important field diagnostic technique (Pappas *et al*., ١٩٨٣). This test was first used by Hawkes *et al*. (١٩٨٢) for screening monoclonal antibodies to rat brain synoptosomal membrane. This technique was used by Srivastava & Singh (١٩٨٨) with modifications, and evaluated for its applicability under field conditions for the diagnosis of kala azar.

In clinically diagnosed group, Dot – ELISA was positive in ٩٠.٣٪ and Enzyme linked immunosorbent assay in ٨٥.٥٪. The sera from healthy controls from the endemic area did not give any positive reaction in concentration sera of ١/١٠٠. Srivastava & Singh (١٩٨٨), also they found that only titer variation was observed with sonicated and whole promastigote antigen . Hoerauf *et al*., (١٩٩٢) recommend the

immunoblot technique as confirmatory test in cases with doubtful indirect fluorescent antibody test or ELISA antibodies titers.

ELISA can be further improved by the use of protein A or protein G conjugates for specific detection of antileishmania antibodies in the sera of patients with visceral leishmaniasis. Enzyme linked protein A gave significantly higher absorbance value for positive sera without corresponding increase in absorbency value from normal individual and this increase the distance between positive and negative value (Reed *et al*., ١٩٩٠).

A high specific and sensitive competitive ELISA based on a specific inhibition of monoclonal antibodies binding to a crude parasite homogenate by serum from patient with visceral leishmaniasis, which was proved to be more specific than traditional ELISA or IFAT (Sundar & Rai, ٢٠٠٢).

The use of amastigote containing ٣٩ amino acids (rk-٣٩) antigen in ELISA technique allow quantitative estimation of the immune response to this antigen which could of value in assessing the severity, follow up of cases. High and point titer predicted increased severity and frequency of clinical symptoms (Sundar *et al*., ٢٠٠٢).

٢.٦. Immunoblotting

This method described so far is particularly useful for measuring levels of certain known antigens or antibodies, but it is often necessary to identify and characterize previously unknown antigens from a complex mixture, in which case immunoblotting is very useful (Baqir & AL-Jeboory, ٢٠٠١).

In leishmaniasis, patients develop specific antibodies that constitute the basis of serodiagnosis. Using western blotting analysis (blotting : is the transfer of electro-phoretically resolved biological samples to immobilizing matrices followed by specific detection) we could determine the specificity of antileishmanial in different patients with VL (Farhat *et al.,* ٢٠٠٢).

Patient's serum with VL recognizes numerous antigens within variable molecular masses depending on the species, state of the disease, immune status and possible other factors. Some of these are specifically recognized in all sera from infected patients (Tiwari *et al*., ١٩٩٥;Farhat *et al.*, ٢٠٠٢), some cross react with sera of patients with other diseases (Rolland *et al.,* ١٩٩٢), whereas others are useful as epidemiologic marker in endemic area (Cardenosa *et al.*, ١٩٩٥). With treatment there was a decrease in intensity or disappearance in some of the diagnostic bands suggesting its use in post treatment control or assessment (Cardenosa *et al.,* ١٩٩٥).

For diagnostic purpose, immunoblotting was more specific than other serological tests as it could discriminate VL from other clinically stimulating diseases as chronic schistosomasis, chaga's disease, autoimmune disease and lymphoproliferative diseases . Moreover, western blotting was found to be more sensitive than IFAT and ELISA (Marcy *et al*., ١٩٩٢). Hoerauf *et al. (*١٩٩٢) recommended the technique as confirmatory test in cases with doubtful or ELISA antibody titer.

٢.٧. Treatment

Treatment of leishmanial infections varies according to the clinical manifestation. In earlier years trivalent antimonial were the only drugs available, but they were so toxic as to be downright dangerous. Pentavalent antimonials have been used extensively, but they also are toxic and usually must be administered under the

care of physician. Two pentavalent preparation are available: Pentostam and Glucantime is available in the United States, through the Centers for Disease Control Parasite Drug Service (Kreisbergs, ١٩٧٨)

Drug resistance had been reported in some strains of some species. Further more, relapses and post kala azar dermal leishmanoid may follow insufficient treatment (Grogl *et al.*, ١٩٩٢)

Some of the most creative although still largely experimental, approaches to treatment involve turning liability into an asset (so to Speak) – the liability being fact that in visceral infections the parasites are located within macrophages. Macrophage will eat foreign particles, so injected drugs such as amphotericine B are bound to artificial particles liposomes or colloidal particles to enhance their efficacy by delivering them to the cells where the amastigotes reside (Croft *et al*., ١٩٩١). It has long been known that tropical forests are a rich source of plant molecules with potential medicinal uses. The rapid disappearance of these forests led to renewed interest in natural plant products, including those that may be effective against leishmanial infections. So far, antileishmanial activity had been found in a number of plant species, including those from the families Apocyanaceae (dogbanes), Gentianaceae (gentians), and Euphorbiaceae (Singha *et al.*, ١٩٩٣).

In late ٢٠٠٢, miltefosine (hexadecylphosphocholine), an orally administered drug originally developed for cancer-patients, was reported to cure ٩٨٪ of VL cases. Miltefosine is now licensed for use in India. An oral, relatively nontoxic drug effective against a virtually fatal parasitic infection is a health professionals dream, miltefosine seem to known and it has not been tested against cutaneous form (Sundar *et al*., ٢٠٠٢).

٢.٨. Immune response to *Leishmania* infection

Leishmania is a protozoan parasites and the causative agent of the disease leishmaniasis. The resistance is conferred by T-helper type -١ (Th١) cells while the susceptibility is conferred by a number of other elements of the immune system. Th١ cells secrete IL -٢ and IFN-γ , but Th٢ cells secrete IL -٤, IL -٥ and IL -١٠. It has been shown that IFN -γ activates macrophages to express $iNOS_{٢}$, the enzyme catalyzing the formation of nitric oxide. Nitric oxide kills the intracellular amastigotes. In contrast, Th٢ immune response limits the action of Th١ functions via IL -١٠ and IL -٤, which deactivated macrophages helping intracellular parasite growth and disease progression. The leishmanial parasite may modulate the host physiology so that becoming vulnerable to infection. A detailed knowledge of this host-parasite interaction would help in designing prophylactic and therapeutic strategies against this infection (Amit *et al.,* ٢٠٠٤).

There was an intricate therapy between host immune response and progression of VL, and the outcome of this potentially deadly contest is likely influenced by host genotype. Clinical VL may not develop for some time, even years, after infection. A symptomatic infections of which there were many, probably resulted from early activation of the Th١ arm of the immune response, while kala – azar results from the proliferation of non protective Th٢ cells (Pearson *et al.,* ١٩٨٦).

The relation between host genetic make up and immunological response to leishmanial infection had been studied extensively in mice. Susceptibility of mice to *L. donovani* infection is controlled by a single gene, *Lsh*, which is inherited in Mendelian fashion and expressed in visceral macrophages (Tabbara *et al.,* ٢٠٠٥).

٢.٨.١. Humoral immunity

Kala – azar characterizes by strong antibody titer (IgG & IgM). Cytokines elaborated by activated T – cells induce the switching of B –lymphocytes to several IgG isotypes and are thus obligatory for some humoral responses. Leishmanial

antibodies could have an opsonizing function leading to enhanced phagocytosis or perhaps were directed against irrelevant bacterial antigens, and it is increased even more by the addition of complements. Antibody are usually useful only for the diagnostic test. Immunological process involved in parasitic disease is a complex one. The control of *Leishmania* infections depend on T-cell mediated immune response. Anti-leishmanial antibodies are produced, but they are not protective. Resolution of infection and protection against infection correlate with proliferation of CD ٤ cell of Th١ type (Bennett & Plum, ١٩٩٦).

Many parasitic infections provoke a non – specific hypergammaglobulinaemia much of which was probably due to substances released from the parasites acting as B-cell mitogens and the level of total immunoglobulins are raised including IgM in visceral leishmaniasis. The relative importance of antibody – dependent and responses of cell –mediated immunity varies with the infection (Casadevall, ١٩٩٨).

Complement activation is an important process, particularly in destruction of bacteria cells, is interaction with complement activated by the classical pathway. The first component in the classical pathway is activated by bound antibody. The end result in both classical and alternative pathway can be the same ; that is, perforation of the foreign cell. Both pathways may also lead to opsonization or enhancement of inflammation. Bind of the complement to antigen – antibody complexes can facilitate clearance of these potentially harmful masses by phagocytic cells (Schalling & Oskam, ٢٠٠٢).

In case of *Leishmania,* such resistance correlates with virulence, *L. tropica* which is easily killed by complement, causes a localized self – healing infection in the skin, whereas *L. donovani*, which is ten times more resistant to complement, becomes disseminated throughout the viscera, causing a disease that is often fatal (Abbas *et al.,* ١٩٩٦).

The mechanisms whereby parasites could resist the effect of complement were different. The lipophosphoglycan surface coat of *L. major* activates complement, but the complex is then shed, so the parasite avoids lysis (Reiner & locksley, ١٩٩٥).

٢.٨.٢. Cellular immunity

٢.٨.٢.١. CD – Marker

CD – markers are glycoproteins characterized in two population of lymphocytes (T and B-lymphocyte) and natural killer cells. Most T-helper cells express CD٤ ,whereas most T – cytotoxic cells express CD٨, NK cells express CD١٦ and CD٥٦, and B cells express CD١٩, CD٢١, CD٢٢, CD٣٢ and CD٣٥. The CD٤ cells are involved in regulation function (helper / inducer) of the immune response and the T – CD٨ cells have suppressive recognition of CD٤ class II molecules which represent their natural ligands. The count of CD٤ and CD٨ lymphocyte in the peripheral blood is a major test in the immunological follow up of the disease with immune dysfunction and patient after organ transplant of marrow graft. In subject with immune deficiency virus (HIV), the count of CD٤ lymphocytes is recommended for the clinical follow-up of patients and it intervenes in the definition of the disease evolution (Levy, ١٩٩٦). The reference technique used for counting T-CD٤ and T-CD٨ in the peripheral blood requires the combined utilization of blood cell counter to determine the number of total lymphocytes, and the flow cytometry which gives access to the percentage of CD٤ or CD٨ cells. The cells numbers for each CD٤ or CD٨ per microliter of blood are calculated from the product of these two measurements.

So , in human, it is determined that the number of CD٤/CD٨ decreases from birth and stabilize in the healthy adult at mean value of ٨٣٠ to ٢٨٨ CD٤/µl of blood and ٣٥٠ to ٢٣١ CD٨/µl of blood (Sagar & Price , ١٩٩٥).

Flow cytometry as well as the alternative evaluation methods, immuno-peroxidase staining and immunofluorescent or lymphocytes enumeration are based on the immunological detection of the markers of the cell surface with labeled specific monoclonal antibodies, however, unlike the flow cytometry, the alternative methods allowed counting the lymphocytes with out requiring heavy laboratory instrumentation (Dhaliwal *et al*., ١٩٩١).

CD٤ and CD٨ cells were within normal range in patients with active visceral leishmaniasis, but in acute visceral leishmaniasis patients had markedly reduced levels of memory T cells (CD٣/CD٤٥ ro) and return to normal level following successful chemotherapy, while another study showed that pretreatment serum level of soluble CD٤ and soluble CD٨ were significantly higher in visceral leishmaniasis patients than in the healthy subjects (Abbas *et al*., ١٩٩٦).

Rohtagi *et al.* (١٩٩٦) reported that, the CD٤ cell count was depressed in peripheral blood of acute and chronic visceral leishmaniansis cases as compared to normal control, while the peripheral blood CD٨ cell was normal in a acute cases, and uniformly low in chronic cases. After treatment, the CD٤ cell count in peripheral blood increased in contrast CD٨ cell count remained unaltered in peripheral blood.

٢.٨.٢.٢. Cytokines

Cytokines are low molecular proteins; they are a soluble mediators produced by a variety of cells of the innate and adaptive immune system and in particular by the cells. Their major functional activities are concerned with the regulation of the development and behavior of the immune effector cells (Benjamini *et al*., ٢٠٠٠).

Cytokines serve as chemical messenger within immune system, although they also communicate certain cells in other systems, including those of the nervous system. Cells regulated by particular cytokine must express a receptor for that factor. Thus; cells are regulated by the quantity and type of cytokines to which they are exposed and by expression up regulation and down regulation of cytokines receptors. Cytokines act in concert with one another to create synergistic effects that reinforce the other actions on given cell. The interaction of multiple cytokines generated during a typical immune response are referred to cytokine cascade (Hunter & Reiner, ٢٠٠٠).

٢.٨.٢.٣. Interleukin – ١٠ (IL -١٠)

Interleukin -١٠ is an MW ١٨٠٠٠ protein; IL -١٠ was produced by Th٢ cells, activated fetal thymocytes, macrophages , and normal B cells. Cells found to have the capacity to produce IL -١٠ in human include : Th٠ , Th١ and Th٢ like CD٤ T-cells clones (Del Prete & Carli, ١٩٩٣).

Interleukin -١٠ can exert either immunosuppressive or immunostimulating effects on variety of cell types. IL -١٠ could inhibit production of a number of cytokines, especially IFN-γ, by Th١ cells responding to antigen in the presence of antigen – presenting cells, IL -١٠ is a potent modular of monocyte /macrophage function. As a down regular of the cell – mediated immune response, IL -١٠ can suppress the production of prostaglandine E٢ and numerous pro-inflammatory cytokines, including TNF-α, IL -١,IL -٦ ,IL -٨ etc by monocyte following activation. It also enhance the release of soluble TNF receptor and inhibition the expression of surface ICAM -١ and B٧. Finally IL -١٠ has been reported to suppress the synthesis superoxide anion plus reactive oxygen intermediate (ROI), and either inhibits or facilitate no synthesis, depending on the time of exposure to activated macrophages (Liew *et al.,* ١٩٩٩).

Interleukin -١٠ can serve as both macrophage activator and deactivator and exhibit potent anti-inflammatory activities. In *vivo*, the induction of IL -١٠ synthesis during certain parasitic infections has been suggested to be an important strategy by which parasite evade IFN-γ dependent, cell mediated immune destruction based on both of the in *vivo* and in *vitro* functions of IL -١٠, it has been suggested that an IL -١٠ antagonist can be applied to boost antiviral immunity against such viruses as EBV and the IL -١٠ may be used as an anti-inflammatory reagent (Mosmann, ١٩٩٤).

٢.٨.٢.٤. Interferon – gamma (IFN –γ)

Interferon – gamma, mainly produced by activated CD٤ and CD٨ T-cells and NK cells, is a homodimeric glycoprotein having two subunits each of ٢١ to ٢٤ k Da is a potent activator of mononuclear phagocytes inducing the respiratory burst, mechanism. IFN – γ also up regulate the expression of MHC class II molecules,leading to amplification the process of CD٤ T-cell activation and promotes their differentiation to Th١ phenotype, and inhibits the proliferation of the Th٢ type cells and thus tends to suppress humoral immunity. It did not only decrease the production of IL- ٤ by Th٢ cells but also potently inhibit the effects of IL-٤ on B-cells, particularly inhibiting IgE production. GKO mice, in which IFN – γ or IFN – γ receptor genes had been disrupted, showing several immunological defects including impaired macrophage activation, reduced NO production, reduced MHC class II molecules expression , reduced IgG٢a and IgG٣ serum levels and defective NK cells function (Benjamini *et al*., ٢٠٠٠). IFN-γ is the primary lymphokine responsible for stimulating macrophage oxidative burst and activating antileishmanial activity in *vitro* (Murray & Delph, ٢٠٠٠). IFN-γ also induces the secretion of TNF – α upon activation of macrophages for intracellular killing of the amastigote by enhancing NO production (Bogdan *et al*., ١٩٩٦).

The efficacy of IFN-γ was tested by gene transfer experiments in which injection of mammalian expression plasmid bearing IFN-γ gene in normal and in IFN-γ gene disrupted infected BALB/C mice lead to controlled visceral leishmaniasis infection and reduced parasite burden (Taylor & Murray, ١٩٩٨).

٢.٨.٢.٥. Tumor necrosis factor – alpha (TNF – α)

TNF-α is a homodimer of ٥١ k Da, which is mainly secreted by the activated macrophages, antigen – stimulated T-cells, activated NK cells and mast cells. It is a key mediator of both innate and specific inflammatory responses. During an inflammatory response, TNF-α induces enhanced expression of cell adhesion molecules on vascular endothelium, production of chemokines by mononuclear phagocytes for leukocyte recruitment, activation of inflammatory leukocytes, as well as, promotes angiogenesis (Titus *et al.,* ١٩٨٩).

Experiments in murine leishmanisis have proved that TNF-α is capable of inhibiting parasite multiplication in *vitro*, as well as control infection in *vivo* mainly due to its ability to cause the activation of infected – macrophages for destruction of intracellular amastigotes (Theodose & Titus, ١٩٩٣). TNF-α production has been found to be absent in susceptible mice but present in *L. major* infected resistant mice. In addition, repeated injection of recombinant human TNF –α in to both strains of mice had a therapeutic effect in the course of infection, while injection of anti-TNF-α antibodies led to marked exacerbation of the infection (Titus *et al*., ١٩٩١). IL-٤ and IFN-γ have been reported to induce TNF-α production in a synergistic manner (Stenger *et al*., ١٩٩١).

Further, TNF-α synergises with IFN-γ for the No-dependent killing of intracellular *L.major* amastigotes (Liew *et a*l., ١٩٩٠).

Recently, it has been shown that endogenous TNF is required to control intracellular *L.donovani* in mice, supports granuloma development, mediates optimal

killing effects of antimony treatment and prevents relapses after ordinary curative amphotericin B treatment (Murray & Delph., ٢٠٠٠).

The level of TNF – α gets elevated in the serum of VL patient and drops rapidly following successful drug treatment, serving as a good parameter to monitor host response to therapy (Barral-Netto *et al.,* ١٩٩١).

٢.٨.٢.٦. Leishmaniacidal activity of eosinophils

Mature eosinophils are characterized by their granules, which have a crystalloid core. Their major function appears to be the secretion of various toxic granules constituents , following activation. The components of the eosinophil granules include major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil – derived neurotoxin (EDN) (Pearson *et al.,* ١٩٨٧).

ECP and EDN are found in the matrix of granules in eosinophils , whereas MBP is found in the core of granules. ECP and EDN are members of the ribonuclease A superfamily and they have highly cytotoxicity. The three eosinophil proteins are highly cationic protein pH ١٠.٨ – ١٠.٩ .MBP has been shown to damage and some times kill, parasites, but also damages host tissue cells (Gleich *et al.,* ١٩٩٢).

EPO is a highly cationic heterodimeric ٧١- ٧٧ k Da. Haemoprotein and oxidize a variety of substrates, including halide ions to produce hypohalite. In deed, this may represent the eosinophil most potent killing mechanism for some parasite (Sandra *et al.,* ١٩٩٧).

ECP an eosinophil – specific toxin which is very potent at killing many parasites. The molecule is a ribonuclease which because of its high charge, binds avidly to negatively charged surfaces. It is possible that it forms membrane channels, which allow other mediators access to the target organisms (Roitt *et al.,* ٢٠٠١)

EDN are molecules produce by eosinophils which are also with strong neurotoxic activity (Roitt *et al.*, ٢٠٠١).

Eosinophil produce nitric oxide (NO) and involved the microbiocidal activity of these cells against *L. major* (Sandra *et al.*, ١٩٩٧).

In general, the microbiocidal activity of eosinophil has been associated with degranulation and release cytotoxic substances to cells and to several parasites in *vitro* (Gleich *et al*., ١٩٩٢) among them, *L. mexicana amazonensis* (Pimenta *et al.,* ١٩٨٧) and *L. donovani* (Pearson *et al*., ١٩٨٧).

٢.٩. Enzymes

Enzymes are specific biologic proteins that catalyze biochemical reactions with out altering the equilibrium point of the reaction or being consumed or under going changes in composition (Bishope *et al*., ٢٠٠٠). Without enzymes, life cannot exist and they are the tools that create life (Marshal, ٢٠٠٠). More than ٣٠٠٠ different enzymes have been identified in the human body. They build new proteins, cells, tissues, and organs, as well as break down diseased tissues. They are found in all tissues and they are present in much higher concentration inside than outside the cell (Benyon, ١٩٩٨).

Enzymes are released into the systemic circulation as a result of :

١. Increased rate of cell turnover during active growth or tissue repair or cancer (Schwartz, ١٩٨٢).
٢. Necrosis or sever damage to cells (Lee & Goldman, ١٩٨٦).
٣. Induction by disease or drugs (Zeltzer *et al*., ١٩٨٦).
٤. Biliary or pancreatic obstruction, as well as renal insufficiency also causes increased enzymes concentration (Moss & Henderson, ١٩٩٩).

In most disease states, in which enzyme elevations occur, the cause is increased membrane permeability, usually secondary to cell injury or necrosis. Occasionally, increased enzyme levels in serum are caused by increased rates of intracellular synthesis and the subsequent diffusion of these secreted enzymes into the circulation (Pincus & Schaffner, ١٩٩٦).

٢.٩.١. Adenosine deaminase (ADA)

Adenosine deaminase enzyme (adenosine amino hydrolase, EC.٣.٥.٤.٤). It is widely distributed in mammalian tissue, including those of human. ADA present in the cytoplasmic fraction of the cell and certain amounts are located in nucleus (Smillie, ١٩٥٧).

ADA is the enzyme that irreversibly catalyzed the hydrolytic deamination of adenosine and deoxyadenosine to inosine and deoxyinosine respectively and ammonia (Dinjens *et al.*, ١٩٨٩). Differences in the molecular characteristic of ADA have been documented in many instances (Ma & Magers, ١٩٧٥).

A heredity deficiency in this enzyme has been associated with sever combined immunodeficiency syndrome (SCID) in which the children so affected lack both T and B lymphocytes and other immune cells function and often have bone abnormalities (Meuwissen *et al.,* ١٩٧٥ ; Polmar, ١٩٨٠).

The activity of ADA seems to vary in a number of diseases. Serum ADA has been reported in carcinoma (Winsten, ١٩٧٤). Patients with acute lymphoblastic leukemia have increased levels of ADA activity in their T-cells (Coleman *et al.,* ١٩٧٨; Hatzistillanou *et al.,* ١٩٩٦).

Other studies have been made to investigate ADA activity in parasitic diseases, and showed that patients infected with schistosomiasis had a decreased

levels of ADA (Juma & AL-Jeboori, ١٩٩٩). In hydatidosis,there was a significant decrease in the levels of ADA enzyme (AL-Qadhi, ٢٠٠٥) while women actually infected with *Trichomonas vaginalis* were show to have elevated levels of ADA (AL-Shawk, ١٩٩٩). However, in visceral leishmaniasis patients, the enzymes levels were significantly increased (AL-Marsomi, ٢٠٠١). Comparative studies on the substrate specificity show differences between the enzymes from different species (Brady, ١٩٤٢). It was found that there are differences in the pH optimum , the electrophoretic mobility and substrate specificity of ADA from many tissues of six mammalian species (Brady & O'Donovan, ١٩٦٥). These finding , however , suggest that the enzymes is the same in different tissues of the same organisms (Giusti, ١٩٨١)

ADA enzyme can be found in human mainly in two isoenzymes from ADA-S with a molecular weight of about ٢٨٠ k Da. Morever, the specific activity of ADA-S isoenzymes is about ٢٠ times higher than that of ADA-L isoenzyme (Akedo *et al.,* ١٩٧٢).

ADA enzyme can be quantitated in serum, RBCs, fibroblast and tissues by several methods which are all, considered reliable. The easier to use and are equally reliable are the measurement of ammonia production (Giusti, ١٩٨١).

A high sensitivity biochemical microassay had been developed for ADA, it is capable of detection a small amount of reaction products (Benson & Monk, ١٩٨٨).

٢.٩.٢. Glutathione reductase

Glutathione, is tripeptide of glycine, glutamic acid, and cysteine, exists in two forms: the antioxidant reduced glutathione abbreviated GHS and the oxidized form, know glutathione disulfied or GSSG. The GSSG/GSH ratio may be a sensitive indicator of oxidative stress (Lomaestro & Malone, ١٩٩٥).

The enzyme that is responsible for maintaining reduced glutathione GSH in the cell is glutathione reductase GHR (EC.١.٦.٤.٢) (Le Trang *et al.,* ١٩٨٣). This enzyme has been studied extensively since reduced GSH plays an important role in a number of diverse system such as detoxification, cell division and stress adaptation.

Reduced glutathione GSH is widely distributed, with intracellular concentration typically in the millimolar range and plays an important role in antioxidant defence by acting in combination with glutathione peroxidase (GSH-px) to breakdown hydrogen peroxide and lipid hydroperoxides (Flohe, ١٩٨٢).

The glutathione peroxidase – glutathione reductase system which is responsible for the detoxification of the H_2O_2 that leak back into cytoplasm during respiratory burst. This system uses H_2O_2 to oxidize the GSH in a reaction catalyzed by glutathione peroxidase enzyme as in the equation:

$$2GSH + H_2O_2 \xrightarrow{\text{GSH-Px}} 2GSSG + 2H_2O$$

The oxidized glutathione GSSG is then reconverted to reduce glutathione by glutathione reductase (Babior, ١٩٧٨).

$$GSSG + NADPH \xrightarrow{\text{GHR}} 2GSH + NADP^+$$

So glutathione reductase uses as its source of electrons the coenzyme NADPH (Nicotinamide adenine dinucleotide phosphate, reduced), therefore NADPH, coming mainly from the pentose phosphate shunt, is the predominant source of GSH power (Cathcart, ١٩٨٥).

Reduced glutathione also reacts readily with a wide variety of free radical species , including carbon centered, peroxy, phenoxy radicals (Baker *et al.,*١٩٨٢) therefore, it is generally considered free radical scavenger and repair of radical mediated biological damage (Kosower & Kosower, ١٩٧٨).

Duke *et al*. (١٩٩٦) showed that intracellular GSH status appears to be sensitive indicator of the cells overall health, and of it's ability to resist toxic challenge and confirmed by experiment that GSH depletion can trigger suicide of the cell by process known as apoptosis , therefore , GSH depletion may be the ultimate factor determining vulnerability to oxidant attack.

Human hereditary GSH deficiency state are not necessarily lethal, probably because some GSH is obtained directly from the diet, as Anderson (١٩٩٧) found that dietary ascorbate can protect against the tissue damage that typically results from depletion of GSH.

GSH depletion had been suggested to represent an important contributory factor to liver injury, and to enhanced morbidity related to liver hypofunction . Also GSH deficiencies have been documented in a number of pulmonary diseases, including asthma, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis (Lomaestro & Malone, ١٩٩٥).

Many theories of aging and disease were based upon the interaction of the formation of free- radicals and the subsequent reduction in glutathione levels which allows for an accumulation of free radicals that remain within cells cause cell damage ,DNA damage and many even cause cell death, cancer transformation or loss of cell immunity. As with other cells is dependent on GSH. Both the T and B lymphocytes require adequate levels of intracellular GSH to differentiate. Healthy humans with relatively low lymphocyte GSH were found to have significantly lower CD٤ counts (Kinscherf *et al.,* ١٩٩٤). Intracellular GSH is also required for the T-cell proliferative response to mitogenic stimulation, for the activation of cytotoxic T killer cells and for

many specific T-cell functions, including DNA synthesis for cell replication, as well as for the metabolism of interleukin -٢ (IL -٢) which is important for mitogenic response (Wu *et al.,* ١٩٩٤).

Experimental depletion of GSH inhibits immune cell functions, some times markedly, and in a number of different experimental systems the intracellular GSH of lymphocytes was shown to determine the magnitude of immunological capacity (Fidelus & Tsan, ١٩٨٧).

٢.٩.٣. Superoxide dismutase

Superoxide dismutase, SOD (EC.١.١٥.١.١) was first isolated by McCord and Fridovich (١٩٦٩) from rabbit RBCs. SOD are metallic enzymes that catalytically scavenge the superoxide radicals ($O^{-}_{٢}$) , which are normally produced in every living cells that is capable of reducing oxygen during aerobic metabolism. The catalyzed reaction can be represented as follows (Southron & Powis, ١٩٨٨)

$$O^{-}_{٢} + O^{-}_{٢} + ٢H^{+} \xrightarrow{SOD - Me^{+٢}} O_{٢} + H_{٢}O_{٢}$$

Three enzymatic with distinct molecular forms have been described with the same kinetic properties**.**The one containing iron in its active site was found in prokaryotids, another with manganess present in both prokaryotic and eukaryotic mitochondria, and the third form with $Cu^{+٢}$ and $Zn^{+٢}$ (Cu – Zn –SOD) exist in the cytoplasm of eukaryotes (Capro & Tirrey, ١٩٧٤ ; Michielis *et al.,* ١٩٩٤), extracellular fluids contain small concentration of SOD. This enzyme binds to endothelial cells (Mundy & Winterbourn, ١٩٨٩). When reactive oxygen intermediates (ROI) are rapidly eliminated, they are capable of producing cellular and tissue damage, generally their activity is prevented by the SOD and other protective scavengers antioxidants (Collier *et al.,* ١٩٩٠).

ROI have been associated with killing of parasites, like *Trypanosoma cruzi* (Nathan *et al*., ١٩٧٩) and *Toxoplasma gondii* (Murray & Cohn, ١٩٧٩). *Leishmania donovani* promastigotes initiate a rapid phagocytic respiratory burst rather than amastigote (Blackwell & Alexander, ١٩٨٣). AL-Marsomi (٢٠٠١) was found a significance increase of superoxide dismutase concentration in the sera of visceral leishmaniasis patients.

٢.٩.٤. Aspartate Aminotransferase (AST)

Aspartate aminotransferase (EC. ٢.٦.١.١) is an enzyme found in red blood cells, liver,skeletal muscle and heart cells. It is also found in other organs such as the pancrease and kidneys. AST formerly was called serum glutamic oxaloacetic transminase (SGOT) (Schwartz, ١٩٨٢).

AST is most commonly used as a marker for body tissue , an organ such as the heart and liver disease or damage and the elevation is usually related to hepatocellular damage. The presence of AST in heart muscle makes it a marker for cardiac disease, but not a good one because of all the other possible sources of an increase. In myocardial infarction, AST peaks at about ٢٤ hours and remains elevated for ٣–٧ days (Marshal, ٢٠٠٠)

AST will be elevated in skeletal muscle disease, including trauma. Hemolysis will lead to increased values of AST. A large number of drugs including isoniazid, phenothiazines, erythromycine, progesterone, steroids, halothan, methyldopa, opiates, indomethacin, and salicylates in children may lead to elevated AST values (Frances & Marshal, ٢٠٠٤).

The AST test might be done at the same time for alanine aminotransferase, or ALT. The ratio of AST to ALT (AST:ALT) some times can help determine the cause of the liver damage. Both ALT and AST levels were reliable indicators of liver damage (Schwartz, ١٩٨٢).

An AST test was done on a blood sample taken from a vein. Some times blood samples for AST testing were collected daily for several days to determine if the AST level is going up or down (Atiah *et al.,* ١٩٩٦).

AL-Saffar & AL-Mudhaffar (١٩٧٩), studied different serum enzyme activities and reported increased serum AST activity in ٩١.٤٪ of the studies patients infected with visceral leishmaniasis.

٢.٩.٥. Alanine Aminotransferase (ALT)

ALT is an enzyme (EC.٢.٦.١.٢) of high concentrations which occurs in the liver, and relatively low concentrations were found in the heart, muscle, and kidney. This test was primarily used to diagnose liver disease and to monitor the course of treatment for hepatitis, active postnecrotic cirrhosis, and the effects of later drug therapy . ALT was more sensitive in the detection of liver disease than in biliary obstraction. ALT also differentiates between haemolytic jaundice due to liver disease (France & Marshal, ٢٠٠٤).

ALT is unbound to ultrastructure, is quite soluble, and apparently leaks through damaged, but viable cell membranes (Edmondson & Peters, ١٩٨٥ ; Podolsky & Isselbacher, ١٩٩١). Elevation of serum ALT activities were rarely observed in condition other than parenchymal liver disease. In extrahepatic and intrahepatic biliary obstruction, moderate increase is seen. Five to ten – fold elevations occured in patients with primary or metastatic carcinoma of liver (Moss & Henderson, ١٩٩٩).

Thirty-five Iraqi patients (ages ranging from ٤ month – ٨ years) infected with visceral leishmaniasis showed increased serum alanine aminotransferase activity (Atiah *et al.*, ١٩٩٦).

٢.٩.٦. Alkaline phosphatase

Alkaline phosphatase, ALP (EC.٣.١.٣.١) belong to a group of enzymes that catalyze the hydrolysis of a wide variety of phosphomonoesters at an alkaline pH (Rod – well,١٩٨١).It is a non specific enzyme capable of reacting with many different substrates (Bishope *et al.*, ٢٠٠٠).

ALP is present in practically all tissues of the body, especially at or in the membrane. It is present at particularly high levels in intestinal epithelium, kidney tubules, bone, liver and placenta (Mc Comb *et al.,* ١٩٧٩). The enzyme requires some divalent ions such as $Mg^{+٢}$, $Co^{+٢}$ and $Mn^{+٢}$ as activators of the enzyme while $Cu^{+٢}$, $Hg^{+٢}$, $Zn^{+٢}$, $Ca^{+٢}$, phosphate, borate , oxalate and cyanide are inhibitors of all forms of the enzyme (Varley *et al.,* ١٩٨٠). Although ALP displays considerable inter-or -intra tissue heterogenecity, rarely are there more than ٢ or ٣ forms found in any one serum sample.

The forms present in sera of normal adults probably originate mainly from the liver, with up to half of the total activity coming from the skeleton; these two forms of ALP are markedly age - dependent . A small amount of intestinal ALP may also be present (Raymond *et al.,* ١٩٩١).

Electrophoresis is considered the most useful single technique for ALP isoenzymes analysis. The liver fraction migrates the fastest followed by bone , placenta and intestinal fraction, there exist some abnormal fractions that are associated with neoplasms (Posen & Doherty, ١٩٨١).

Smith *et al.* (٢٠٠٠) listed the physiological conditions that are associated with increased plasma ALP activity, which are:

١. Normal pregnancy as a result of release of ALP from the placenta which may cause its total activity in placenta to rise within second and third trimester of about twice normal adult levels.

٢. Infancy and childhood, the upper reference values here being as much as ٣ – ٤ times the adult values. This increase is a direct result of the intestine osteoplastic activity associated with normal bone growth.
٣. Meals containing fat which cause transient small increase in plasma intestinal ALP activity.

The bulk of ALP in the serum of normal patients is made up of liver and bone ALP. The normal ALP values in the plasma are ٣٠ - ٨٥ IU/L in the adult (Martin, ١٩٨١). Elevated levels of ALP are seen in a variety of bone disorders (Warren, ١٩٨١).

Variety of malignancies including ovarian, lung, and gastrointestinal cancers, were found to have elevated levels of ALP enzyme, such as leishmaniasis (Yousif, ١٩٨١) and schistosomiasis (Juma & AL-Jeboori, ١٩٩٩).

ALP levels are significantly decreased in the inherited condition of hypophosphatasia. Subnormal activity is due to the absence of the bone isoenzyme and results in adequate bone calcification (Bishope *et al.,* ٢٠٠٠).

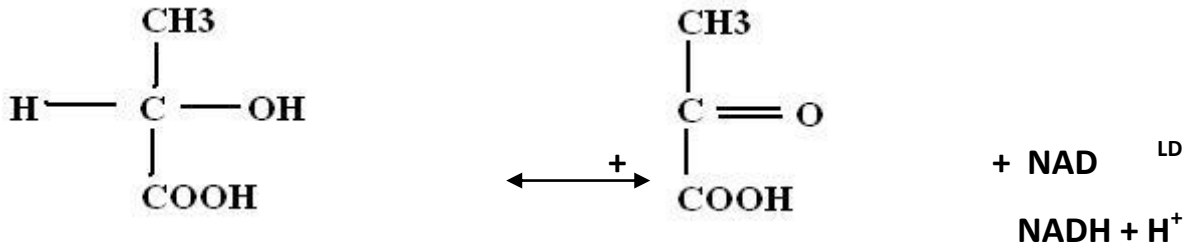
The precise metabolic function of the enzyme is not understood, it appears that the enzyme is associated with lipid transport in the intestine and with the calcification process in bone (Smith *et al.,* ٢٠٠٠).

Measurement of ALP was usually done by using enzymatic colorimetric methods, through the use of buffers and chromogenic substrate in commercial kits, used for this purpose (Mc Comb *et al.,* ١٩٩٧; Varley *et al*., ١٩٨٠). Immunoassay had also been used for bone ALP (Broyles *et al.,* ١٩٩٨) and immunofluorescence techniques (Shibano *et al.,* ١٩٩٩).

٢.٩.٧. Lactate dehydrogenase (LD)

Lactate dehydrogenase (EC.١.١.١.٢٧) is cytoplasmic enzyme that transfers H^+ and plays a role during the anaerobic glycolytic pathway. LD catalyzes the

oxidation of Lactate to pyruvate in the presence of nicotinamide adenine dinucleotide (NAD) as hydrogen acceptor (Bishope *et al.,* ٢٠٠٠) as follows:



(Lactate) **(Pyruvate)**

The enzymatic activity of LD is proportional to the rate of production of NADH (Mc Comb, ١٩٨٣; Alacam *et al.,* ١٩٩٦). This enzyme is widely distributed in the body.Very high activities are found in the heart, liver, skeletal muscle, kidney and erythrocytes. It is present in lesser amount in the lung, smooth muscle and brain (Bruns *et al.,* ١٩٨١).

LD is released to peripheral blood in case of excess heart or cold, starvation, dehydration and injury expose to bacterial toxins. The serum LD is highly sensitive but not specific test and this could be enhanced by isoenzymes analysis (Lott & Nemensanszky, ١٩٨٧).

The enzyme has a molecular weight of ١٢٨.٠٠٠ daltons, and is composed of four polypeptide chains with molecular weight of ٣٢.٠٠٠ daltons each.

Two different polypeptide chains, designated H (heart) and M (muscle), combined in five arrangements to yield the five major isoenzymes each under

separate genetic control (Mackawa & Kanno, ١٩٨٩). The five major fractions each comprise four subunits, which are LD-١ (H^4), LD-٢ (H^3M), LD-٣ (H^2M^2), LD-٤ (HM^3) and LD-٥ (M^4). LD-٦ had been identified in the sera of severely ill patients its identify remain uncertain (Moss & Henderson, ١٩٩٩). LD-١ migrates most quickly towards the anode, followed in sequence by the other fractions with LD-٥ migrating the slowest (Mercer, ١٩٧٨).

Enzyme value may be moderately elevated in myocarditis and cardiac infarction with hepatic congestion, sever shock and in anorexia (Lee & Goldman, ١٩٨٦). In malignancies, LD is one of the enzyme systems preferentially produced and retained by cancer cells, being necessary to maintain tumor growth (Rotenberg *et al.,* ١٩٨٨).

The highest levels of LD were seen in pernicious anemia and hemolytic disorders. Liver disorders, such as viral hepatitis and cirrhosis, show slight elevations of two or three times (Bishope *et al*., ٢٠٠٠).

In parasitic disease, elevated serum LD levels have been reported in patients with sarcocytosis (Prass & Fayer, ١٩٨١), toxoplasmosis (Sacks *et al.,* ١٩٨٣) and leishmaniasis (Akrawi, ١٩٨٥).

By measuring NADH consumption, this method was used to measure total LD activity (Moss & Henderson, ١٩٩٩). Several methods have been developed to study isoenzymes pattern of LD, these include: Electrophoresis, this procedure is most widely used, after electrophoretic separation, the isoenzymes can be detected either fluorometrically or colorimetrically , or by chemical inhibition methods, ion exchange chromatography and immunoprecipitations (Meckenzie & Henderson, ١٩٨٣ ; Paz *et al.,* ١٩٩٠).

٢.١٠. Trace elements

The importance of trace elements is of far greater magnitude than concentrations in *vivo* suggest. They are found mg / kg amount or less as essential component of biological enzyme systems or of structural portion of biologically active constituents (Schwartz, ١٩٧٥). In modern medicine, trace elements are receiving considerable attention as the two extremes of great excess and gross deficiency continue to be recognized. The importance of trace elements metabolism had only become appreciated in the past thirty years (John & Christy, ٢٠٠٠).

The mineral elements that the body requires are frequently classified as either macro or micro nutrients, depending on the amount of each that is need in the diet (Anderson *et al.*, ١٩٨٧).

The seven minerals needed by the human body in relatively large amounts are referred to as macrominerals which include (Calcium, Chlorine, Magnesium, Phosphorus, Potassium, Sodium and Sulfur). Twelve minerals (Arsenic , Chromium, Copper, Fluorine, Iodine, Iron, Manganese, Molybdenum, Nickel, Selenium, Silicon, and Zinc that are need in only small quantities for maintenance of good health, these are referred to as microminerals (trace elements or trace minerals). Many nutritionists now believe that there are three other trace elements that should be added to this list (Boron, Tin, and Vanadium) (John & Christy, ٢٠٠٠).

The action of very small amounts of trace elements is necessary for optimal performance of the whole organism. Lack of a small amount of trace element (e.g iron) can result in clinical abnormalities (anemia) seemingly disproportionate to the amount of elements (Levander & Chang, ١٩٨٠). The basis for the amplification of trace elements action is related to their interaction with enzymes and hormones that regulate the metabolism of larger amount of such biochemical substrate (Nielsen, ١٩٨٢).During the past many years there was recognition that metal compounds are important classes of environmental and occupational carcinogens. So it has become well established that many trace

elements play an essential role in a number of biological processes through their action as activators or inhibitors of enzymatic reaction by competing with other elements and proteins for binding sites, by influencing the permeability of cell membranes, or through other mechanisms (Sherif & Howard, ١٩٨٤).

Homeostatic regulations of trace elements depends on their absorption, storage and excretion (Levandr & chang, ١٩٨٠). The principle excretory route for elimination of most trace elements is via the feces; relatively small amounts of trace elements are excreted via the urine. The loss of trace elements through other routes such as hair, nails, skin cell desquamation and sweat are generally relatively minor (Jacob *et al.,* ١٩٨١). Menstrual iron loss and seminal zinc loss are also minor but can be significant in some cases (Milne, ١٩٩٩). The importance of micronutrients in protecting the living organism against the potentially lethal effects of reactive oxygen species and toxic environmental chemicals has recently been realized (Podracka *et al*., ١٩٩٩).

The biological antioxidant defense system is an integral array of enzymes, antioxidant and free radical scavengers like, glutathione reductase, superoxide dismutase, catalase, vitamins A,C and E. However the emerging newer concepts on the role of trace elements and other dietary components in antioxidant defence intake of some trace elements has been associated with different disease (ischemic heart disease, arthritis, stroke and cancer) where pathogenic role of free radicals is suggested (Lalle *et al*., ١٩٩٩). Wound healing and immune function also depend on adequate levels of trace elements (Demling & De Biase, ١٩٩٥). On the other hand, excessive intake of trace elements to disease and toxicity, therefore a fine balance is essential for health (Jacob, ١٩٨٦).

Analytic methods used for the determination of trace elements in biological specimens should be sensitive, specific, precise and relatively fast. Atomic absorption spectrophotometry (AAS) is the method of choice without access to advanced instrumental techniques. AAS technique detects only one element at a

time, elemental determination is quick and accurate, besides offering good specificity and sensitivity (Christian & Feldman, ١٩٧٠).

٢.١٠.١. Copper

Copper is an essential trace element that is an integral component of certain metalloenzymes and proteins such as ceruloplasmin the major Cu – containing protein in plasma, superoxide dismutase and cytochrome – C oxidase (Uary *et al*., ١٩٩٨).

It is a heavy metal whose unbound ions are toxic. Almost all copper in the body is present as a component of copper proteins, thereby reducing the in *vivo* concentration of unbound copper ions almost to zero (Berkow, ٢٠٠٠).

The body contains about ١٠٠ mg of copper, widely distributed in various tissues but with the highest concentrations in liver and brain (Gowenlock *et al.*, ١٩٨٨). The recommended daily dietary allowance for copper is about ٢ – ٥ mg and this intake is often exceeded in a normal diet, though bio-availability varies and presence of other metal ions may inhibit absorption (Causins, ١٩٨٥).

Plasma copper concentrations were regulated by a strong homeostatic mechanism and were maintained within a relatively narrow range within an individual. Plasma copper falls only after stress and severely depleted with an increased infection and increased susceptibility to bacterial pathogens (Milne *et al*., ١٩٩٠). About one to two thirds of ingested copper is absorbed in the small intestine probably by a mechanism closely related to that for zinc. After transport across the brush border, copper and zinc may pass from the intracellular pool to combine with plasma albumin in the portal blood, or remain in the enterocyte bound to intestinal

metallothionein. Absorption is impaired in conditions of mucosal damage (Gowenlock *et al.,* ١٩٨٨).

Copper deficiency is rare in healthy people, but low serum copper concentration (hypocupremia) has been observed in nephrosis, cystic fibrosis, celiac disease and other malabsorption syndromes (Premakumer *et al*., ١٩٧٥).

Accidental ingestion of copper components may cause acute copper toxicity and associated epigastric pain, nausea and vomiting (Olivares, ١٩٩٦). High concentration of copper is found in the plasma proteins in a number of disorders including cancer , infection and inflammation condition (Terao & Owen, ١٩٧٧).

The principle role of copper excretion is by secretion in to the bile (Linder & Moryan, ١٩٩٦), thus faeces contain both unabsorbed and excreted copper. There is very low concentration of copper in urine (Gowenlock *et al*., ١٩٨٨).

٢.١٠.٢. Magnesium

Magnesium is not a true trace element, it is considered as macronutrient, depending on the relatively large amount that is needed in the diet. Adult human body contains ٢١ – ٢٣ gm of Mg, ٦٠٪ of which is combined with calcium and phosphorus in the structure of bone, ٢٠٪ in skeletal muscles, ١٩٪ in other cells and about ١٪ in extra cellular fluid (Ryan, ١٩٩١). Magnesium in serum exists in several forms; protein bound (١٩ – ٣٤٪ of total), free $Mg^{+٢}$ ion (٦١ – ٦٧٪ of total), and complexed to certain anions (٥.٥ – ١٤٪ of total) (Altura & Altura, ١٩٩٤). The major biological functions of Mg are :

a. Maintains the structural integrity of cellular membrane and macromolecules such as DNA and RNA (Rude & Singer, ١٩٨١).
b. Interacts with calcium to affect the permeability of excitable membranes and neuromuscular transmission (Lowenstein & Stanton, ١٩٨٦).

c. It is an activator of alkaline phosphatase, an enzyme involved in calcium and phosphorus metabolism (Pennington & Wilson, ١٩٩٠).
d. The synthesis of both DNA, RNA and proteins depends on Mg (Gullestad *et al*., ١٩٩٤).
e. Magnesium plays structural role in bone and teeth development (Benyon, ١٩٩٨).
f. Co-factor for more than ٣٠٠ enzymes in the body including phosphotransferase and kinase, (Smith *et al.,* ٢٠٠٠).

Magnesium is absorbed mainly in the jejunum and the ileum and is effected by malabsorption syndrome (Milne, ١٩٩٤). The major excretory pathway for absorbed Mg is through kidney, which is the main organ for Mg homeostasis in maintaining plasma concentration, which is normally kept within narrow limits (Smith *et al*., ٢٠٠٠).

Increased sever magnesium concentration has been observed in dehydration, sever diabetic acidosis and Addison's disease (Ryan, ١٩٩١).

Magnesium deficiency leads to impairment of calcium and potassium homeostasis, magnesium depletion may occur in various conditions that either impair it's intestinal absorption or increase it's urinary secretion, like in chronic diarrhea , statorrhea, chronic alcoholism, acute renal failure and malabsorption syndrome (Shils, ١٩٩٠).

٢.١٠.٣. Zinc

Zinc is an essential trace element. It is a co-factor for more than ٣٠٠ enzymes, and zinc containing enzymes are found in every enzyme classes, alkaline phosphatase, alcohol dehydrogenase, carbonic anhydrase, DNA polymerase and superoxide dismutase contain zinc (Vallee & Falchuk, ١٩٩٣). It is necessary for structure, stability and catalytic function in these enzymes. Certain hormones ,

thymosine and testosterone are zinc dependent, which may accounts of the hormonal abnormalities arising with zinc deficiency (Carl & Edward, ١٩٩٩).

The human body contains about ٢ -٣ gm of zinc highly concentrated in the kidney, retina, semen, hair, nail, skin, and prostate. Muscle and bone tissue contain about ٩٠٪ of the total body zinc (Benyon, ١٩٩٨).

Zinc influences many body systems and functions, including growth, bone formation, brain development, reproduction, fetal development, sensory functions (like taste and smell), immune mechanism, membrane stability, wound healing, preserves the integrity of sub cellular organelles and protection from free radical damage(Linder, ١٩٩١; Vallee & Falchuk, ١٩٩٣; Cunningham – Rundle, ١٩٩٦).

The absorption of zinc mostly occurs in the duodenum and proximal jejunum, the major route of zinc excretion via the feces. The maintenance of zinc homeostasis appears to be chiefly gastrointestinal. With increasing intake, fecal loss of zinc increases with stable urinary excretion (Milne, ١٩٩٩).

Zinc is transported in blood plasma by albumin (٦٠-٧٠٪) and α-٢ macroglobulin (٣٠ – ٤٠٪), with small amount associated with transferring and free amino acid (Milne *et al*., ١٩٩٣). Malabsorption, chronic disease of liver and kidney, alcoholism, and diets high in phosphate or low in zinc are associated with zinc deficiency (Linder, ١٩٩١ ; Vallee & Falchuck, ١٩٩٣). Tissue zinc is preserved at the expense of growth or by decreased excretion until the deficiency is severe. Plasma zinc concentration does not change significantly until zinc deficiency is pronounced (King, ١٩٩٠).

Symptoms of deficiency include skin lesions, diarrhea, dwarfism, sensory alterations and susceptibility to infection (Prasad, ١٩٦٣; Vallee & Falchuck, ١٩٩٣). Injury, surgery, infection and variety of acute illness are often accompanied by a fall in plasma zinc due to stimulation of hepatic metallothionien synthesis. This is one of the many components of the acute phase response and an important intracellular

storage site for zinc and other essential trace elements (Cherian *et al.,* ١٩٩٤). Reduced immune function of T-Cells has been associated with deficiency of zinc, which acts as a growth factor (Prasad, ١٩٩٦).

Ingestion of zinc in a large amount (٢٠٠ to ٨٠٠ mg/day), usually by consuming acidic food or drink from a galvanized container, can cause vomiting and diarrhea (Goldman & Beanett, ١٩٩٩). Doses of zinc ranging from ١٠٠ to ١٥٠ mg/day interfere with copper metabolism and cause hypocupremia (Berkow, ٢٠٠٠).

٢.١١. Vaccination

Due to the increasing number of resistant isolates to the known antimonial compounds, the toxicity of the used drugs (antimony compounds and amphotericin), in addition to the high cost of these therapeutic regimens, a safe, effective and non-species specific vaccine will be the ideal solution. Conventional ways of developing vaccine against infection on pragmatic grounds using killed parasite with or without an adjuvant has been tried in animal models (Dube *et al.,* ١٩٩٨).

- Studies with first generation vaccine

Most of the clinical trials using killed vaccine, including all trials in the old world, have been sponsored and coordinated by the special programme for research and training in tropical disease (TDR) and the WHO. The overall of the TDR/WHO leishmaniasis vaccine program has been to develop a vaccine(s) for the prevention of all forms of leishmaniasis (Modabber, ١٩٩٥).

It was decide to proceed to field efficacy trials with simplest and most cost effective protocol for the first generation vaccine (i.e. single injection) in the initial step, following by trials with booster injections later, a preliminary clinical evaluation of a merthiolate – killed vaccine (KLM) mixed with BCG was conducted in a non – endemic area. Later, phase I–II randomized, double–blinded controlled trials were done comparing KLM with a newly produced and more stable autoclaved–killed

preparation (ALM), which showed similar a profile of safety and immunogenicity (Alexander *et al.,* ١٩٩٨).

Multicenter trial of a single injection (١ mg of ALM vaccine plus one tenth dose of BCG used for vaccination against tuberculosis) began in ١٩٩٥ in two sites in Iran. The results of the trials in Isfahan and in Bam, Iran involving the vaccination of over ٦٠٠٠ individuals against zoonotic and arthroponotic CL, respectively, have been reported In each case, the vaccine was safe; side effects consisted only of those associated with BCG vaccination. However, the overall - ٢ year incidence of CL did not differ statistically between the groups vaccinated with ALM + BCG alone. Safety, immunogenicity, and efficacy field trials involving three injections of ALM + BCG were recently completed in two different sites in Iran. Again, no difference was observed in the over all -٢ year incidence of CL compared with the control group receving BCG alone (Sharifi *et al.,*١٩٩٨).

A non- human primate model for VL has been developed in Lucknow India, where langure monkeys are used to evaluate various vaccine and / or drug preparation for leishmaniasis caused by *L.donovani* . In preliminary experiment, three doses of ALM +BCG (the vaccine as used in clinical trials in Iran for cutaneous disease) were shown to be protective against a lethal challenge with *L.donovani* amastigotes given interavenously (Dube *et al*., ١٩٩٨).

The observations were in line with the notion that, because of the tremendous cross reactivity amongst different species of *leishmania* and *L.major* vaccine can protect against *L.donovani*. The results of prospective study in Sudan had indicated that individuals exposed to *L.major* may have some immunity against VL. For this reason, as well as the sever nature of the current epidemic in eastern Sudan, the ALM+BCG vaccine (٣ doses) was tested ăğainst VL in the Quadaref area of Sudan. The results of this trials were also disappointing ; the overall - ٢ year incidence of VL did not differ statically between the ALM+BCG and BCG alone group and a new

formulation of the Indian heat killed vaccine includes alum as an adjuvant has recently been evaluated in *Rhesus macaque* model of infection (Khalil *et al.,* ٢٠٠٠).

The combination of two adjuvants, BCG and alum, in an intradermal injection has not been previously evaluated. Following a single high dose (٠.٥ – ١٠ mg) intradermal injection, all monkeys had strong in duration and a smaller degree of ulceration at the vaccination site. All monkeys immunized with ALM/BCG were completely protected against intradermal challenge. Langur monkeys vaccinated with an identical high dose combination of alum absorbed ALM plus BCG had an unacceptable local reaction to the vaccine, but they were also completely protected against the development of visceral disease following with *L.donovani* (Misra *et al*., ٢٠٠١). A safe low dose determination and immunogenicity trials involving a single injection of alum adsorbed ALM plus BCG were recently completed in Sudan. In comparison, the skin test conversion rates with the three doses of ALM+BCG , the greater immunogenicity has been confirmed . Efficasy trials have been initiated in Iran (Misra *et al*., ٢٠٠١).

- Studies involving second generation vaccine

The generation of safe, live attenuated vaccines using molecular genetic approaches has been accomplished by targeted deletion of genes involved in parasite survival or virulence. Promastigotes lacking the DHFR gene were able to persist in macrophages for several days and provided partial protection against CL(Titus *et al.,* ١٩٩٥). When genes encoding a family of cysteine proteinase were deleted, promastigotes were completely attenuated and produced partial protection against cutaneous disease (Alexander *et al.*, ١٩٩٨).

To date, at least ١٠ different recombinant leishmanial antigens had been used to vaccinate mice against CL, including gp٦٣, gp٤٦, gp٤٢, LACK and HSP٧٠ (reviewed in Handman,٢٠٠١). Adjuvants were always required to elicit an appropriate immune response, and in each case the protection observed was partial

in that all vaccinated mice developed lesions that were either delayed in their appearance and/or healed more rapidly. Of the adjuvants used, only interleukin – ١٢ (IL – ١٢) potentiated vaccine efficacy when delivered in a subcutaneous as opposed to systemic site and only IL-١٢ might be considered suitable for human use (Muyomwe *et al.,* ١٩٩٨).

Finally, it should be noted that, in virtually all of these experimental vaccination models, efficacy was evaluated in a short time (٢-٦ weeks) following administration of the vaccine, and thus may not have provided a stringent test of the durability of immune responses that will be required in a field setting.

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Chapter Three

Materials And Methods

Materials and Methods

This study was conducted from May, ٢٠٠٣ to December, ٢٠٠٥. Follow up was done in Medical Research Center / Al-Nahrain Medicine College and Central Public Health Labortory / Baghdad.

٣.١. Subject selection

Blood samples were collected from children under six years old in Al-Zahraa Children and Maternity Hospital / Najaf and Babil Maternity and Children Hospital, AL- Khadhmiya Teaching Hospital, Central Children Hospital, Al – Ilwia Children Hospital, AL – Mansoor Children Hospital and Central Public Health Laboratory.

The patients were divided into three groups :

a) Confirmed VL group: eighty-six patients with splenomegaly, positive (microscopic examination or culture) bone marrow aspirate and positive dipstick assay for visceral leishmaniasis. These samples were used as confirmed samples in this study.

b) Healthy control: thirty-six blood samples were collected from children under six years old from different primary health centers with no history of living in endemic areas with VL and who were apparently healthy by physical examination.

c) Infection control: twenty infected children with disease other than VL. These were as follows : Tuberculosis, Typhoid fever, Toxoplasmosis and Brucellosis.

Some information was taken for each patients such as name , sex, age , address and other subjects (Appendix ١).

٣.٢. Sample collection

A sufficient amount of blood could be collected in an anticoagulant container and plain tube for humeral and cell-mediated immunity from sixty-two children with disease (before administration of sodium stibogluconate injection), and fifty five children who were followed up until the end of therapy (٢٨ days of sodium stibogluconate therapy). Each blood sample obtained in a plain tube centrifuge as soon as possible and serum separated, liquated into portions to avoid repeat freezing and thawing then stored at -٢٠ ^{o}C until used.

٣.٣. Parasitological diagnosis

Bone marrow samples (biopsies) were aspirated from children who were suspected of being infected with VL by specialists. The patient was usually antisepticized locally with iodine followed by spirit. A puncture was made by means of a dry sterile bone marrow needle. About ١ ml of bone marrow was aspirated; portion of the aspirate were introduced aseptically into different culture media, the process being carried out to a flame on a clean sterile table in a room where the door and windows were kept closed during the operation to eliminate any possible source of draft ; talking was not allowed. All these precautions were taken so that possible sources of contamination were cut down to the lowest level. (Rassam & AL-Mudhaffar, ١٩٧٩) .

Bone-marrow sample was smeared on to a clean slide, then air dried and fixed with methyl alcohol for ١-٢ minutes. The slide was stained with ١٠ -١٥٪ Giemsa

stain for ٣٠ minutes, washed in tap water and air dried. The slides were examined microscopically under oil – immersion lens for LD bodies, the smears were considered negative for LD if these bodies were not seen after examination.

٣.٤. Culture media used for the primary isolation

The remaining part of the bone marrow aspirate was inoculated onto semisolid medium, then incubated at ٢٥ ^{o}C .The culture was examined microscopically for the presence of the promastigote stage. It was considered negative in the absence of this stage during the first four weeks (Rassam & AL-Mudhaffar, ١٩٧٩). The culture media used during this study were :

٣.٤.١. Semisolid medium

This medium was used for primary isolation of the parasite from the aspirate bone marrow and for subculturing of the parasite. This medium was prepared according to AL-Hussayni *et al.* (١٩٨١).

Each liter contained :

NaCl	٦.٩١ gm	BDH
$CaCl_{٢}.٢H_{٢}O$	٠.٢٢ gm	BDH
$NaHCO_{٣}$	٠.١٠ gm	BDH
KCl	٠.٢٩ gm	BDH
D-glucose	٠.٧٧ gm	BDH
Agar	٤.٠ gm	Difco

Pepton	١.٠	gm	Oxoid
Beef extract	٠.٣٠	gm	Oxoid
Distilled water	٨٠٠	ml	
pH:	٧.٤ ± ٠.١		

٣.٤.٢. Liquid medium (liquid cell free medium)

This medium had been used by AL-Bashir *et al.* (١٩٩٢). It was used for growing parasites in large numbers, which were used for antigen preparation and for isoenzymes study.

Each liter contained :

NaCl	٦.٩١	gm	BDH
$CaCl_{٢}.٢H_{٢}O$	٠.٢٢	gm	BDH
$NaHCO_{٣}$	٠.١٠		BDH
KCl	٠.٢٩	gm	BDH
D-glucose	٠.٧٧	gm	BDH
Pepton	١.٠٠		Oxoid
Beef extract	٠.٣٠	gm	Oxoid
Brain – heart infusion	١٠.٠	gm	Difco
Distilled water	٨٠٠	ml	
pH :	٧.٤ ± ٠.١		

Each components of the two media was sterilized by autoclaving at ١٢١ ^{o}C for ٢٠ minutes, cooled to ٥٠ ^{o}C and supplemented with ٢٠٪ defibrinated rabbit blood

(obtained by cardiac puncture of the animals; defibrinated was facilitated by shaking with glass beads in a sterile – cap bottle). Gentamycine, ٥٠ mg were added to autoclaved media. Two or five ml of medium was dispensed into ١٠ or ٢٥ ml capacity screw – cap bottles. All the media were tested for sterility either by incubation at ٣٧ ^{o}C for ٢٤ hours or at ٢٥ ^{o}C for ٤٨ hours. The media stored at ٤ ^{o}C until used.

٣.٥. The parasites source

The following table was showing strains designation:

Species	References	Local reference code
L. tropica	MHOM/SU/٥٨/OD	(MHOM/IQ/١٩٨٤/BRC١٨)
L. major	MHOM/SU/٧٣/٥ASKH	(MHOM/IQ/١٩٨٤/BRC١٩)
L. donovani	MHOM/ET/٦٧/HU٣	(MHOM/IQ/١٩٨٢/BRC١)

They were obtained from Medical Research Center / Leishmania Unit in AL – Nahrain College of Medicine. This parasite had been serially passed in semisolid medium and laboratory Balb/c mice.

٣.٦. Cultivation and maintenance of parasite

Promastigotes in semisolid media could be cultivated on liquid media to obtain large numbers of the parasites; this was done as follow: After the parasite culture contained heavy growth, it was transferred to liquid media in shaking incubator at ٢٥ ^{o}C. The culture were examined every two days to observe the growth of the parasite and absence of contamination. When a heavy growth was observed more liquid medium was added to the bottles. This step was repeated every two

days until the culture reached the required number (١ x ١٠^{٧} cell / ml) at the end of logarithmic phase (AL-Bashir *et al.*, ١٩٩٢).

٣.٧. Protein measurement of *Leishmania* antigens

Protein content was measured by Folin-Phenol method (Lowery *et al*., ١٩٥١) as follows:

١. Mix ١٥ ml of reagent A, ٠.٧٥ ml reagent B and ٠.٧٥ ml reagent C (Appendix ٦).

٢. Take one ml of the sample and one ml of the mixture above, mix thoroughly and wait for ١٥ minutes at room temperature.

٣. Add ٣ ml of ١: ٩ ٢N folin phenol : D.W. to the mixture above.

٤. Mix well . Wait for ٤٥ min. at room temp. and read by spectrophotometer at ٥٤٠ nm .

٥. Compare to standard protein of ٠.٣ mg bovine serum albumin in one ml D.W.

٣.٨. Enzyme immunoassay for qualitative determination of antibodies against *Leishmania Infantum* in human serum

This assay was done by using ELISA kit (Vircell, S.L. spain) as recommended by the manufacturer.

a. Principle :

The ELISA method is based upon the reaction of antibodies in the sample tested with the antigen adsorbed on the polystyrene surface. Unbound immunoglobulins were washed off. An enzyme – labelled anti-human globulin binds the antigen – antibody complex in a second step. After a new washing step, bound

conjugate was developed with the aid of a substrate solution (TMB) to render a blue coloured soluble product which turns into yellow after adding the acid stopping solution.

b. Kit contents :

١. Leishmania plate : ٩٦ – wells plate coated with antigen of *L. infantum* .

٢. Serum diluent : ١٥ ml of serum dilution : a blue colored phosphate buffer containing protein stabilizers and proclin. Ready to use.

٣. Positive control (IgM + IgG) : ٢٥٠ ml of positive control serum containing proclin.

٤. Cut off control: ٢٥٠ ml of cut off control serum containing proclin.

٥. Negative control: ٢٥٠ ml of negative control serum containing proclin.

٦. Conjugate : ١٥ ml of anti-human peroxidase conjugate dilution in a red – coloured proclin – containing buffer. Ready to use.

٧. Substrate solution : ١٥ ml of substrate solution containing tetramethylbenzidine (TMB). Ready to use.

٨. Stop reagent : ١٥ ml of stopping solution : ٠.٥ M sulphuric acid.

٩. Wash buffer : ٥٠ ml of ٢٠x washing solution : a phosphate buffer containing tween® – ٢٠ and proclin.

c. Assay procedure :

١. Set incubator / water bath to ٣٧ ± ١ ^{o}C.

٢. Bring all reagents to room temperature before use (approximately one hour), with out removing the plate from the bag.

٣. Shake all components.

٤. Remove the plate from the package. Determine the numbers of wells to be employed, counting in four wells for the controls: two for the cut off serum and one each for the negative and positive sera. Wells not required for the test should be returned to the pouch, which should then be sealed.

٥. Prepare a serial dilution (٨ , ٣٢ , ١٢٨ , ٥١٢ and ٢٠٤٨) for serum samples and cut off serum (duplicate).

٦. Cover with a sealing sheet and incubate at ٣٧ ± ١ °C for ٤٥ min.

٧. Remove the seal, aspirate liquid from all wells and wash five times with ٠.٣ ml of washing solution per well. Drain off any remaining liquid.

٨. Immediately add ١٠٠ ml of conjugate solution into each well.

٩. Cover with a sealing sheet and incubate in incubator / water bath at ٣٧ ± ١°C for ٣٠ min.

١٠. Remove the seal, aspirate liquid from all wells and wash five times with ٠.٣ ml of washing solution per well. Drain off any remaining liquid.

١١. Immediately add ١٠٠ µl of substrate solution into each well.

١٢. Cover with a sealing sheet and incubate at room temperature for ٢٠ min. protected light.

١٣. Remove the seal and immediately ٥٠ µl of stopping solution into all wells.

١٤. Read with a spectrophotometer at ٤٥٠ nm within one hour of stopping.

d. Interpretation of the results :

Calculation the mean O.D for cut off serum.

Antibody index = (sample O.D / cut off serum mean O.D) x ١٠

Index	Interpretation
< ٩	Negative
٩ – ١١	Suspect
>١١	Positive

• Samples with equivocal result must be retested and / or a new sample obtained for confirmation.

• Samples with indexes below ٩ were considered as not having antibodies against *Leishmania.*

• Sample with indexes above ١١ were considered as having antibodies against *Leishmania*.

٣.٩. Dot – blot immunobinding assay ٤٤

This technique was used to detect the specific antibodies of *L. donovani* in the sera of patients.

a. Antigen preparation :

L. donovani was cultured on liquid medium for ٣ – ٤ days in shaking incubator at ٢٦ ^{o}C. Promastigotes were harvested , then washed three times with PBS, centrifuged at ١٢٠٠ x g for ٢٠ minutes at ٤ ^{o}C , PH was adjusted to ٧.٢. The pellets were stored at -٢٠ ^{o}C until used. The organisms in the pellets were disrupted by sonication for three times of one minute duration each and one minute rest

between the runs. The sample was kept cool by surrounding it's container with crushed ice. Saline was added to the sonicated promastigotes and the suspension was centrifuged at ٣٠٠ x g for ٣٠ minutes at ٤ ^{o}C. The supernatant fluid represent the soluble antigen (Baqir *et al.,* ٢٠٠١). This was divided into small aliquots and stored at – ٢٠ ^{o}C until required. The protein content was determined by Lowery *et al*. (١٩٥١) method.

b. Preparation of the strips :

Schliecher and Schwell (S&S) Nytrane membrane was soaked for ١٠ minutes in Tris – Buffer Saline (TBS) solution (٢٠٠ mM NaCl, ١٥ mM Tris – HCl, pH ٧.٤), before it was placed in the Bio – Dot apparatus. The appropriate wells were filled with the soluble antigen diluted with TBS to obtain a final concentration of ١٠ µg/ml. Then the entire sample was allowed to filter through the membrane by gravity flow for one hour , then the vacuum pump was applied to ensure complete drainage of the sample from the wells. The coated membrane with the antigen was removed from the apparatus and blocked with ١٠٪ fat free milk in TBS with continuous shaking overnight.

The next day the membrane was removed from the blocking solution, dried and cut directly into strips and store at ٤ ^{o}C and used within three days from the time of preparation (Ibrahim , ١٩٩٦).

c. Serodiagnosis :

١. The antigen coated strips were incubated with serially double diluted sera (٨ ,٣٢ , ١٢٨, ٥١٢ and ٢٠٤٨) in ٣٪ BSA in TBS with continuous shaking for ٢ hours at room temperature.

٢. The antigen coated strips were washed with ٠.١٥٪ (v/v) Triton X-١٠٠ in TBS three times for ١٠ minutes with constant agitation.

٣. After washing the antihuman IgG alkaline phosphatase conjugate diluted ١/١٥٠٠ in ٢٪ BSA in TBS was added to the strips for one hour at room temperature with continuous shaking.

٤. Washed as in step (٢).

٥. Exposed the strips to ٥-Bromo-٤-Chloro-٣-Indyol phosphate (BCIP) and Nitro Blue Tetrazolium (NBT) for ٢٠ – ٢٥ minutes in dark place and read immediately by naked eye.

٦. Serum dilution that gave blue color was considered positive, and the serum with no color was considered negative. Each sample was performed in duplicates.

d. Sensitivity and specificity

Sensitivity was measured by dividing true-positive test results over all patients with the disease = { a / (a + c)}.

Specificity was measured by dividing true-negative test results over all patients with out the disease = { d / (b + d)}.

Positive predictive value was measured by dividing true-positive test results over all positive test results = { a / (a + b)}.

Negative predictive value was measuring by dividing true-negative test results over all negative test results = { d / (c + d)}.

Overall accuracy was measured by dividing true-positive + true negative test results over all tests {(a + d) / (a + b + c + d)}

Whereby:

a = true positive

b = false positive

c = false negative

d = true negative

e. Antigen titration

To obtain the ideal concentration of antigen which differentiates between positive and negative results (the term negative used as indicating the absence of specific antibodies above the background noise). Check board titration was done using a series of antigen concentration ranging from ٥ -٥٠ µgm protein/ml with a known positive and negative reference serum in a serial dilution from

One antigen – antiserum combination consistently giving the best differentiation between positive and negative sera was used throughout the study. The optimal antigen concentration of ١٠-١٥ gm protein / ml and a single serum dilution at ١:١٢٨ were selected (Baqir *et al.,* ١٩٩٩).

٣.١٠. Immunoglobulins & complements levels

The concentration of immunoglobulins IgG , IgM , IgA , the complement C٣ and C٤ were estimated in the sera of VL patients and healthy control using a single radial immunodiffusion assay (RID) by immuno-kits (Biomaghreb , Tunissia) . According to the manufacturer information , the plates contained the following components : buffered saline, agarose , sodium azide and monospecific antiserum .The assay was done in serology Unit / Medical Research Center, Al – Nahrain College of Medicine .This test was based on the formation of an immunoprecipitin ring in an agarose gel .The test was done as follows :

١. After removing the serum samples and immuno-kit from the refrigerator , they were left at room temperature (about ٢٠ °C) for ١٥ minutes .

٢. The plate was removed from the ziplock bag , and the lid was removed if moisture was present , the uncovered plate was allowed to remain at room temperature for approximately ١٥ minutes until moisture had evaporated.

٣. Five µl from control serum were dispensed into well No.١ ,and ٥ µl of each serum sample were dispensed into the following wells.

٤. The lid was replaced and the plate was incubated at ٢٣ ± ٢ °C on a level surface for ٤٨ hours for the plates of IgG , IgA , C٣ & C٤ and ٧٢ hours for the plates of IgM .

٥. The immunoprecipitin ring diameters were measured with the viewing device which was capable of measuring the percipitin ring diameter in ٠.١ mm increments .

٣.١١ Western blotting

٣.١١.١. Preparation of the parasite antigens

The test was done as described by Al-Bashir *et al.* (١٩٩٢) as follows :

a. The promastigotes of different *leishmania* species were harvested from the liquid media by centrifugation at ١٢٠٠ xg for ١٥ minutes at ٤ °C. The pellets were washed ٣ times (١٢٠٠ xg /١٥ minutes) in PBS, pH ٧.٢. The pellets were mixed with an equal volume of triton X-١٠٠ (٠.٠٣٪) and disrupted by sonication ٣ times for ٢ minutes each time, with a break of ٤٠٧ second in between the runs. This was done under cooling conditions. The sonicated promastigotes were centrifuged at ١٢٠٠ xg for ٢٠ minutes at ٤ °C . The supernatant (crude antigens) was stored at -٢٠ °C until used. The protein content of the crude antigens extract were determined as described below.

b. The protein content in the antigens extract was determined according to the method of lowery *et al*. (١٩٥١).

The crude extract of *Leishmania* antigens were separated on a horizontal SDS – polyacrylamide gel (SDS-PAGE), with a stacking gel of ٤٪ and separating gel of ١٠٪, standards of known molecular weight were included, and the separated proteins were electrophoretically transferred to ٠.٤٥ µm nitrocellulose paper using semi-dry blotter (Towbin *et al.,* ١٩٧٩).

٣.١١.٢. SDS-PAGE electrophoresis

This is one dimensional gel electrophoresis under denaturing conditions (in the presence of ٠.١٪ SDS) which separates proteins based on molecular size.

Preparation of the SDS – gels

a. The separating gel :

The separating gel was prepared as follows:

١٧.٥ ml of ٣٠٪ acrylamide / ٠.٨٪ bisacrylamide + ١٣.١٢٥ ml of ٤x tris. HCl/SDS pH ٨.٨ , and ٢١.٨٧ ml of deionized D.W. was added .Then ١٧٥ µl of ١٠٪ ammonium persulfate and ٣٥µl TEMED was added to the solution and gently swirled to mix.

The glass plate sandwich was assembled, using two clean glass plates and two ٠.٧٥ mm spacers, and then the sandwich was locked to the casting stand. ٥٢.٥ ml of the separating gel with final acrylamide ١٠٪ was poured. Using a Pasture pipette , the separating gel solution was transferred to the center of the sandwich. Using another Pasture pipette, the top of the gel was slowly covered with a layer (~١ cm thick) of isopropyl alcohol by gently squirting the isopropanol against the edge of

one of the spacers, and then the gel was allowed to polymerize ١ hour at ambient temperature.

b. The stacking gel:

The layer of isopropyl alcohol was completely poured off and the gel washed ٣ times by D.W. The stacking gel was prepared as follows :

Mix ٢.٦ ml of ٣٠٪ acrylamide/٠.٨٪ bisacrylamide solution, with ٥ ml of ٤x Tris. HCl/SDS pH ٦.٨, and ١٢.٢ ml of deionized D.W. was added, then ١٠٠ µl of ١٠٪ ammonium persulfate and ٢٠ µl TEMED. The mixture was gently swirled to mix and use immediately. Using a Pasture pipette, the stacking gel solution was slowly allowed to trickle into the center of the sandwich along the edge of one of the spacers. The stacking gel was allowed to polymerize ٦٠ – ٩٠ minutes at room temperature.

c. Loading the gel:

The protein sample was dissolved in ٥٠ µl of ٢xSDS / sample buffer at a concentration of ٣٠٠ µg / ml, and boiled for ٥ minutes at ١٠٠ ^{o}C. Using micropipette, the protein samples were loaded on the stacking gel using sample applicator. Then the wicks (Whatman MM filter paper) were soaked into electrophoresis buffer and attached to the stacking gel from one side and the separating gel from the other side. The electrophoresis apparatus was then covered.

d. Running the gel:

The power supply was connected to the cell and run at ٥٠ mA of constant current. After the bromophenol blue tracking dye had reached the bottom of the separating gel, the power supply was disconnected. The total run time for gel was ٦ hr. after disassembling the gel, the part of gel for detection of protein bands by coomassie blue staining was cut and separated from the other part of gel Western blotting, and placed in plastic box.

e. Coomassie blue staining:

The polyacrylamide gel that was placed in a plastic box, was covered by fixing solution for ٢ hr. and agitated slowly on a shaker, then the fixing solution was poured out, and the gel was covered with coomassie blue solution for ١ hr. and slowly agitated. The staining solution was then poured out and the gel rinsed briefly with fixing solution, and covered with destaining solution over night, slowly agitated, and the destaining staining solution was then poured out.

f. Molecular weight determination

Low molecular weight calibration kit (LKB ١٨٦٠١٠٢) was used. This kit provided ٨ protein standard covering molecular weight ranges from ١٢٠٠٠ – ٢٣٢٠٠٠ Dalton as follows:

Protein	Mol.wt. (Da.)
Catalase	٢٣٢.٠٠٠
Lactate dehydrogenase	١٤٠.٠٠٠
Ovotransferrin	٧٨.٠٠٠
Albumin	٤٥.٠٠٠
Dehydrogenase	٣٦.٠٠٠
Carbonic anhydrase	٢٩.٠٠٠
α-lactalbumine	١٤.٠٠٠
Cytochrome	١٢.٠٠٠

The kit was used as follows:

The protein in the calibration kit was dissolved in a sample buffer by applying ١٠٠ µl of the buffer per vial, it was gently swirled and heated at ١٠٠ ^{o}C for ٥ -١٠ min, then ١٠ µl of the sample were applied and electrophoresis was carried out. The protein bands were stained and the migration distance of the calibration proteins were measured, the relative mobility (RM) was measured :

$$RM = \frac{\text{Distance of protein migration}}{}$$

Then calibration curve was constructed by plotting RM versus log molecular weight for calibration proteins, and the molecular weight of proteins of interest was determined from the position of its RM value on the calibration curve (Fig. ٣.١).

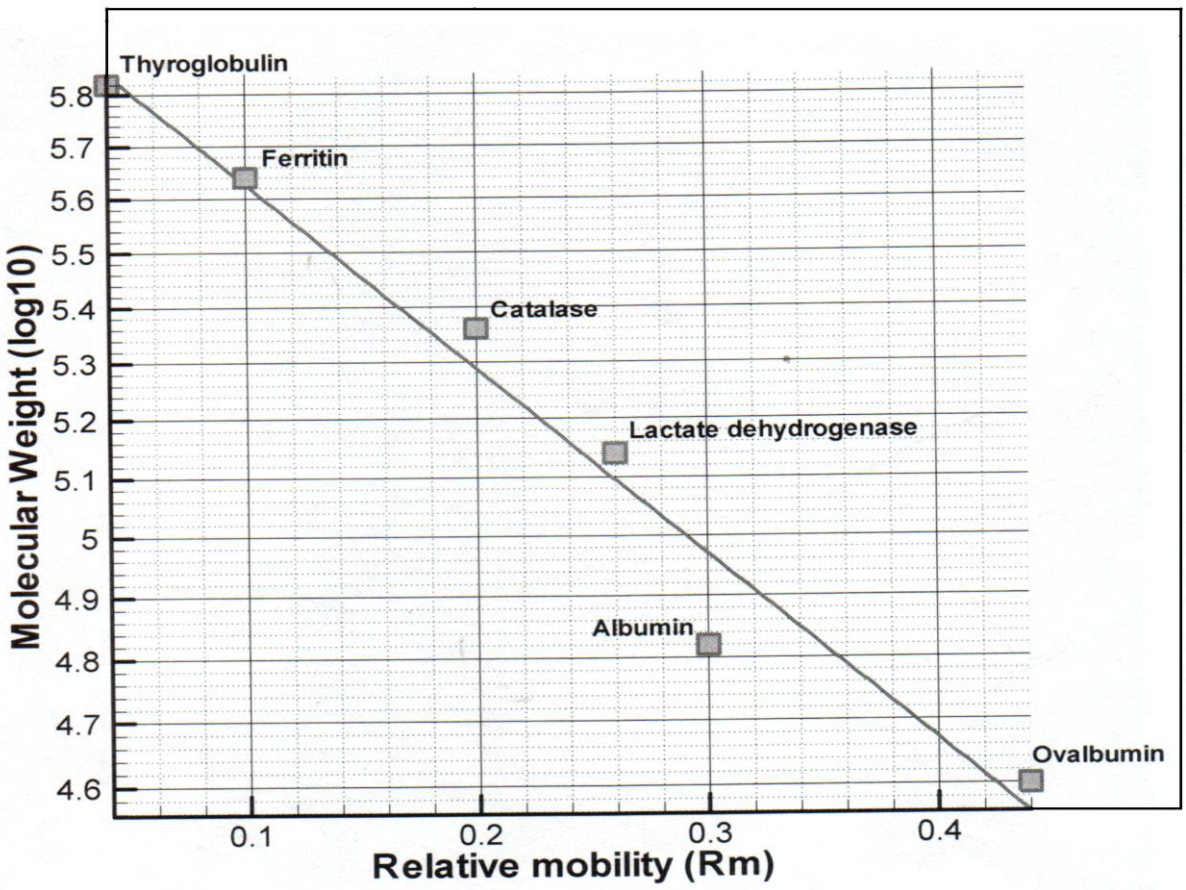


Fig. (٣.١) : Calibration curve for approximate estimation of molecular weight of protein.

٣.١١.٣. Immunoblotting

The separated components in the gel were immediately transferred electrophoretically onto nitrocellulose membrane (٠.٤٥ µm) with the use of transport apparatus and blotting buffer (pH ٨). The procedure went as follows :

A piece of Whatman ٣ MM filter paper, cut to the same size as the gel and pre wetted with electro-blotting buffer was placed on the blotter being near the anode, then a piece of cut, marked and wetted nitrocellulose membrane was directly placed on the filter paper facing on the anode, all air bubbles between the filter paper and the membrane were removed by gently pushing with gloved fingers. The gel was then placed on the membrane and the surface of the gel was moistened with electro-blotting buffer. Any air bubbles were also removed as above. Another piece of wetted Whatman filter paper was placed on the cathode side of the gel, also all air bubbles were removed, and then the cover of the blotter was applied.

The current was adjusted so as to be ٠.٨ x surface area of the gel; transfer time is ٣ hr. Following the blotting, the membrane was stained with ٠.٥٪ ponceau-s solution for five minutes and destained with distilled water for ٢ minutes to reversibly stain the transferred proteins. It was completely destained by soaking in water for ١٠ minutes, then strips of nitrocellulose membrane were cut out longitudinally and incubated overnight with blocking buffer using constant agitation with rocking platform , to block non specific binding sites.

Patients serum samples were diluted ١:٥٠ in blocking buffer . The strips were incubated with the diluted serum for ١ hr., at room temperature using constant agitation with rocking platform , then non specifically bound primary antibody was washed away by washing for three times using PBS for minutes each wash.

The bound antibody was detected by HRP-anti-IgG conjugate diluted ١:٢٥٠ in blocking buffer. It was also incubated with the strips for ١ hr. at room temperature using constant agitation with rocking platform, then the strips were also washed ٣ times by agitation with PBS , ١٥ minutes each time. Finally, the strips were stained with freshly prepared DAB (diamino benzidine) substrate solution (١٠ – ١٥ min.) and the reaction was stopped by rinsing briefly with water. All solution used in electrophoresis and WB are in (appendix ٢).

٣.١٢. Analysis of lymphocyte subsets

Reagents and substances :

١) Monoclonal antibodies (CD-Marker)

* CD٣ (clon ab) is suited for use as a T-cell reagent.

* CD٤ (clon ab) is reactive with T-helper / T-inducer antigens.

* CD٨ (clon ab) is reactive with a toxic T-cell antigens.

* CD٢٢ (clon ab) is reactive with B-cell antigens.

All primary antibodies are mouse antihuman antigens (Immunotech Abeckman Coulter Company,France).

٢) HRP-kit (which contains biotinylated secondary anti-mouse immunoglobulin and streptavidin-peroxidase (Immunotech Abeckman coulter Company,France).

٣) Power block reagent (Abeckman Coulter Company,France).

٤) DAB (Chromogen kit) (BioGenex).

٥) Total antihuman immunoglobulin FITC conjugate F(ab)٢ (Sanofi –Diagnostic Pasteur ,France).

٦) Counter stain: Hematoxylin (BDH).

٧) Glycerin (BDH).

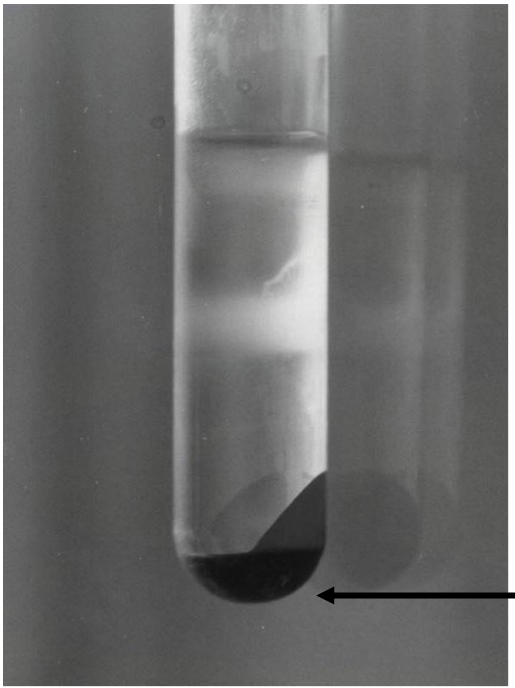


Fig .(٣.٢) :Lymphocytes separation according to the isopaque technique.

٣.١٢.١. Lymphocyte Separation

The Isopaque-Ficol technique originally described by Boyum (١٩٦٨) was used for Isolation of lymphocytes as follows:

Five ml of heparinized blood was diluted with an equal volume of PBS then layered over ٥ ml of lymphocyte separation medium in a sterile glass tube. After centrifugation at ١٥٠٠ rpm for ٣٠ minutes in a centrifuge (١٨ °C). The lymphocyte formed a grey layer at the interface of the blood plasma and the separation medium ; the lymphocyte layer was then aspirated (Fig. ٣.٢).

The lymphocytes were transferred to a centrifuge tube containing at least three times the volume of PBS and suspend evenly. Then lymphocytes suspension was centrifuged at ١٠٠٠ rpm for ١٠ minutes and the supernatants were aspirated and discarded and cell pellet was resuspended in PBS by gently drawing the cells in and out of a Pasteur pipette , repeating centrifugation another two times at ١٥٠٠ rpm, then resuspended in the PBS for subsequent experimentation.

٣.١٢.٢. Cell counting and viability assessment

The Cells counting and viability can be calculated by counting the cells in hemocytometer and trypan blue. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do and staining facilitates the visualization of cell morphology. Cells counting in this study were done as follows:

A cell suspension was prepared in PBS , then ٠.٢ ml of this cell suspension were transferred to a test tube containing ٠.٥ ml of trypan blue solution and ٠.٣ ml of PBS (i.e dilution factor : ٥) and mixed thoroughly , allowed to stand for minutes , then with the cover – slip in place , a Pasteur pipette was used to transfer a small amount of trypan blue cell suspension mixture to chamber of the hemacytometer . Cell number was calculated by following :

Cell per ml = The average count per square × dilution factor × $١٠^{٤}$

Cell viability (%) = Total viable cells / Total cells × ١٠٠

Cell number was adjusted to contain ١ × $١٠^{٦}$ cells /ml. Ten µ١ of lymphocyte suspension were placed per well of an immunofluorescent slide then allowed to air – dry at room temperature , ١٠ µl acetone as a fixative was added , allowed to air – dry at room temperature , wrapped in foil and stored at -٧٠ ^{o}C until used .

٣.١٢.٣ Immunoperoxidase Staining for counting lymphocyte subsets

a. Principle :

Precoated wells were rehydrated and treated with the protein blocking agent to reduce non-specific binding of antibodies . The precoated wells are then incubated sequentially with :

١. Primary antibody which binds to specific cell antigens .
٢. Biotinylated secondary antibody which was bound to the primary antibody ; it is polyvalent and universal ; it will be bound to primary antibodies derived from rabbit, mouse and rat.

٣. Streptavidin-peroxidase reagent,which was bound to the secondary antibody. The streptavidin was bound to the secondary antibody ; the streptavidin binds to the biotin on the secondary antibody. The peroxidase then served as the indicator enzyme.

٤. Addition of peroxidase substrate (hydrogen peroxidase and of the chromogen resulted in the formation of the colored precipitate at the cell antigen sites. Visualization is aided by counter staining with hematoxylin .

b. Assay procedure :

The precoated IF-slides with lymphocytes were removed from freezer , allowed to reach room temperature unwrapped and then fixed by dipping the slides in pre-cooled acetone at – ٢٠ ^{o}C for ٣٠ second and allowed to dry , after that slides were washed with distilled water and then with PBS. After fixation , slides were placed on a flat level surface (avoid attachment of slide to each other and should not allowed to dry out at any time) , then enough drops of ٣٪ hydrogen peroxidase were dropped to cover each well and incubated for ٥ minutes at room temperature then rinsed with PBS from a wash bottle , slides then placed in PBS wash bath for ٢ minutes and excess buffer were taped and wipped around wells , enough power block reagent were applied for ٥ minutes and excess blocking reagent were tapped but not washed , the coated lymphocytes were probed with ١٠ µl of human monoclonal Ab (primary Ab) raised against a specific human CD-maker (CD٣ , CD٤ , CD٨ , and CD٢٢). Slides then incubated at ٣٧ ^{o}C for ١ hour, unreacted monoclonal Ab were removed by three cycle of washing with PBS , after washing enough solution of biotinylated secondary antibody (anti-human Ab) were applied to cover each and distributed evenly over the precoated cells. Slides were placed in humidified chamber for ١٠ minutes at ٣٧ ^{o}C and washed in buffer , bathed in PBS for ٢ minutes and wipped around well . Enough solution of streptavidin conjugated peroxidase were applied to cover the well and the slides were placed in humidity chamber for ١٠ minutes for ٣٧ ^{o}C, then washed in buffer and bath in PBS for ٢ minutes and wipped around the wells . Slides developed in a substrate (DAB) washing solution which

prepared by addition of ٠.٥ ml substrate buffer to ٤.٥ ml deionized D.W. and ٤ drops of chromogen (DAB) after mixing well, ٢ drops of hydrogen peroxidase substrate solution were added. Enough drops of freshly prepared DAB working solution were applied to cover the wells at room temperature for ٥ -١٠ minutes or until the color is observed . The reaction terminated by rinsing gently with D.W. from washing bottle.

Slides were placed in bath Mayer's hematoxylin for ١ minute at room temperature and rinsed gently with D.W. from washing bottle then rinsed under gently running water for ٥ minutes.

c. Calculation of the Results :

Slides were examined under ٤٠x - magnification power of light microscope (Olympus). The dark brown staining identified positive labelled cells , ٢٠٠ cells were counted to determine the percentage of reactivity of each of the tested monoclonal Abs.

٣.١٢.٤. Indirect fluorescent antibody test (IFAT) for counting lymphocyte subsets

a. Principle :

Indirect immunofluorescence for the detection of CD-antigen depends on two steps: the first step leads to the binding of primary antibody to specific cell antigen. The second step allows the detection of specific CD-antigen when antimouse immunoglobulin IgG fluorescinated conjugate was added to be examined by immunofluorescent microscope. The positive samples showed an apple green fluorescence corresponding to areas of cell surface where primary antibody bound.

b. Assay procedure :

١. Lymphocyte suspension was adjusted to contain ٢x١٠^٦ cells / ml, ٤٥ µl of lymphocyte suspension was transferred in tube and ٥µl of monoclonal antibody (CD٣,CD٤,CD٨ and CD٢٢) was added, mixed well and incubated at ٢-٨ °C for ٣٠ minutes.

٢. Lymphocyte suspension was centrifuged two times at ١٥٠٠ rpm for ٥ minutes and the supernatants was aspirated and discarded and cell pellet was resuspended in PBS/BSA.

٣. Fifty µl of fluorescent conjugate (diluted ١:٨٠ in PBS/BSA) was added and incubated for ٣٠ minutes at ٢-٨ °C in the dark.

٤. Washing was repeated as in the step ٢.

٥. The cell pellets were resuspended in ٢٠٠ µl of PBS / BSA , a drop was delivered by Pasteur pipette and placed in the center of a clean slide with cover slip .

c. Calculation of the results :

Slides were examined under ٤٠x-magnifcation of a fluorescent microscope . Their dark green staining identified positively labelled cells ; ٢٠٠ cells were counted to determine percentage of reactivity of the tested monoclonal antibodies .

٣.١٣. Determination of cytokines

٣.١٣.١. Interferon -γ and interleukin -١٠

Serum levels of IFN-γ and IL-١٠ were measured by means of enzyme immunoassay using ELISA kits (Mabtech AB, Sweden) as recommended by the manufacturer. The assays had detection limits of ٢ pg / ml for IFN-γ and ٠.٥ pg / ml for IL-١٠.

a. Assay procedure :

١. A cytokine-specific MoAb was coated onto the microtitre plate by adding ١٠٠ µl/well and was incubated overnight at ٤-٨ ^{o}C.

٢. Wash twice with PBS (٢٠٠ µl /well)

٣. Block plates by adding ٢٠٠ µl /well of blocking solution e.g. PBS with ١٪ bovine serum albumin (BSA) . Incubate for ١ hour at room temperature.

٤. Wash five times with PBS containing ٠.٠٥ % Tween .

٥. Prepare hIFN –γ and hIL-١٠ standard by reconstituting contents of recombinant human IFN –γ and recombinant human IL-١٠ standard vials in ٢٠ µl with suitable buffers (appendix ٤) to a concentration of ٠.١ mg/ml . Dilute in PBS in ٠.١٪ BSA to make up a stock solution of ١٠ µg/ml. Prepare dilutions of the stock using the standard rang as a guideline in the kit leaflets.

٦. Add ١٠٠ µl/well of samples or standards diluted in PBS Tween containing ٠.١٪ BSA (incubation buffer) and incubate for ٢ hours at room temperature .

٧. Wash as in step ٤ .

٨. Add ١٠٠ µl/well of biotinylated antibodies at ١ µl/ml in the incubation buffer . Incubate for ١ hour at room temperature .

٩. Wash as in step ٤ .

١٠. Add ١٠٠ µl/well of streptavidin –ALP diluted ١:١٠٠٠ in the incubation buffer . Incubate for ١ hour at room temperature .

١١. Wash as in step ٤ .

١٢. Add ١٠٠ µl/well of appropriate substrate solution e.g.١ tablet of phosphatase substrate (sigma) =٢mM $MgCl_2$ in ٥ ml.

١٣. Measure the optical density at ٤٠٥ nm in an ELISA reader after a developing time of ١٥ minutes.

١٤. The concentrations of IL-١٠ and IFN-γ were calculated by referring to standard curves constructed with known amounts of recombinant human IL-١٠ and IFN-γ standard (fig. ٣.٣ and ٣.٤) respectively. Both assay results were expressed as the mean of duplicate determinations in pg/ml

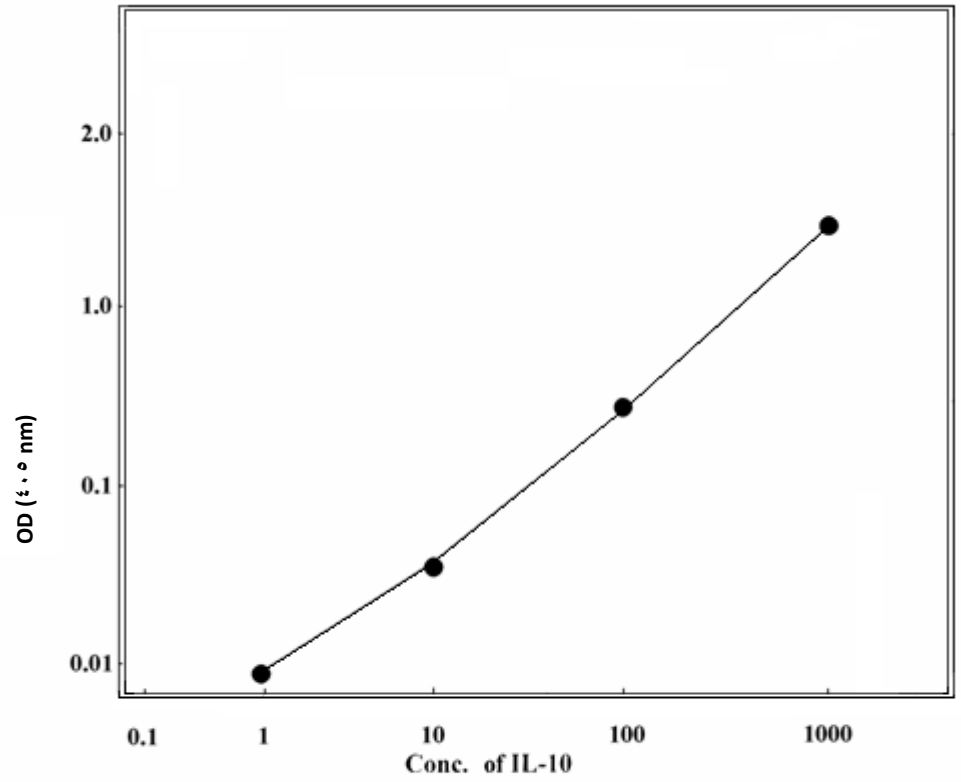


Fig. (3.3): Standard curve for estimation of interlukin-10 by ELISA technique.

Conc. of IL-١٠

Fig. (٣.٣): Standard curve for estimation of IL-١٠ by ELISA technique.

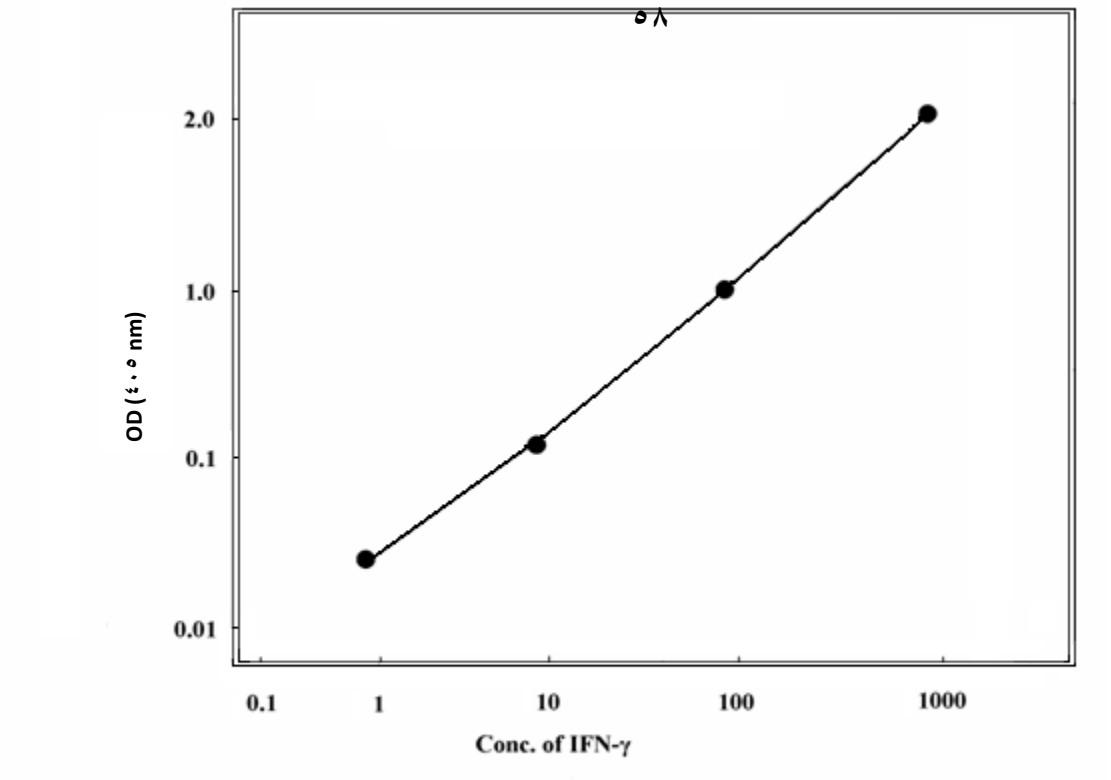


Fig. (3.4): Standard curve for estimation of interferon-γ by ELISA technique.

hnique.

٣.١٣.٢. Tumor necrosis factor – alpha (TNF – α)

Serum levels of TNF – α was measured by means of enzyme immunoassay using ELISA kit (Mabtech AB , Sweden) as recommended by the manufacturer , the assay had detection limits of ٨ pg/ml.

Procedure :

١. Coat a high protein binding ELISA plate with mAb TNF α-١ , diluted to ٢ µg/ml in PBS, pH ٧.٤, by adding ١٠٠ µl/well . Incubate overnight at ٤-٨ °C.
٢. Wash twice with PBS (٢٠٠ µl /well)
٣. Block plate by adding ٢٠٠ µl /well of PBS -Tween contain ٠.١٪ BSA (incubation buffer*) . Incubate for ١ hour at room temperature.
٤. Wash five times with PBS containing ٠.٠٥ % Tween.
٥. Prepare h TNF – α standard by reconstituting contents of vial ٤ in ٢٠٠ µl pure water to a concentration of ١٠ µg/ml .The stock solution should be used immediately or stored in aliquots at – ٢٠ °C. for future use . We recommend the aliquots not be refrozen after initial use . For the test , prepare dilution of the stock using the standard range as a guideline .

٦. Add ١٠٠ µl/well of samples or standards diluted in incubation buffer and incubate for ٢ hours at room temperature .
٧. Wash as in step ٤.
٨. Add ١٠٠ µl/well of mAb TNFα-II-biotin at ١ µg/ ml in the incubation buffer . Incubate for ١ hour at room temperature .
٩. Wash as in step ٤ .
١٠. Add ١٠٠ µl/well of streptavidin –ALP diluted ١:١٠٠٠ in the incubation buffer . Incubate for ١ hour at room temperature .

١١. Wash as in step ٤ .

١٢. Add ١٠٠ µl/well of appropriate substrate solution e. g .p-nitrophenyl - phosphate (pNPP).

١٣. Measure the optical density (٤٠٥ nm for pNPP) in an ELISA reader after a suitable developing time.

١٤. The concentrations of TNF-α were calculated by referring to standard curve (fig. ٣.٥). * The same buffer is used for blocking and for dilution.

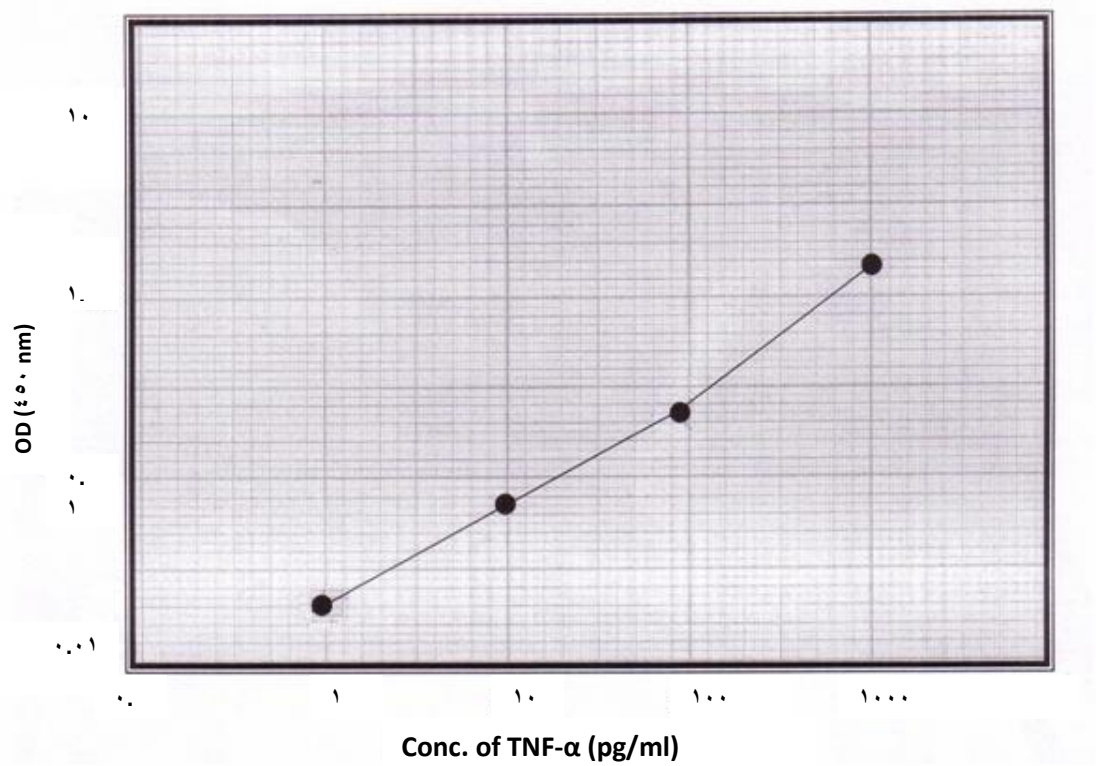


Fig. (٣.٥) : Standard curve for the estimation Tumor Necrosis Factor (TNF-α) .

٣.١٣.٣. Eosinophil Cationic Protein (ECP)

a. Principle :

ECP test is a quantitative assay which measures human ECP specifically with high sensitivity in serum by sandwich ELISA (MBL , Japan) ; this ELISA detects human ECP with a minimum detection limit of ٠.١٢٥ ng/ml.

b. Assay procedure :

STEP ١. (Sample incubation)

(١) Add ١٥٠ µl of prepared samples and standards (do not dilute standard) to ٩٦-well polyvinyl preparation plate as the same order of assay run. Then transfer ١٠٠ µl of each sample to the antibody coated microwell using multichannel pipette and mix well.

(٢) Incubate for ٦٠ minutes at room temperature (٢٠-٢٥ ^{o}C)

STEP ٢. (Washing)

Aspirate or discard the well contents. Fill the well with wash solution and then completely aspirate or discard the contents . Wash the well ٤ times with wash solution using washing bottle .

- Each laboratory is recommended to confirm its own appropriate washing times and set-up.
- Washing buffer should be used at room temperature (٢٠-٢٥ ^{o}C)
- Remove excess wash solution by gentle tapping or aspiration .

STEP ٣. (Conjugate incubation)

(١) Pour conjugate reagent (ready to use) into a reservoir . After removing wash solution completely, pipette ١٠٠ µl of conjugate reagent to each well with multichannel pipette .

(٢) Incubate for ٦٠ minutes at room temperature (٢٠-٢٥ ^{o}C).

STEP ٤. (Washing)

Wash the microplate following the STEP ٢.

STEP ٥. (Substrate incubation)

(١) Pour substrate reagent into the reagent reservoir. After removing wash solution completely, pipette ١٠٠ µl of substrate reagent to catch well with multichannel pipette .

(٢) Incubate for ١٠ minutes at room temperature (٢٠-٢٥ ^{o}C).

STEP ٦. (Stopping reaction)

Pour stop solution into a reservoir . Pipette ١٠٠ µl of stop solution to each well with multichannel pipette.

STEP ٧. (Reading)

Read the absorbance of each well at ٤٥٠ nm . If a dual wavelength plate reader is available , set the test wave length at ٤٥٠ nm and the reference at ٦٢٠ nm .

- Reading should be done within ٣٠ minutes after stopping the reaction .

STEP ٨. (Calculation of results)

Calculate the mean absorbance value of each standard . Plot on the semi-log graph and construct a standard curve [Absorbance on the vertical axis . Concentration (ng/ml) on the horizontal axis]. (Fig. ٣.٦).

Report the ECP concentration of samples of multiplying the value read from the standard curve by dilution.

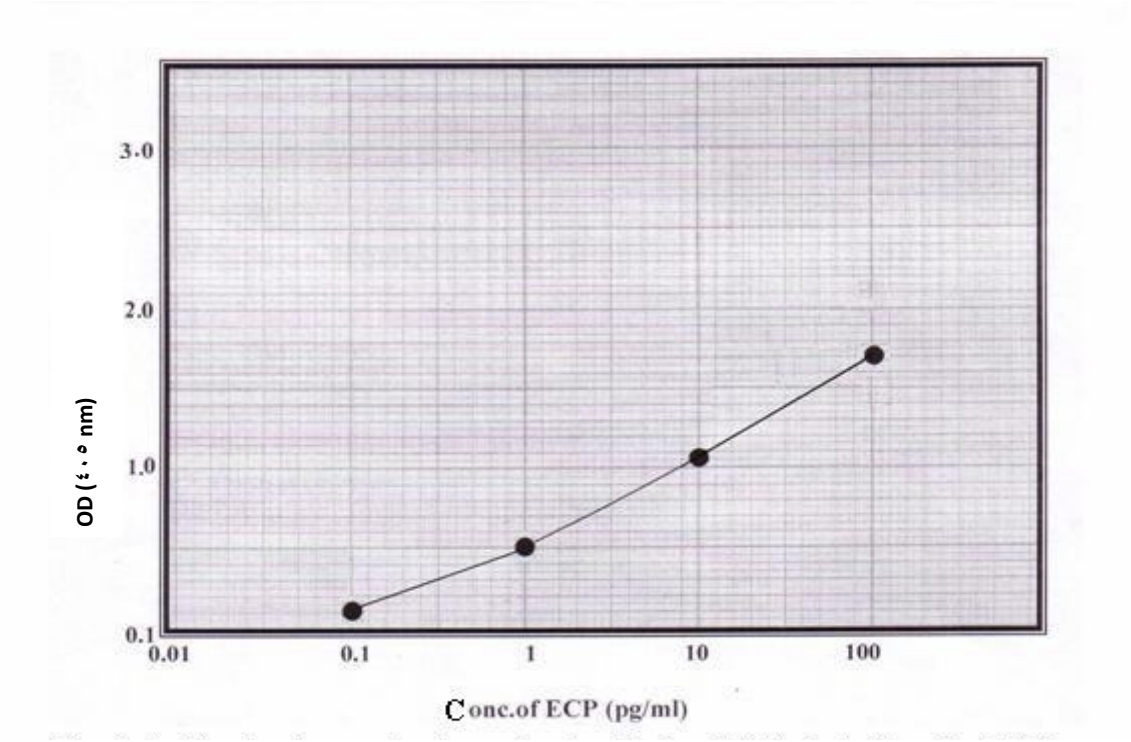


Fig. (٣.٦) : Standard curve for the estimation Eosinophil Cationic Protein (ECP) .

Conc. of E.C.P. (pg / ml)

٣.١٤. Biochemical tests

٣.١٤.١. Superoxide dismutase (SOD) assay

The SOD was determined in the serum of all patients and controlled according to the method of Ying *et al*. (١٩٨٨).

A. Reagents :

- The stock solution consisted of ٢ mg of Cu , Zn SOD (Sigma) dissolved in ٢٥ ml D.D.W.; this solution was refrigerated until used . Before use in the assay , the stored solution was diluted to ٦٠٠ µg/ml with D.D.W.

- Xanthine Oxidase solution (XO) :

This solution was prepared freshly by mixing ٥٠ µl XO (stock solution ١.٨ µg/ml) (Sigma) with ٠.٤٥ ml of PBS .

- Xanthine (٠.٣ mM) :

١.٣ mg Xanthine powder (Sigma) was dissolved in ٥٠ ml D.W.

- EDTA (٠.٠٦ mM) :

٠.٠١١ gm EDTA powder (Fluka) was dissolved in ٥٠ ml D.W.

- NBT (١٥٠ µ mol/l) :

٣.٠٦ mg NBT (BDH) was dissolved in ٢٥ ml D.W.

- Sodium Carbonate (٤M١)(pH ١٠.٢) :

١.٠٦ gm $Na_٢CO_٣$ (Fluka) was dissolved in ٢٥ ml D.W.

- Bovine serum albumin (BSA) (g/l)(Sigma) :

- $CuCl_٢$ (٠.٨ mM).

B. Preparation of the samples :

٠.٣ ml of chloroform and ٠.٥ ml of ethanol were added to the serum samples to remove the hemoglobin . The samples were centrifuged at ٣٠٠ xg for ٦٠ minutes . The supernatant fluid was diluted by a factor of ١٠٠ . ٠.٥ ml from this dilution was used in the assay .

C. The SOD assay :

Each tube contained ١.٢٢٥ ml of the SOD assay reagent and ٠.٥ ml of diluted serum or Cu , Zn SOD standard (١٠-٩٠ ng/ml) were incubated in a water bath at ٢٥ ^{o}C (blank without serum) . Then ٥٠ µl of xanthine oxidase solution was added. The sample was incubated for ٢٠ min. To terminate the reaction, ١ ml of ٠.٨ mM/l $CuCl_{٢}$ solution was added .

The absorbance for each tube was measured at ٥٦٠ nm .

The percent of inhibition was calculated by the equation :

$$\% \text{ Inhibition} = \frac{(A_{blank} - A_{sample})}{} \times ١٠٠ \%$$

Using the standard inhibition curve the SOD concentrations were determined (Fig. ٣.٧).

Log Inhibition

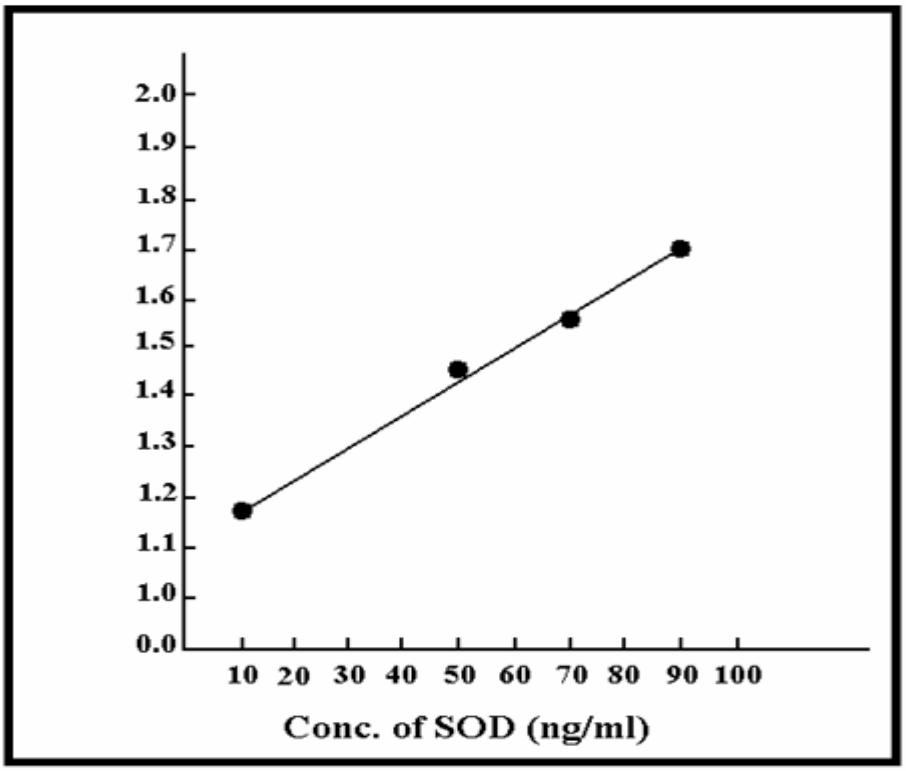


Fig. (3.7): Superoxide dismutase (SOD) standard curve.

٣.١٤.٢. Adenosine deaminase (ADA) activity

The activity was determined in the serum according to the method of Giusti (١٩٨١) . The method was based on measuring the rate of ammonia consumption at ٦٢٠ nm.

a. Reagents :

All reagents were prepared at the time of assay initiation to avoid any loss or gain of ammonia in any of the reagents which may interfere with the results :

- Alkaline hypochlorite solution (١١ mM NaOCl ; ١٢٥ mM NaOH) :
  Sodium hydroxide ١٢٥ ml of ١ N solution and ١٦.٤ ml of sodium hypochlorite were mixed with DDW bringing the final volume to one liter .

- Ammonium sulphate stock solution (١٥ mM):
  ١.٩٨٢ gm of anhydrous ammonium sulphate were dissolved in DDW bringing the final volume to one liter.

- Ammonium sulphate standard solution :
  ٠.٥ ml of ammonium sulphate stock solution was diluted to ١٠٠ ml with phosphate buffer .

- Buffered adenosine solution (٢١ mM adenosine , ٥٠ mM phosphate) :
  ١٥ ml of phosphate buffer was added to ١٤٠ mg of adenosine and warmed in a water bath at ٣٧ ^{o}C for ١٠ minutes then cooled under running water . The pH was adjusted to ٦.٥ with phosphoric acid and the volumes were brought to ٢٥ ml with phosphate buffer .

- Phenol / Nitro prusside solution (١٠٦ mM phenol , ٠.١٧ mM sodium nitro prusside) :

١٠ gm of phenol and ٥٠ mg of sodium nitro prusside were dissolved in DDW bringing the final volume to one liter .

- Phosphate buffer (٥٠mM , pH ٦.٥) :

  ٤.٧٣ gm of hydrated sodium dihydrogen phosphate and ٥.٦٢ gm of hydrated disodium hydrogen phosphate were dissolved in DDW , bringing the final volume to one liter and adjusting the pH to ٦.٥ with phosphoric acid.

- Sodium hydroxide solution (١N) :

  ٤٠ gm of sodium hydroxide were dissolved in DDW bringing the final volume to one liter .

b. Assay procedure :

One adenosine blank, one reagent blank , and one standard were prepared for the whole series. A corresponding number of sample blank (one for each sample, without adenosine) were also prepared according to tab. (٣.١).

Tab. (٣.١) : Experimental design for ADA estimation in patients with VL disease .

Tubes / Reagents	Reagent Blank ml	Standard ml	Adenosine Blank ml	Sample blank ml	Sample ml
Phosphate buffer	١.٠	-	-	١.٠	-
Buffered adenosine solution	-	-	١.٠	-	١.٠
Ammonium sulphate standard solution	-	١.٠	-	-	-
Sample	-	-	-	٠.٠٥	٠.٠٥
DDW	٠.٠٥	٠.٠٥	٠.٠٥	-	-

The contents are mixed in stoppered tubes and incubated for ٦٠ minutes in a water bath , at ٣٧ °C .

Phenol / nitro prusside solution	٣.٠	٣.٠	٣.٠	٣.٠	٣.٠
Alkaline hypochlorite solution	٣.٠	٣.٠	٣.٠	٣.٠	٣.٠

The last two solutions were added in the order given and the contents of the tubes were mixed before pipetting into the next tube.

The tubes were incubated for ٣٠ minutes in a water bath , at ٣٧ °C . The absorbency (E) of the samples was read at ٦٢٨ nm against DDW.

c. Calculations :

The calculations were made according to the following equation :

E sample – E sample blank = A

E adenosine blank – E reagent blank = B

E standard – E reagent blank = C

$$\text{Volume activity} = \frac{(A - B)}{} \times ٥٠ \text{ (u/l) ; } ٣٧ \text{ °C}$$

٣.١٤.٣. Glutathione reductase (GHR) activity

The activity of this enzyme was determined in the serum according to the method of Le Trang *et al*. (١٩٨٣).

$$NADPH\ (NADH) + H^{+} + GSSG \xrightarrow{\text{GHR}} NADP + (NAD^{+}) + ٢GSH$$

a. Reagents :

١. Potassium phosphate buffer (KH_2PO_4) ٠.١ mol / l , pH ٧.٤ (appendix ٥).

٢. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was prepared daily by dissolving ١٦.٦ mg of NADPH in ١٠ ml of ١ % sodium bicarbonate .

٣. Oxidized glutathione (GSSG) was prepared daily by dissolving ٤٦ mg of GSSG in ١٠ ml D.D.W. then one drop of ١ M NaOH to adjust the pH.

٤. Ethylene diamine tetra acetic acid (EDTA) ٨٠ mmol / l was prepared by dissolving ١.٥ gm of dipotassium salt in ٥٠ ml of D.D.W.

b. Procedure :

GHR estimation was prepared according to tab.(٣.٢).

Tab. (٣.٢) : Experimental design for GHR estimation in VL patients .

Test	Control
٢.٠٠ ml buffer	٢.٠٠ ml buffer
٠.٠٥ ml EDTA	٠.٠٥ ml EDTA
٠.١٠ ml serum	٠.١٠ ml D.W.
٠.١٠ ml GSSG	٠.١٠ ml GSSG

Equilibrate tubes and contents at ٣٧ °C for ٨ min. and then add to each ٠.١ ml NADPH . Measure the decrease in absorbance at ٣٤٠ nm . over ١٠ min.

c. Calculation :

The calculations were made according to the following equation:

$$\text{Activity } \mu \text{ mol / ml} = \frac{\Delta A}{} \times \frac{١٠٠٠}{} \times \frac{V_t}{}$$

Δ A = absorbance

T = test

V_t = total volume

$$\Delta A = \frac{A_{T١٠\ min} - A_{T\ ٠\ min}}{}$$

٣.١٤.٤. Alkaline phosphatase (ALP) activity assay

The activity was determined in the serum and the assay was carried out using bioMerieux phosphatase alkaline – kit, according to the following reaction:

$$\text{Phenylphosphate} \xrightarrow{\text{ALP}} \text{Phenol} + \text{Phosphate}$$

The phenol liberated was measured in the presence of amino-٤-antypyrine and potassium ferricyanide. The presence of sodium arsenate in the reagent stopped the enzyme reaction.

a. Reagents

Reagent ١: Substrate buffer, which contains :

- Dinatrium phenylphosphate ٥ mmol/l
- Carbonate – bicarbonate buffer pH١٠ ٥٠ mmol/l

Reagent ٢: Standard solution, which is made of :

- Phenol equal to ٢٠ kind and king Unit.

Reagent ٣: Inhibitor, which is composed of :

- Amino- ٤-antipyrene ٦٠ mmol/l
- Sodium arsenate ٧٥ g / l

Reagent ٤: Color reagent, which is made of :

- Potassium ferricyanide ١٥٠ mmol/l

b. Procedure

Set up the following tubes according to tab. (٣.٣).

Tab. (٣.٣) : Experimental design for ALP estimation in VL patients .

	Tested sample	Serum blank	standard	Reagent blank
Reagent ١	٢ ml	٢ ml	٢ ml	٢ ml
	Incubate for ٥ minutes at ٣٧ °C			
Serum	٥٠ µl	-	-	-
Reagent ٢	-	-	٥٠ µl	-
	Incubate for exactly ١٥ minutes at ٣٧ °C			
Reagent ٣	٠.٥ ml	٠.٥ ml	٠.٥ ml	٠.٥ ml
	Mix well			
Reagent ٤	٠.٥ ml	٠.٥ ml	٠.٥ ml	٠.٥ ml
Tested samples	-	٥٠ µl	-	-
Distilled water	-	-	-	٥٠ µl

- Mix , let stand for ١٠ minutes in the dark .
- Measure at wave length ٥١٠ nm (Hg ٤٩٢[٦٨]); for zero adjustment we used reagent blank.

C. Analysis of samples:

The activity was determined by the following equation:

$$\text{Calculation} = \frac{\text{OD serum sample} - \text{OD serum blank}}{} \times n$$

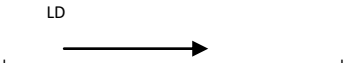
OD : optical density

n: ١٤٢ u/l

٣.١٤.٥. Lactate dehydrogenase (LD) activity assay

The activity was determined in the serum; the assay was carried out using RANDOX kit for colorimetric determination of LD.

This determination was based on the reduction of pyruvate to lactate in the presence of NADH by the action of LD :



The pyruvate that remained unchanged reacted with ٢,٤-dinitrophenylhydrazine to give the corresponding phenylhydrazone which was determined colorimetrically in an alkaline medium.

a. Reagents:

١. Buffer which is made of:

Phosphate buffer (pH ٧.٤٥)	١٠٠ mmol/l
Sodium pyruvate	٠.٨ mmol/l

٢. NADH ١ mg/ml

٣. Color reagent, which is made of:

٢,٤-dinitrophenylhydrazine	١ mmol/l
HCl	١ mmol/l

٤. NaOH ١.٦٪ (٠.٤ N)

The specimen (serum) should be freshly collected and free from hemolysis.

c. Procedure :

Pipette in cuvette according to tab. (٣.٤).

Tab. (٣.٤) : Experimental design for LD estimation in VL patients .

٠.٩ ml	NADH
Place in a water bath at ٣٧ ^{o}C for ٢-٣ min.	
٠.١ ml	Sample
Place in a water bath at ٣٧ ^{o}C for exactly ٣٠ min.	
١.٠ ml	Colour reagent
Mix , allow to stand at room temp . for ٢٠ min.	
١٠ ml	Sodium hydroxide

-Mix , allow standard for ١٠ min.

-Read absorbance of samples against distilled water at wave length ٥٢٠ nm.

-Enzyme activity was determined by reference to the calibration curve, the corresponding values in U/L.

d. Calibration curve :

Absorbance	٠.٥٠	٠.٤٢	٠.٣٣	٠.٢٨	٠.١٩	٠.١١
U/L	٠	١١١.٤	٢٢٣.١	٣٣٤.٩	٣٩٠.٦	٤٤٦.٣

Calculation of values was done by interpolation with calibration curve Fig. (٣.٨).

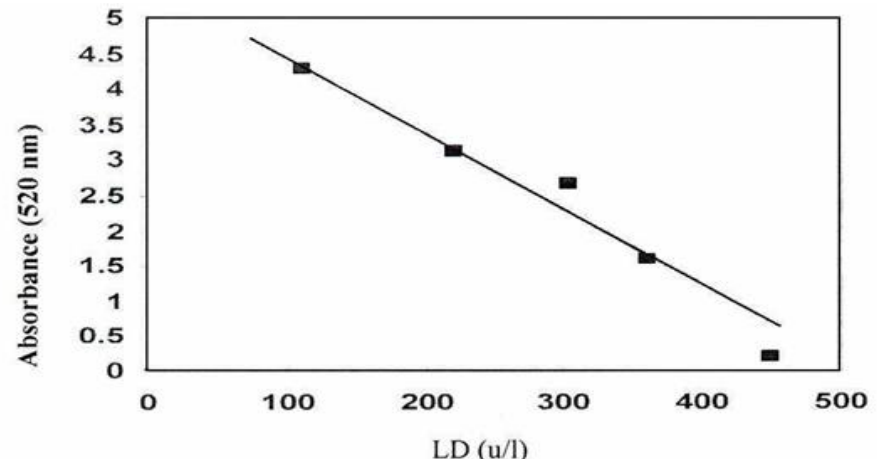


Fig. (٣.٨) : Calibration curve for lactate dehydrogenase enzyme.

٣.١٤.٦. Alanine aminotransferase (ALT) activity assay

The activity was determined in the serum ; the assay was carried out using RANDOX kit for colorimetric determination of ALT (Reitman *et al.*, ١٩٥٧).

Principle :

$$\alpha - \text{Oxoglutarate} + \text{L-alanine} \xrightarrow{\text{ALT}} \text{L-glutamate} + \text{Pyruvate}$$

Alanine aminotransferase (ALT) was measured by monitoring the concentration of pyruvate hydrazone formed with ٢,٤-dinitrophenyl-hydrazine.

Sample: serum

a. Reagents :

- Buffer which is made of :

  Phosphate buffer (pH ٧.٤) ١٠٠ mmol/l

  L-alanine ٢٠٠ mmol/l

  α- oxoglutarate ٢.٠ mmol/l

- ٢,٤-dinitrophenylhydrazine ٢.٠ mmol/l
- Sodium hydroxide ٤.٠ mmol/l
- Pyruvate standard ٢.٠ mmol/l

b. Procedure :

١. Measurement against reagent blank .

Pipette into test tubes :

	Reagent blank	Sample
Sample	……..	٠.١ ml
Buffer	٠.٥ ml	٠.٥ ml
Distilled water	٠.١ ml	……..
Mix , incubate for exactly ٣٠ min. at ٣٧ ^{o}C		
٢,٤ – DNP	٠.٥ ml	٠.٥ ml
Mix, allow to stand for exactly ٢٠ min at ٢٠ to ٢٥ ^{o}C.		
Sodium Hydroxide	٥.٠ ml	٥.٠ ml

Mix, read the absorbance of sample (A $_{sample}$) against the reagent blank after ٥ minutes at wave length ٥٤٠ nm.

٢. Measurement against sample blank

Pipette into test tubes:

	Sample blank	Sample
Sample	……..	٠.١ ml
Buffer	٠.٥ ml	٠.٥ ml
Mix , incubate for exactly ٣٠ min. at ٣٧ ^{o}C		
٢,٤ – DNP	٠.٥ ml	٠.٥ ml
Sample	٠.١ ml	……
Mix, allow to stand for exactly ٢٠ min. at ٢٠ - ٢٥ ^{o}C.		
Sodium hydroxide	٥.٠ ml	٥.٠ ml

Mix, read the absorbance of sample (A $_{sample}$) against the sample blank after ٥ minutes at wave length ٥٤٠ nm.

c. Calculation :

Obtain the activity of ALT in the serum from the table :

Absorbance	**U/L**	**Absorbance**	**U/L**
٠.٠٢٥	٤	٠.٢٧٥	٤٨
٠.٠٥٠	٨	٠.٣٠٠	٥٢
٠.٠٧٥	١٢	٠.٣٢٥	٥٧
٠.١٠٠	١٧	٠.٣٥٠	٦٢
٠.١٢٥	٢١	٠.٣٧٥	٦٧
٠.١٥٠	٢٥	٠.٤٠٠	٧٢
٠.١٧٥	٢٩	٠.٤٢٥	٧٧
٠.٢٠٠	٣٤	٠.٤٥٠	٨٣
٠.٢٢٥	٣٩	٠.٤٧٥	٨٨
٠.٢٥٠	٤٣	٠.٥٠٠	٩٤

٣.١٤.٧. Aspartate aminotransferase (AST) activity assay

The activity was determined in the serum ; the assay was carried out using RANDOX kit for colorimetric determination of AST (Reitman *et al.*, ١٩٥٧).

Principle

$$\alpha - \text{oxoglutarate} + \text{L} - \text{aspartate} \xrightarrow{\text{AST}} \text{L} - \text{glutamate} + \text{Oxaloacetate}$$

Aspartate aminotransferase was measured by monitoring the concentration of oxaloacetate hydrazone formed with ٢, ٤ – dinitrophenyl – hydrazine .

Sample : Serum

a. Reagents :

- AST Buffer :

  Phosphate buffer (pH ٧.٤) ١٠٠ mmol / l

  L – aspartate ١٠٠ mmol / l

  α – Oxoglutarate ٢.٠ mmol / l

- ٢,٤ – dinitrophenylhydrazine ٢.٠ mmol / l
- Sodium hydroxide
- Pyruvate Standard :

  Pyruvate ٢.٠ mmol / l

b. Procedure :

١. Measurement against reagent blank

Pipette into test tubes :

	Reagent blank	Sample
Sample	……..	٠.١ ml
ALT buffer	٠.٥ ml	٠.٥ ml
Distilled water	٠.١ ml	……..
Mix , incubate for exactly ٣٠ min. at ٣٧ ^{o}C		
٢,٤ - dinitrophenylhydrazine	٠.٥ ml	٠.٥ ml
Mix , allow to stand for exactly ٢٠ min. at ٢٠ – ٢٥ ^{o}C		
Sodium hydroxide	٥.٠ ml	٥.٠ ml
Mix , read the absorbance of sample (A_{sample}) against the reagent blank after ٥ minutes at wave length ٥٤٠ nm .		

٢. Measurements against sample blank pipette into test tubes :

	Sample blank	Sample
Sample	……	٠.١ ml
AST buffer	٠.٥ ml	٠.٥ ml
Mix , incubate for exactly ٣٠ min. at ٣٧ ^{o}C		
٢,٤ – dinitrophenylhydrazine	٠.٥ ml	٠.٥ ml
Sample	٠.١ ml	……
Mix , allow to stand for exactly ٢٠ min. at ٢٠ to ٢٥ ^{o}C		
Sodium hydroxide	٥.٠ ml	٥.٠ ml
Mix , read the absorbance of sample (A_{sample}) against the sample blank after ٥ minutes at wave length ٥٤٠ nm .		

c. Calculation :

Obtain the activity of AST in the serum from the table :

Absorbance	U/L	Absorbance	U/L
٠.٠٢٠	٧	٠.١٠٠	٣٦
٠.٠٣٠	١٠	٠.١١٠	٤١
٠.٠٤٠	١٣	٠.١٢٠	٤٧
٠.٠٥٠	١٦	٠.١٣٠	٥٢
٠.٠٦٠	١٩	٠.١٤٠	٥٩
٠.٠٧٠	٢٣	٠.١٥٠	٦٧
٠.٠٨٠	٢٧	٠.١٦٠	٧٨
٠.٠٩٠	٣١	٠.١٧٠	٨٩

٣.١٥. Polyacrylamide gel electrophoresis for isoenzymes

a. Equipments :

The LKB multiphore system with LKB ٢١٩٧ power supply was used (Fig.٣.٩) .

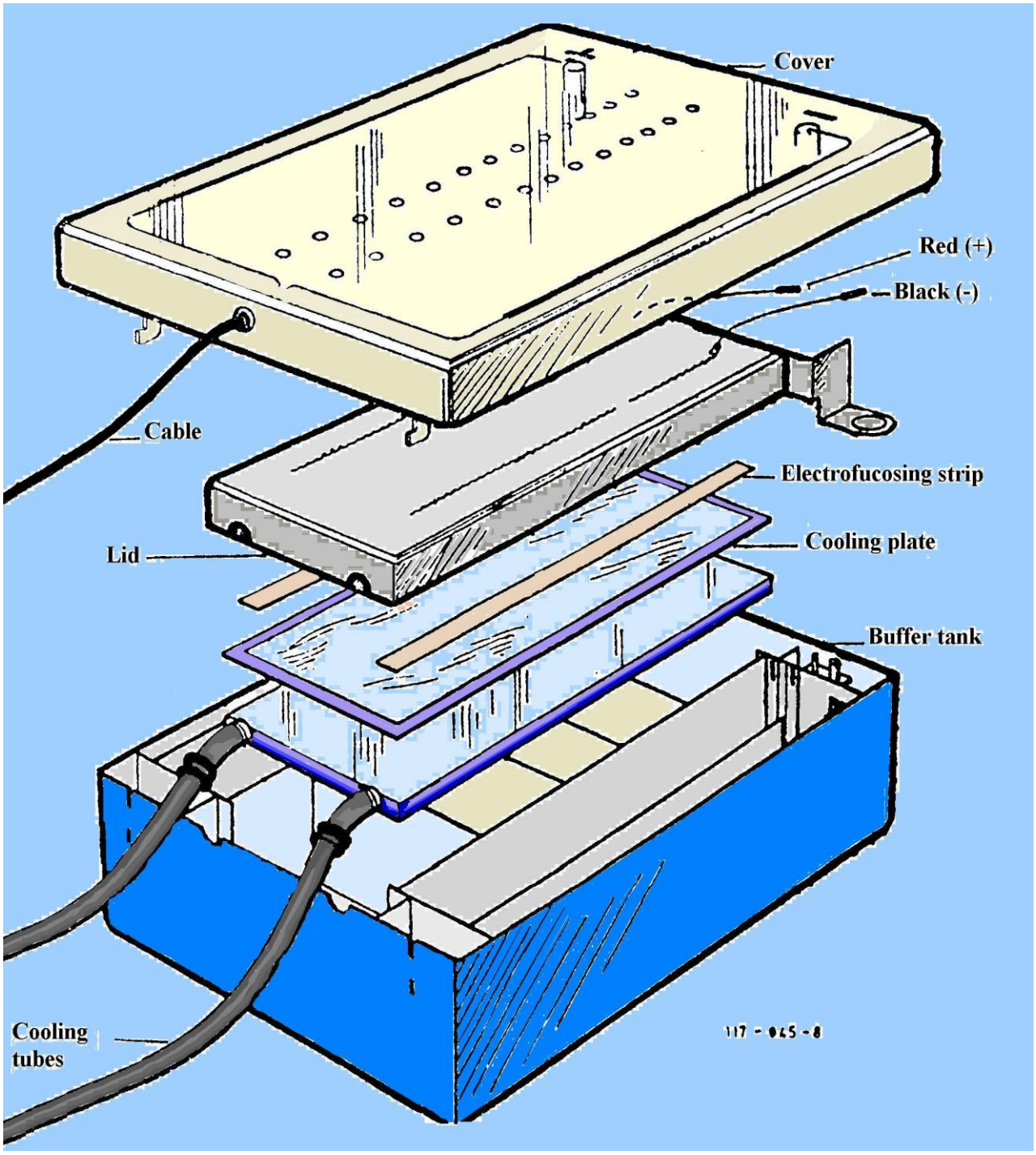


Fig. (٣.٩): Diagramatic representation of LKB ٢١١٧ Multiphor Instrument used for SDS & Conventional Polyacrylamide Gel Electrophoresis.

b. Solutions :

١. Tris – glycine buffer stock (٠.٢ M) pH ٨.٩.

Each liter contained :

١٥.٠٢ gm glycine

٠.٥ gm sodium azide

Titrate with tris to pH ٨.٩

٢. Electrode buffer :

١ part buffer solution plus ١ part distilled water .

٣. Ammonium persulphate solution (٠.٠٧ M) :

١.٥ gm ammonium persulphate was dissolved in ١٠ ml D.W. and kept in dark bottle at ٤ ^{o}C . This solution was prepared immediately.

٤. Acrylamide solution :

٢٢.٢ gm acrylamide together with ٠.٦ gm Bis were dissolved into ١٠٠ ml D.W. The solution was filtered through Whatman No. ١ filter paper .

٥. Bromophenol blue solution ٠.٢٥ % ($^{w}/_{v}$) :

٠.٠٢٥ gm bromophenol blue was dissolved in ١٠ ml D.W.

c. Preparation of the gel :

The gel solution was made by mixing the following :

Distilled water	٧.٥ ml
Tris – glycine buffer stock	٣٣.٣ ml
Acrylamide solution	٢٢.٢ ml
Ammonium per sulphate	٣.٢ ml
TEMED	٠.١ ml

Final volume	٦٦.٠ ml

The solution must be immediately poured into the moduling set with the help of plastic funnel . The gel was left for ٤٥ minutes . After that the gel was kept at ٤ ^{o}C for ٢٤ hrs. (LKB Application Note ٣٠٦ , ١٩٧٧).

d. Electrophoresis :

١. The gel plate was placed on a cooling plate , ٨ – ١٠ layers of electrode wicks (Whatman No. ١ filter papers) were soaked with the tris – glycine buffer . One edge in the buffer tank .

٢. Pre-electrophoresis should be preformed with a pre set constant current (٥٠ mA) for ٣٠ minutes.

٣. ١٠ µl of tested samples and ١ µl bromophenol were placed in each slot of the gel.

٤. Electrophoresis have started by switching on the power supply immediately after application of the samples in the slot with current of ٢٠ mA for ١٠ minutes , then adjusted to ٥٠ mA , stopping electrophoresis when the bromophenol blue band was reached to the end of the gel , then the position of bands were detected by using the proper stain .

٣.١٥.١. Isoenzymes of Alkaline phosphatase (ALP)

ALP isoenzymes were developed after electrophoresis as described by Rassam *et al*. (١٩٩٦).

* Solutions for developing ALP isoenzymes :

٠.٠١	Nitroblue trizolium (NBT)
gm	٥-bromo-٤-chloro-٣-indolyl phosphate
٠.٠٠٥	$MgCl_2$
gm	Tris glycine buffer
٠.٠٧	
gm	
٢٠.٠	
ml	

Kept in ٤٠ ^{o}C till bands appeared.

٣.١٥.٢. Isoenzymes of lactate dehydrogenase (LD)

* Solutions for developing LD isoenzymes :

١٠	ml	Tris glycine buffer
٥	ml	Sodium lactate
٣	ml	PMS
٣	ml	NBT
٠.٠٥	gm	NAD

Kept in ٤٠ ^{o}C till bands appeared.

٣.١٦. Determination of trace elements

Atomic absorption spectrophotometer (Shimadzu A.A , ٦٤٦) was used for determination of Copper (Cu) , Zinc (Zn) and Magnesium (Mg) in serum.

Assay :

٠.١ ml of serum dilution to total volume of ١ ml using ٦٪ n-butanol solution with a Copper and Zinc hollow cathode lamps were used at wave length of ٣٢٤.٧٥ nm. respectively. While the assay for magnesium estimation was carried out by adding ٤.٩ ml of (١٪ lanthanum chloride) solution to ٠.١ ml of serum. These solutions were aspirated directly into air-acetylene flame and the magnesium hallow cathode was used at wave length ٢٨٥.٣ nm.

٣.١٧. Statistical analysis :

The data were analyzed using the available soft-ware package. The results were presented as number, percentage, and mean ± SD whenever possible. The data were analyzed by using analysis of variance (ANOVA) test taking $p<٠.٠٥$ as the lowest limit significance. These manipulations were carried out according to Statistical Analysis System (SAS, ٢٠٠١).

* * * * * * * * * * * * * *

Chapter Four

Results

Results

٤.١. Serological finding by ELISA assay

Serum antibodies (IgG & IgM) in visceral leishmaniasis patients were tested by using ELISA technique which measures the optical density (OD) of patients sera, the higher OD, the higher level of anti-leishmanial immunoglobulins. By using promastigote antigen, ٤٨ patients (٩٢.٣٪) were positive with a titer range from ١/٨ to ١/٥١٢ for antibodies and ٤ patients (٧.٦٩ %) were sero-negative depending on ELISA cut off value (٠.٧٥), so any OD value above the cut off point was considered as positive (tab. ٤.١).

٤.٢. Serological finding by DIBA assay

Another technique was used to measure serum antibodies in visceral leishmaniasis. Using promastigote antigen, ٤٨ patients (٩٢.٣٪) were positive for leishmanial antibodies with a titer range from ١/٣٢ to ١/٢٠٤٨ and ٤ patients (٧.٦٩٪) were sero-negative depending on the results obtained were read by naked eye, the dilution that gave blue color was considered positive and with no color considered negative (fig. ٤.١). In order to know exactly which type of technique shows the highest sero-positive and more sensitivity and specificity results, LSD test was used

which has revealed that there is no significant differences between ELISA and DIBA technique (tab. ٤.١) .

٤.٣. Results of DIBA and ELISA assay with heterologus antisera against *L. donovani* antigen

Twenty children sera which were from other clinical infection other than kala - azar who were confirmed clinically, and ten sera of healthy children . All sera were subjected to test by Dot – blot immunobinding assay. Cross – reaction with *L. donovani* antigen was observed only in two patients, one with tuberculosis and the other with typhoid fever and negative results with other tested diseases. Sera from healthy children gave negative results for *L. donovani* assay (tab. ٤.٢). The same subject to commercial ELISA test was applied. Cross – reaction was observed in two patients one with tuberculosis and other with toxoplasmosis.

Tab. (٤.١): Comparison between two results obtained by DIBA and ELISA assay for serum sample from ٥٢ patients with visceral leishmaniasis.

Titer / Type of assay	No. of samples with indicator reciprocal titer					No. of positive sample	Sensitivity %	Specificity %	P-value
	٨	٣٢	١٢٨	٥١٢	٢٠٤٨				
DIBA	٠	١٠	١٢	١٣	١٣	٤٨	٩٦.٥	٩٢	≥ ٠.٠٥
ELISA	٨	١٤	١٤	١٢	٠	٤٨	٩٧	٩٩	≥ ٠.٠٥

Tab. (٤.٢): Results of Dot-blot immunobinding assay with homologous and heterologous antisera against *leishmania donovani* antigen.

Disease	No. of sera examined	Positive results
Brucellosis	٥	٠
Tuberculosis	٥	١
Typhoid fever	٥	١
Toxoplasmosis	٥	٠
Sera from healthy children	١٠	٠
Total	٣٠	٢

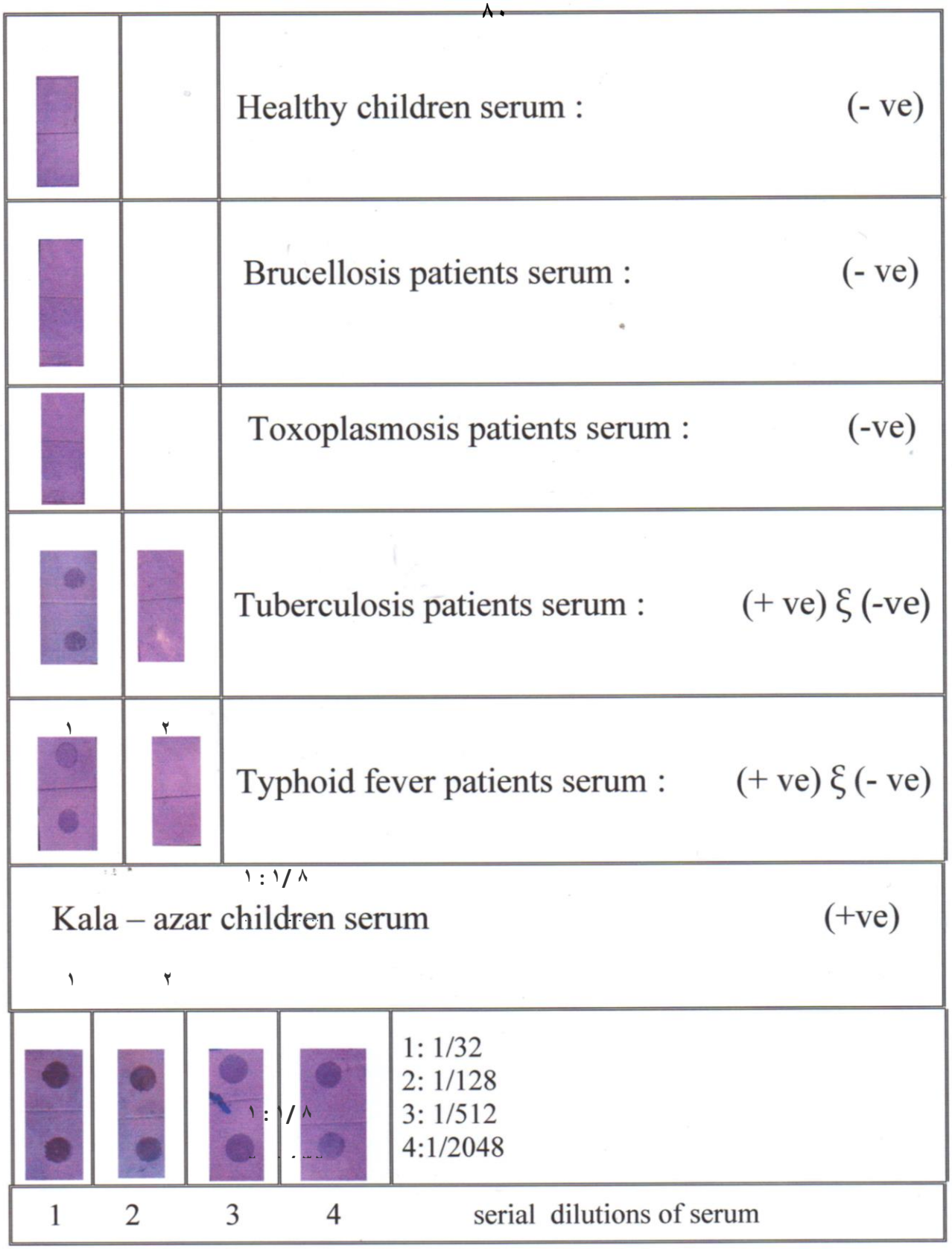


Fig .( ) : Dot – blot immunobindinding assay with homologous and heterologous antisera against leishmania donovani .

Fig.(٤.١): Dot-blot immunobinding assay with homologous & heterologous antisera against *Leishmania donovani*.

٤.٤. Immunoglobulin levels

٤.٤.١. Serum level of IgG

The mean of IgG level in patients with VL cases (١٧٧٧.٨ ± ٤٧٦.١ mg/dl) was significantly higher than mean value of healthy control (٨٢٥.٠ ± ١٨٣.٦ mg/dl) and significantly important (p < ٠.٠٥) . The mean levels after treatment with sodium stibogluconate therapy (٨٥٠.٨ ± ٩٠.١ mg/dl) which though reduced, but still slightly higher than that in healthy control group (p ≥ ٠.٠٥) , (tab. ٤.٣ and ٤.٤).

٤.٤.٢. Serum level of IgM

The mean level of IgM was (١٧٤.٣ ± ٢٥.٤ mg/dl) higher in patients with VL than control group (١٢٨.٠ ± ٢٤.٥ mg/dl) with a significant difference (p < ٠.٠٥). After treatment the mean level of IgM (١٣٠.٦ ± ١٧.٥ mg/dl) was significant between pretreated and post treated group (p < ٠.٠٥). There were no significant differences between post treated and control group (p > ٠.٠٥) , (tab. ٤.٣ and ٤.٤).

٤.٤.٣. Serum level of IgA

The mean value of IgA (١٠٥.٥ ± ٢٨.٠ mg/dl) did not show any significant differences statistically from healthy control (١١٠.٥±٤٠.٣ mg/dl) (p > ٠.٠٥) and there

are no significant differences between post treated and control groups (p> ٠.٠٥), (tab.٤.٣ and ٤.٤).

٤.٥. Complement component levels

٤.٥.١. Serum level of C٣ complement

The mean value of C٣ complement was (١٧٢.٤ ± ٢٠.١ mg/dl) higher in patients with VL than control group (١٢٥.٢ ± ٢٣.٧ mg/dl) (p < ٠.٠٥) and there are no significant differences between post treated and control groups (p > ٠.٠٥) , (tab. ٤.٣ and ٤.٤).

٤.٥.٢. Serum level of C٤ complement

The mean value of C٤ complement was (٤٨.٤ ± ١٢.٧ mg/dl) higher in patients with VL than control group (٢٧.٢ ± ٤.٨ mg/dl) (p < ٠.٠٥) and there are no significant differences between post treated and control groups (p > ٠.٠٥) , (tab. ٤.٣ and ٤.٤).

Tab. (٤.٣): Mean concentration ± SE (mg/dl) of immunoglobulins (Igs) and complements (Cs)in the sera of ٥٢ patients infected with visceral leishmaniasis & healthy control.

Immunoglobulins & Complements	Control	Patient	P- value
IgG	٨٢٥.٠ ± ١٨٣.٦	١٧٧٧.٨ ± ٤٧٦.١	< ٠.٠٥
IgM	١٢٨.٠ ± ٢٤.٥	١٧٤.٣ ± ٢٥.٤	< ٠.٠٥
IgA	١١٠.٥ ± ٤٠.٣	١٠٥.٥ ± ٢٨.٠	> ٠.٠٥
$C_٣$	١٢٥.٢ ± ٢٣.٧	١٧٢.٤ ± ٢٠.١	< ٠.٠٥
$C_٤$	٢٧.٢ ± ٤.٨	٤٨.٤ ± ١٢.٧	< ٠.٠٥

Tab.(٤.٤): Mean concentration ± SE (mg/dl) of immunoglobulins (Igs) and complements (Cs) in the sera of ٥٢ patients infected with visceral leishmaniasis in relation to the period of therapy.

Immunoglobulins & complements	Days of therapy			
	٠	٧	١٤	٢١
IgG	١٨٢٠.٥ ± ٥٣.٦	١٦٤٠.٣ ± ٦٠.٢	١١٢٠.٤ ± ٤٨.٣	٨٥٠.٨ ± ٩٠.١
IgM	١٧٦.٨ ± ١٥.٩	١٥٠.٧ ± ١٨.١	١٣٩.٦ ± ١٣.٨	١٣٠.٠ ± ١٧.٥
IgA	١٠٥.٤ ± ٢٠.٧	٩٥.٨ ± ٢٢.٦	٩٨.٣ ± ١٣.٤	٩٨.٢ ± ١٨.٩
$C_٣$	١٧٢.١ ± ١٠.٣	١٥٠.٠ ± ١٣.٨	١٣٠.٤ ± ١٢.٨	١٢٠.٥ ± ٩.٥
$C_٤$	٥٢.٣ ± ٦.٨	٤٦.٣ ± ٨.٣	٣٠.٦ ± ٩.٤	٢٥.٠ ± ٣.٢

٤.٦. SDS – PAGE and Western blot (WB)

The whole protein profile and molecular weight of different leishmanial antigens were obtained in SDS-PAGE (fig. ٤.٢). To facilitate the calculation of molecular weight for these subunits, zymograph for different bands were done as shown in fig.(٤.٣). Molecular weight of each band was arranged according to their antigenic groups:

- Lane (١) : ١٣, ١٨, ٢٨, ٣٠, ٤٥, ٥٣, ٦٦ and ٧٤ k Da.
- Lane (٢) : ١٨, ٣٠, ٤٠, ٤٥ and ٥٣ k Da.
- Lane (٣) : ١٨ and ٢٥ k Da.
- Lane (٤) : ١٨ k Da.
- Lane (٥) : ١٨ ,٢٠ ,٢٨ ,٣٦ ,٤٥ and ٥٣ k Da.

The serum IgG recognition of the different antigens in WB was based on the banding patterns that appeared on nitrocellulose paper strips (fig. ٤.٤). The results showed that sera from patients with active VL recognize numerous antigens with a molecular weight ranges from ١٢ k Da to more than ٧٠ k Da ; these include ٧٤ ,٦٦ ,٥٣ ,٤٥ ,٤٠ ,٣٦ ,٣٠ ,٢٨ ,٢٥ ,٢٠ ,١٨ and ١٣ k Da. Many bands were recognized in practically all active cases of disease, those include ١٨,٤٥ and ٥٣ k Da. Other bands were recognized in various percentages of cases including: ٧٤ (٩٥٪), ٦٦ (٨٦٪), ٤٠ (٩٢٪), ٣٦ (٨٩٪) , ٣٠ (٩٢٪), ٢٨ (٨٥٪), ٢٥ (٨٢٪), ٢٠ (٩٣٪) and ١٣ (٩٦٪) k Da, so we listed the molecular weights of different subunits of VL that were recognized by IgG antibodies according to antigenic groups (fig. ٤.٤).

- Freezing and thawing of VL and CL cocktail subunits at ١٣, ١٨, ٢٨, ٣٠, ٤٥, ٥٣, ٦٦ and ٧٤ k Da (strips number ٢, ٣ and ٤).
- Autoclaved CL subunits at ١٨, ٣٠, ٤٠, ٤٥ and ٥٣ k Da (strips number ٥, ٦ and ٧).
- Autoclaved VL subunits ١٨ and ٢٥ k Da (strips ٨, ٩ and ١٠).

- Freezing and thawing VL subunits at ١٨ k Da (strips ١١, ١٢ and ١٣).
- Autoclaved cocktail of VL and CL subunits at ١٨, ٢٠, ٢٨, ٣٦, ٤٥ and ٥٣ k Da (strips ١٤,١٥, and ١٦).
- Control strips (١٧, ١٨ and ١٩): the results did not show any banding pattern appeared on nitrocellulose paper strips, that the sera which were applied in WB obtained from healthy children were confirmed by physical examination.

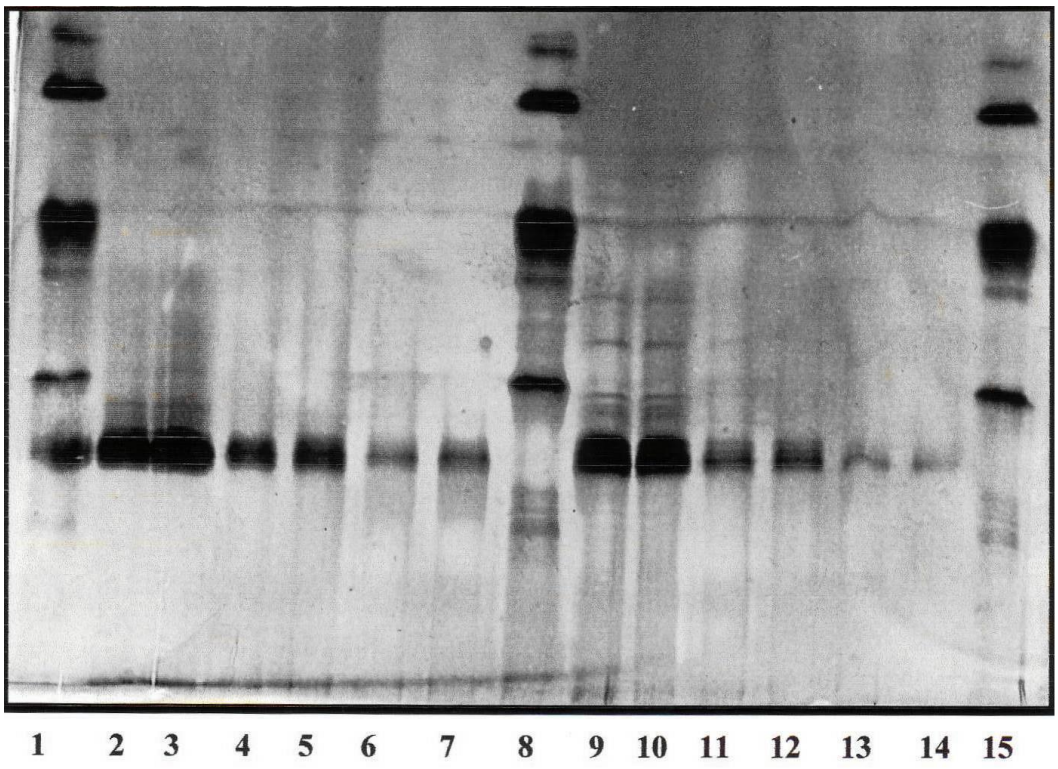


Fig. (٤.٢): Electrophoretic photograph (Conventional Technique) of leishmanial promastigote.

Lane (١,٨ & ١٥): Autoclaved cocktail of vis. & cut. leishmanial antigens.

Lane (٢ & ٣): Autoclaved vis . leishmanial antigens.

Lane (٤,٥,١١ & ١٢): Freezing and thawing vis . leishmanial antigens.

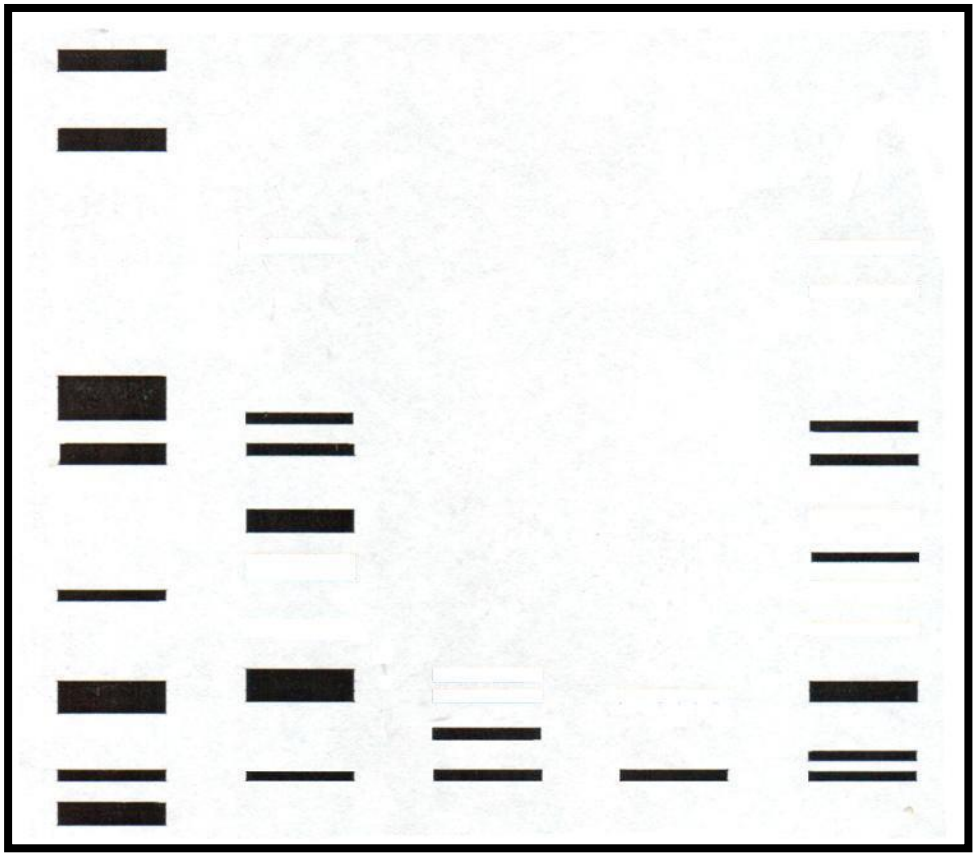


Fig. (٤.٣) : Zymograph of different bands of leishmanial promastigote antigens obtained by conventional technique .

Lane (١): Autoclaved cocktail of vis. & cut. leishmanial antigens .

Lane (٢): Autoclaved vis . leishmanial antigens.

Lane (٣): Freezing and thawing vis . leishmanial antigens.

Fig. (٤.٤) : Description of serum IgG

Lane (١) strip: St

Lane (٢,٣, & ٤) s

Lane (٥,٦, & ٧) s

Lane (٨,٩, & ١٠

٤.٧. Lymphocyte subsets (CD-marker)

Phenotyping of peripheral blood lymphocyte (PBL) subsets of healthy control had been done by immunofluorscence (IFAT) and immunoperoxidase (IP) methods (fig ٤.٥ and ٤.٦). The comparison between the two methods revealed that the number of CD٣,CD٤,CD٨ and CD٢٢ cells counted by IFAT were higher than those found in IP method with significant difference (p<٠.٠٥) , (tab.٤.٥).

٤.٧. ١. Total T-lymphocytes (CD٣)

Visceral leishmaniasis children had shown that CD٣ cells percentage (٤٦.٠ ± ٢.٠٪) which was significantly lower (p<٠.٠٥) than the control group (٧٠.٥ ± ٣.٥٪) (tab.٤.٦). In the post treated group CD٣ cells percentage was (٦٣.٢ ± ١.٣٪) significantly higher than that in the pretreated group (p<٠.٠٥). There was a significant difference between post treated and control group (p<٠.٠٥) (tab. ٤.٦). But, there was no significant difference (p>٠.٠٥) in CD٣ cells percentage between VL patients in relation to some clinical parameters such as sex and age (tab.٤.٧).

٤.٧. ٢. T- helper/ inducer lymphocytes (CD٤)

The percentage of CD٤ cells in VL patients (٤٥.٠ ± ١.٣٪) was significantly lower (p<٠.٠٥) than that of the control group (٥٢.٦ ± ١.٤٪)(tab.٤.٦). CD٤ cells in the post treated VL group was (٤٩.٠ ± ٠.٣٪) significantly higher in comparison with pretreated group (p<٠.٠٥). There was a significant difference between post treated and control group (p<٠.٠٥)(tab.٤.٦). However, there were no significant differences (P>٠.٠٥) in CD٤ cells percentage between VL patients in relation to sex and age (tab.٤.٧).

٤.٧. ٣. T- Cytotoxic/supressor lymphocytes (CD٨)

The percentage of CD٨ cells (٦١.٠ ± ١.٠) of VL patients was significantly higher (p<٠.٠٥) than that of the control group (٤٠.٣ ± ٢.٠ %) (tab. ٤.٦). In the post treated VL group of CD٨ cells, there was (٤٧.٣ ± ٠.٣٪) significant difference in comparison with pretreated group (p<٠.٠٥). There was a significant difference between post treated and control group (p<٠.٠٥) (tab.٤.٦), but there was no significant difference in (p>٠.٠٥) in CD٨ cells percentage between VL patients in relation to sex and age (tab.٤.٧).

٤.٧. ٤. CD٤/CD٨ ratio

The CD٤/CD٨ ratio was of special importance because it represents an index that refers to the immunological balance between T-helper cells and T-cytotoxic cells in the immune system, in the higher CD٤/CD٨ ratio the nearer balance point would be to T-helper cells, which means lower cytotoxic activity and higher other form of CMI and humoral immunity. CD٤/CD٨ ratio was lower in VL patients (٠.٧٥) than that of the control group (١.٣٠) while in the post treated group the ratio was (١.٠٣) higher than that in pretreated group and lower than that in control (tab.٤.٦).

٤.٧. ٥. B-lymphocytes (CD٢٢)

There was a relative increase in CD٢٢ cells percentage of VL patients (١٣.٤ ± ٠.٥٪) in comparison to CD٢٢ cells percentage of the control group (١٠.٢ ± ٣.٦٪) but, this increase was not significant (p>٠.٠٥) (tab.٤.٦). In the post treated VL group

CD٢٢ cells were (١١.٢ ± ١.٣٪) lower in comparison with pretreated group (p>٠.٠٥). There was a relative increase in post treated in comparison with control group (tab.٤.٦).

٤.٨. Cytokine levels

The serum levels (pg/ml) of IFN-γ, IL-١٠ and TNF-α in ١٥ patients with visceral leishmaniasis before, during and after therapy were recorded (fig.٤.٧, ٤.٨ and ٤.٩).

٤.٨.١. Human interferon – gamma (IFN – γ) level

A highly significant increase (p<٠.٠٥) of serum IFN-γ was recorded in patients with VL (١١٢.١ ± ٤.٦ pg/ml) in comparison to that of control group (٣.٢٢ ± ١.١ pg/ml)(tab.٤.٨). There was a significant decrease in serum levels of IFN-γ in post treated group (٥.٠٢ ± ٢.٠ pg/ml), which were significantly lower than that in pretreated group (tab.٤.١٠). There was no significant difference between post treated and control group (p>٠.٠٥).

Tab. (٤.٥): Mean percentage ± SE of lymphocyte subsets for healthy patients

Group / Assay	CD٣	CD٤	CD٨	CD٢٢	CD٤/CD٨
IFAT	٧٠.٥ ± ٣.٥	٥٢.٦ ± ١.٤	٤٠.٣ ± ٢.٠	١٠.٢ ± ٣.٦	١.٣٠
IP	٦٨.٦ ± ٢.٤	٥٠.٤ ± ٠.٦	٣٧.٠ ± ١.٨	٨.٣ ± ١.١	١.٤٢

counted by Immunoflurescence & Immunoperoxidase technique .

Tab. (٤.٦): Mean percentage ± SE of lymphocyte subsets in blood of ٥٢ visceral leishmaniasis patients and healthy control.

CD% / Group	$CD_{٣}$	$CD_{٤}$	$CD_{٨}$	$CD_{٢٢}$	$CD_{٤}/CD_{٨}$ Ratio
Control	٧٠.٥ ± ٣.٥	٥٢.٦ ± ١.٤	٤٠.٣ ± ٢.٠	١٠.٢ ± ٣.٦	١.٣٠
Patients	٤٦.٠ ± ٢.٠	٤٥.٠ ± ١.٣	٦١.٠ ± ١.٠	١٣.٤ ± ٠.٥	٠.٧٥
Post therapy	٦٣.٢ ± ١.٣	٤٩.٠ ± ٠.٣	٤٧.٣ ± ٠.٣	١١.٢ ± ١.٣	١.٠٣

Tab. (٤.٧): Mean percentage ± SE of lymphocyte subsets in blood of ٥٢ visceral leishmaniasis patients in relation to sex and age.

CD-marker typing %	Sex		P-value	Age (year)			P-value
	Males	Females		< Year	Year	>Year	
CD_3	٤٨.٨٣ ± ٢.٤٠	٤٦.٧٣ ± ٢.٤٥	> ٠.٠٥	٤٧.٢٤ ± ٣.٢٨	٤٦.٢٨ ± ٢.٣٦	٤٦.١٢ ± ٢.٨٣	> ٠.٠٥
CD_4	٥٤.٨٠ ± ٢.٣٠	٥٢.١٨ ± ٢.٦٣	> ٠.٠٥	٥٤.٢٧ ± ٢.٣١	٥٥.٣١ ± ٢.٣٥	٥٦.٠ ± ١.٨٨	> ٠.٠٥
CD_8	٦٣.٢٤ ± ٠.٨٢	٦٤.٧٦ ± ٣.٣٣	> ٠.٠٥	٦٥.٥ ± ٢.٦٤	٦١.٨٤ ± ٠.٨٨	٦٠.٦٣ ± ١.٢٣	> ٠.٠٥
CD_{22}	١٣.٥٢ ± ٠.٩٤	١٢.٩٧ ± ٠.٧٠	> ٠.٠٥	١٢.٩ ± ٥.٧٨	١٤.٤ ± ١.٦٢	١٣.٩٨ ± ٣.٥٢	> ٠.٠٥

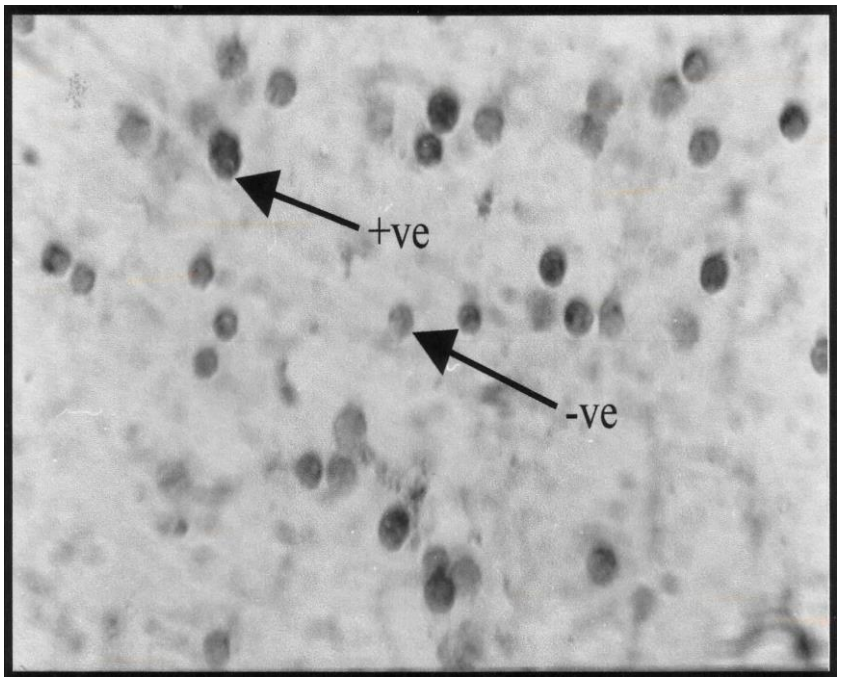


Fig. (٤.٥): Peripheral blood lymphocytes stained with CD markers Immunoperoxidase by DAB staining show positive and negative cells (power X 40).

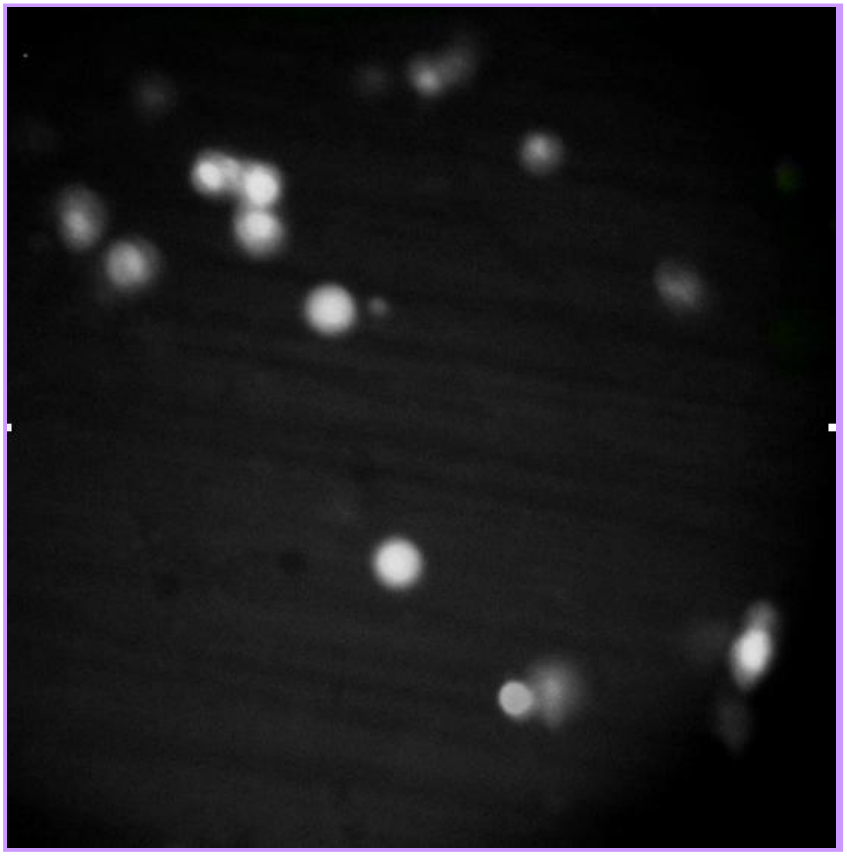


Fig. (٤.٦): Slide of indirect immunofluorescence microscope at 490 nm. Positive cells give green-apple when with FITC-labeled antibodies after exposure to UV-light to see lymphocytes subsets .

٤.٨.٢. Interleukin – ١٠ (IL – ١٠)

There was an increase of IL-١٠ levels in the sera of patients with VL (٢٠٢.٣ ± ٩.٨ pg/ml) when compared to that of the normal control group (٥.٤٥ ± ٣.٤ pg/ml)(tab.٤.٨). In the post treated VL group the levels were (٩.٠٤ ± ٤.٦ pg/ml) significantly lower than pretreated VL group (P< ٠.٠٥). There was no significant difference between post treated and control group (p> ٠.٠٥) , (tab.٤.١٠).

٤.٨.٣. Tumor necrosis factor (TNF-α)

There was an increase of TNF-α levels in the sera of patients with VL (٥٩.٣ ± ٦.٧ pg/ml) when compared to that of the normal control group (١٦.٢٣ ± ١.٢ pg/ml)(tab.٤.٨). In the post treated VL group the levels were (١٨.٢ ± ٣.٤ pg/ml) lower than pretreated VL group (p<٠.٠٥). There was no important difference between post treated and control group (tab.٤.١٠).

٤.٨.٤. IL-١٠/ IFN-γ ratio

Visceral leishmaniasis patients exhibited higher production of IL –١٠ and lower IFN –γ levels, resulting in higher ratio of IL-١٠ : IFN– γ (١.٨٠) compared to that of the control group (١.٦٨) , (tab.٤.٩).

٤.٩. Eosinophil Cationic Protein (ECP)

There was an increase of ECP levels in the sera of patients with VL (٢٧.٢ ± ٣.٤ ng/ml) when compared to that of the normal control group (٨.٩ ± ٦.٨ ng/ml) (tab.٤.١١) . In the post treated VL group the levels were (٨.٧ ± ٣.٢ ng/ml) significantly lower than pretreated VL group (p < ٠.٠٥) (tab. ٤.١٣) . There was no

important difference between post treated and control group and also no significant changes in relation to sex and age (tab. ٤.١٣).

٤.١٠. Enzymes

٤.١٠.١. Superoxide dismutase (SOD)

The activity of SOD enzyme in VL group was higher (٤٦.٣٨ ± ٦.٩٠ µg/l) as compared with its control group (٤٢.٣٦ ± ٧.٥ µg/l) . The differences between the two groups were significant at the P<٠.٠٥ level, but there were no significant changes (p>٠.٠٥) in relation to some clinical parameters including sex and age (tab.٤.١٤ and ٤.١٥).

Tab. (٤.٨): Mean cytokines concentration ± SE of ٥٢ sera in patients infected with visceral leishmaniasis and healthy control.

Type of cytokine	Patients	Controls	P- Value
IFN- γ (pg/ml)	١١٢.١ ± ٤.٦	٣.٢٢ ± ١.١	< ٠.٠٥
IL-١٠ (pg/ml)	٢٠٢.٣ ± ٩.٨	٥.٤٥ ± ٣.٤	< ٠.٠٥
TNF- α (pg/ml)	٥٩.٣ ± ٦.٧	١٦.٢٣ ± ١.٢	< ٠.٠٥

Tab. (٤.٩):The ratio of IL-١٠/IFN- γ in the sera of visceral leishmaniasis patients compared to healthy control.

Groups	Mean of IL-١٠ (pg/ml)	Mean of IFN- γ (pg/ml)	IL-١٠/IFN- γ ratio
Controls	٥.٤	٣.٢	١.٦٨
Patients	٢٠١.٧	١١٢.٠	١.٨٠

Tab. (٤.١٠): Serum levels ± SE (pg/ml) of IL-١٠, IFN- γ and TNF- α of ٥٢ patients infected with visceral leishmaniasis measuring by ELISA before, during and after therapy.

Type of cytokines	Days of therapy			
	٠	٧	١٤	٢١
IL-١٠	٢٠٣.٣ ± ٩.٨	٤٦.٠٥ ± ٨.٣	١٧.٣٦ ± ٧.٨	٩.٠٤ ± ٤.٦
IFN- γ	١١٢.١ ± ٤.٦	١٨.٤٩ ± ٤.٣	٧.١٩ ± ٣.٦	٥.٠٢ ± ٢.٠
TNF- α	٥٩.٣ ± ٦.٧	٢١.٤٠ ± ٩.٧	١٩.١٨ ± ٩.٦	١٨.٢ ± ٣.٤

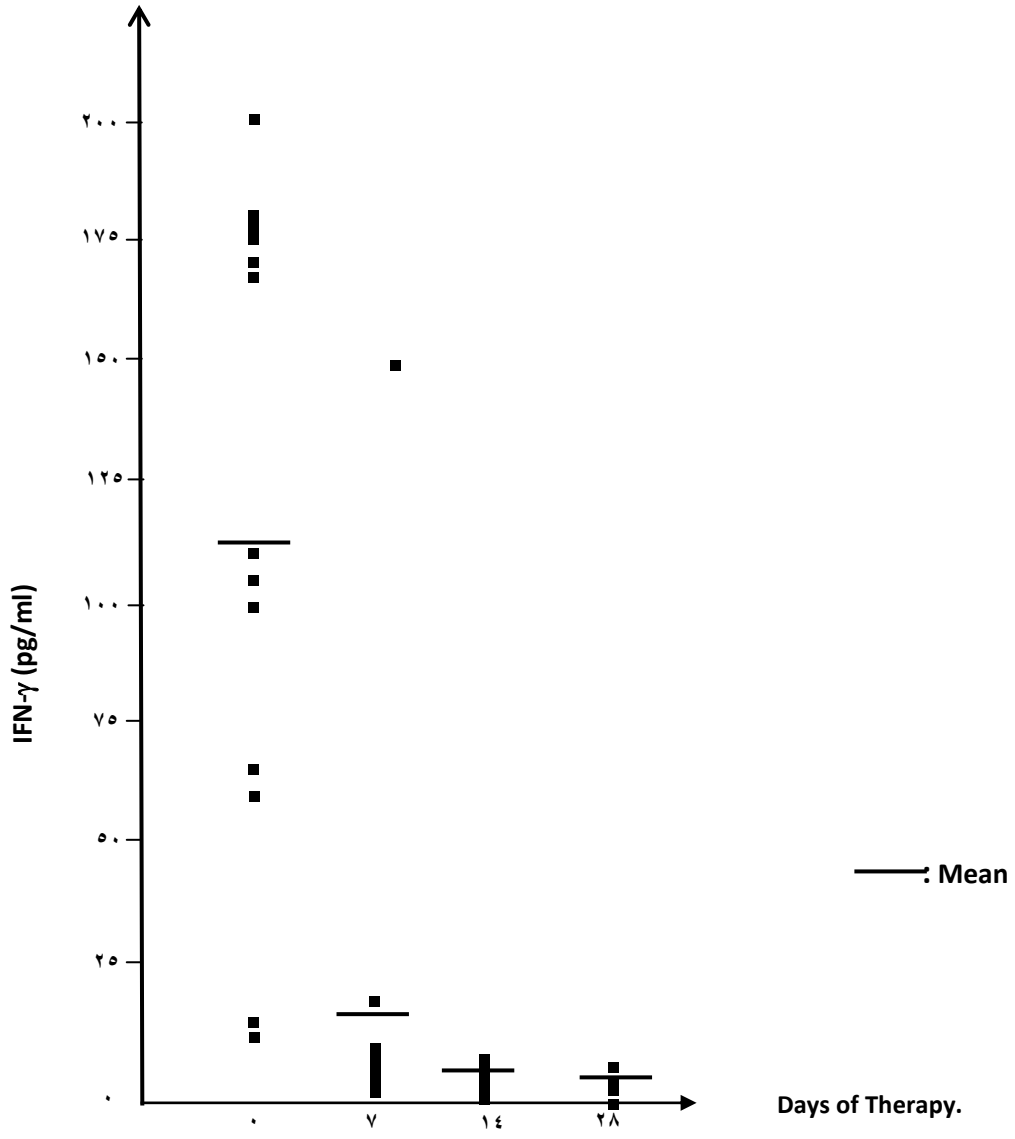


Fig.(٥) :Serum levels of IFN-γ (pg/ml),measured by ELISA, in ٥ patients with visceral leismaniasis befor, during & after therapy.

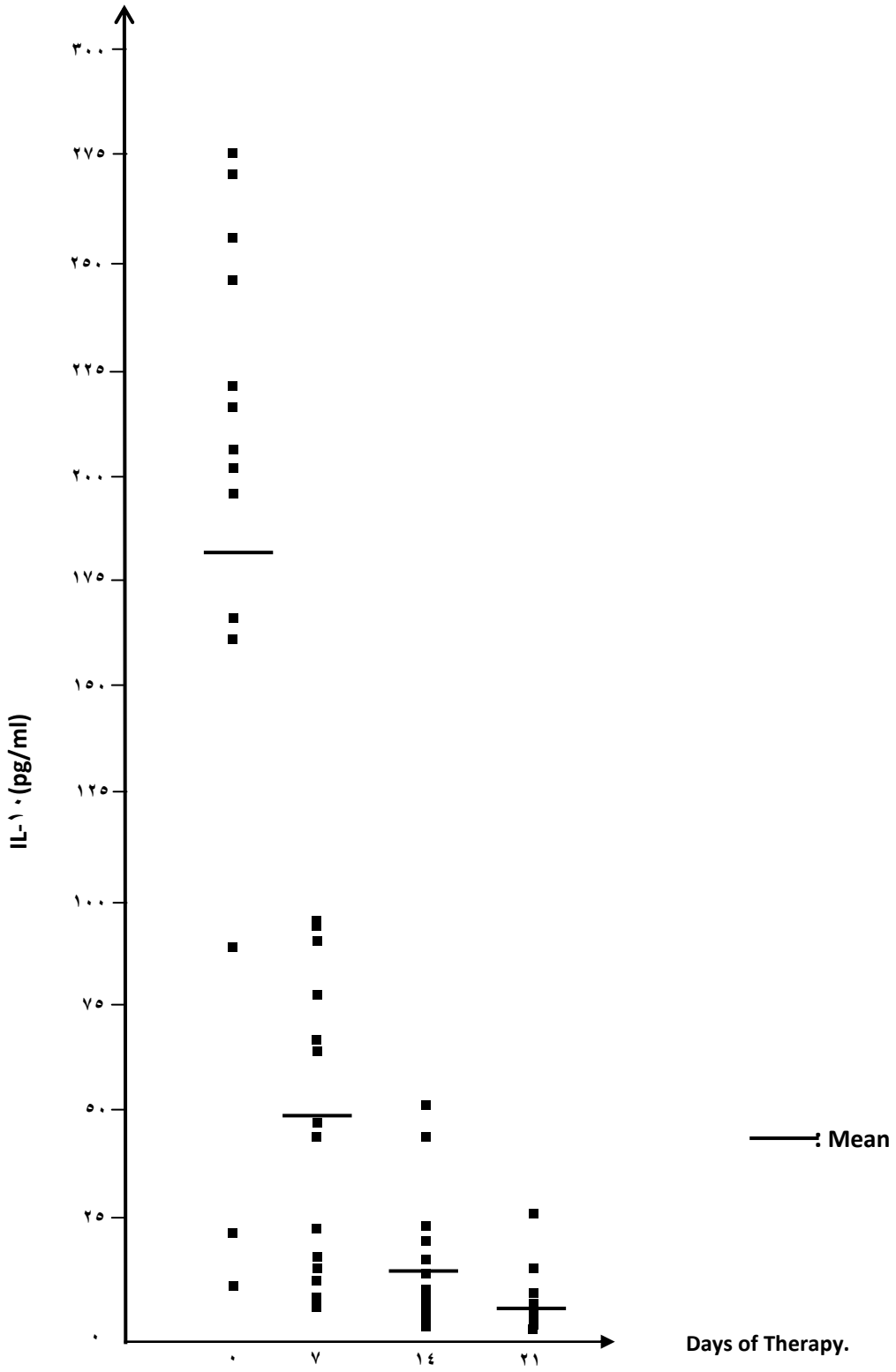


Fig.( ) :Serum levels of IL- (pg/ml) measured by ELISA, in patients with visceral leismaniasis befor, during & after therapy.

Fig.( ) :Serum levels of TNF-α (pg/ml),measured by ELISA, in patients with visceral leismaniasis befor, during & after therapy.

Tab. (٤.١١) : Mean concentration ± SE (ng/ml) of Eosinophil Cationic Protein (ECP) in the sera of ٥٢ patients infected with visceral leishmaniasis & healthy control.

Controls	Patients	P- value
٨.٩ ± ٦.٨	٢٧.٢ ± ٣.٤	< ٠.٠٥

Tab. (٤.١٢): Mean serum level ± SE (ng/ml) of Eosinophil Cationic Protein (ECP) in the sera of ٥٢ patients infected with visceral leishmaniasis measuring by ELISA before, during and after therapy.

Days of therapy			
٠	٧	١٤	٢١
٢٧.٣ ± ١.٤	١٧.٢ ± ١.٩	١١.٨ ± ٣.٧	٨.٧ ± ٣.٢

Tab. (٤.١٣) : Mean concentration ± SE (ng/ml) of Eosinophil Cationic Protein (ECP) in the sera of ٥٢ patients infected with visceral leishmaniasis in relation to the sex and age.

Sex		P-value	Age (year)			P- value
Male	Female		< Year	Year	> Year	
٩.٦ ± ٢.٣	٨.٩ ± ٢.٤	> ٠.٠٥	٩.٠ ± ٣.١	٩.١ ± ٢.٤	٨.٦ ± ١.٨	> ٠.٠٥

٤.١٠.٢. Adenosine deaminase (ADA)

The mean concentration of ADA of healthy control (٣٤.٩٨ ± ٤.٣ u/l) was significant lower (p<٠.٠٥) than that of patients with VL (٨٥.٦٩ ± ٤.٠٣ u/l). In relation to the clinical parameters evaluated in this study (sex and age) patients showed no significant differences (p>٠.٠٥) (tab.٤.١٥).

٤.١٠.٣. Glutathione reductase (GHR)

The mean concentration of GHR of healthy control (٧.٢٠ ± ٢.٥ µmol/ml) was significant lower (p<٠.٠٥) than that of patients with VL (١٥.١٧ ± ٢.٣ µmol/ml) (tab. ٤.١٤). In relation to the clinical parameters evaluated in this study (sex and age) patients showed no significant differences (p>٠.٠٥) (tab.٤.١٥).

٤.١٠.٤. Alkaline phosphatase (ALP)

A highly significant increase(p<٠.٠٥) in the levels of VL patients was seen , compared to that of the normal healthy control group (١٧٧.١٧ ± ٧.٦٨ versus ٨٠.٤٥ ± ٦.٥٠ u/l)(tab.٤.١٦). The ALP activity was also assessed in relation to some clinical parameters (sex and age) and there were no significant changes in its value (p>٠.٠٥)(tab.٤.١٧).

٤.١٠.٥. Lactate dehydrogenase (LD)

There was highly significant increase (p<٠.٠٥) in serum LD mean level of VL patients compared to the healthy control group (٣٤١.٨٨ ± ٩.٦٢ versus ١٤٤.١٨ ± ٧.٧٥ u/l) (tab.٤.١٦).There were no significant changes in relation to some clinical parameters (sex and age) in its value (p>٠.٠٥)(tab.٤.١٧).

٤.١٠.٦. Alanine aminotransferase (ALT) or GPT

A highly significant increase (p<٠.٠٥) in the levels of ALT of VL patients was seen, compared to that of the normal healthy control mean (٣١.٥٠ ± ٣.٥٢ versus ٦.٥٩ ± ٣.٨٦ u/l) (tab.٤.١٦). The ALT activity was also assessed in relation to some clinical parameters (sex and age) and there was no significant changes in its value (p>٠.٠٥)(tab.٤.١٧).

٤.١٠.٧. Aspartate aminotransferase (AST) or GOT

There was highly significant increase (p<٠.٠٥) in serum AST mean level of VL patients compared to healthy control group (٥٧.٠ ± ٥.٩٨ versus ٨.٦٣ ± ٤.٧٨ u/l) (tab.٤.١٦) whereas non- significant differences (p>٠.٠٥) were observed when comparing in relation to sex and age.(tab.٤.١٧).

Tab. (٤.١٤): Mean concentration ± SE of ADA, GHR & SOD enzymes in the sera of ٥٢ patients infected with visceral leishmaniasis & healthy control.

Enzyme	Control	Patients	P-value
ADA (u/l)	٣٤.٩٨ ± ٤.٣	٨٥.٦٩ ± ٤.٠٣	< ٠.٠٥
GHR(µ mol/ml)	٧.٢٠ ± ٢.٥	١٥.١٧ ± ٢.٣٠	< ٠.٠٥
SOD(µ g/l)	٤٢.٣٦ ± ٧.٥	٤٦.٣٨ ± ٦.٩٠	< ٠.٠٥

Tab. (٤.١٥): Mean concentration ± SE of ADA, GHR & SOD enzymes of visceral leishmaniasis patients in relation to sex and age.

Enzyme	Sex		P-value	Age (year)			P-value
	Male	Female		< year	year	> year	
ADA(u/l)	٨٦.٥٧±١.٣٤	٨٧.٢٨±١.٧٥	> ٠.٠٥	٨٤.٣٢±٠.٠١	٨٣.٩٦±٢.٧٤	٨٥.٨٢±٢.٧٣	> ٠.٠٥
GHR(µmol/ml)	١٤.٥٧±١٢	١٦.٦٧±١.٨٠	> ٠.٠٥	١٥.٢٦±٠٦٢	١٥.٥±٣.١٦	١٦.٠±١.٣٣	> ٠.٠٥
SOD(µ g/l)	٤٣.٢٣±٤.٥٢	٤٤.٥٣±٣.٢١	> ٠.٠٥	٤٢.٣١±٠٦٥	٤٤.٢٣±٤.٥٣	٤٦.٣±٢.٩١	> ٠.٠٥

Tab. (٤.١٦): Mean concentration ± SE of ALP, LD, ALT & AST enzymes in the sera of ٥٢ visceral leishmaniasis patients & healthy control.

Enzymes (u/l)	Control	Patients	P- value
ALP	٨٠.٤٥ ± ٦.٥٠	١٧٧.٧١ ± ٧.٦٨	< ٠.٠٥
LD	١٤٤.١٨ ± ٧.٧٥	٣٤١.٨٨ ± ٩.٦٢	< ٠.٠٥
ALT(GPT)	٦.٥٩ ± ٣.٨٦	٣١.٥٠ ± ٣.٥٢	< ٠.٠٥
AST(GOT)	٨.٦٣ ± ٤.٧٨	٥٧.٠ ± ٥.٩٨	< ٠.٠٥

Tab. (٤.١٧): Mean concentration ± SE of ALP, LD, ALT & AST enzymes in the sera of ٥٢ visceral leishmaniasis patients in relation to sex & age.

Enzymes (u/l)	Sex		P-value	Age (year)		P-value
	Male	Female		< year	> year	
ALP	٨١.٢ ± ٤.٣	٨٠.٥ ± ٣.٢	> ٠.٠٥	٨٣.٣ ±٣.٨	٧٥.٦ ± ٢.١	> ٠.٠٥
LD	١٤٦.٧ ± ٣.٥	١٤٥.٢ ± ٤.٨	> ٠.٠٥	١٥٥.٢ ± ٤.٦	١٣٥.٣ ± ٣.٤	> ٠.٠٥
ALT	٧.٢ ± ٣.٨	٧.٩ ± ٣.٤	> ٠.٠٥	١١.٢ ± ٣.٨	٦.١ ± ١.٤	> ٠.٠٥
AST	٨.٥ ± ١.٧	٨.٣ ± ١.٤	> ٠.٠٥	١٠.٠ ± ١.٥	٦.١ ± ١.٦	> ٠.٠٥

٤.١١. Isoenzymes

٤.١١.١. Isoenzymes of alkaline phosphatase (ALP)

There are three banding patterns for ALP enzyme activity which were discerned among the sera of ٨٦ patients, that differ from three zymodemes which were obtained from ١٨ healthy control, these differences due to isoenzymes activity of patients with VL before and after therapy (fig.٤.١٠ and ٤.١١).

a. Control group

Alkaline phosphatase enzyme of ١٨ healthy control sera (١٠٠٪) were separated into ٣ major fractions ; liver, bone and intestine. Liver ALP normally runs as an α-٢ band , bone runs in the α-٢/β- region and intestinal isoenzyme was usually found in the β-٢/γ- region (fig.٤.١٠ and ٤.١١).

b. ALP of VL patients before treatment

Fourty patients (١٠٠٪) had different bands (with excess amount) between α-٢ and β- region towards the normal liver band, in addition to the presence of a thick band of skeletal isoenzyme. The intestinal band had a thin band in comparison with control group (fig.٤.١٠ and ٤.١١).

c. ALP of VL patients after treatment

Fourty six patients (١٠٠٪) showed a thin liver band of ALP enzyme, but relatively thicker than that of skeletal and intestinal isoenzyme band (fig.٤.١٠ and ٤.١١).

٤.١١.٢. Isoenzymes of lactate dehydrogenase (LD)

There are five banding patterns for LD isoenzyme activity in VL patients differ from the control group. These differences mainly occurred due to the ALP isoenzyme activity in patients with VL before and after therapy (fig.٤.١٢ and ٤.١٣).

a. Control group

Twenty children (١٠٠٪) of normal healthy control group, sera LD enzyme was separated in to five major fractions (LD١ , LD٢ , LD٣ , LD٤ and LD٥) , LD-١ migrates most quickly towards the anode, followed in sequence by the other fractions, with LD-٥ migrating the lowest. All the LD isoenzymes showed the greatest amount (thick bands) except LD٤ characterized by a decrease in amount (faint band) (fig.٤.١٢ and ٤.١٣).

b. LD of VL patients before treatment

Thirty five patients (٨٥٪) showed a similar LD banding pattern to the control group, with the greatest amount (thick bands) in LD٤ and LD٥. The other fractions (LD١, LD٢ and LD٣) had a diminished quantities (thin bands) in the order : LD٢, LD١ and LD٣ (fig.٤.١٢ and ٤.١٣).

c. LD of VL patients after treatment

Twenty patients (٨٣٪) showed similar banding patterns to the control group with two clear thin bands of LD١ and LD٢. The other fractions had a slight elevated quantity (but, thin bands) in comparison with LD٤ and LD٥ isoenzymes(fig.٤.١٢ and ٤.١٣).

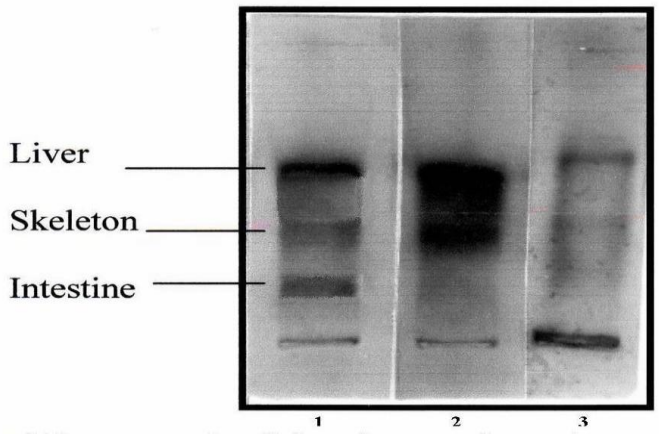


Fig . (4.10) : Photograph of the electrophoretic patterns obtained with kala-azar disease for alkaline phosphatase (ALP) isoenzymes.
Strip (1) : control sera (healthy children) .
Strip (2) : kala – azaric sera .
Strip (3) : sera from children after treatment .

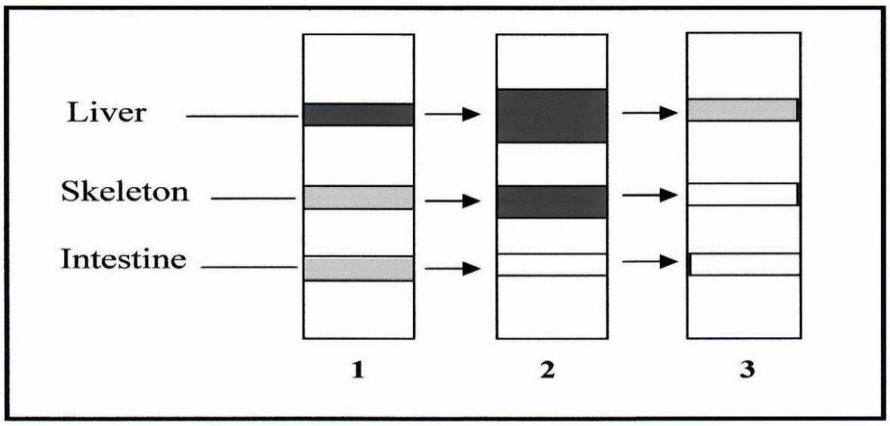


Fig . (4.11) : Zymograph of ALP (alkaline phosphatase) isoenzymes , obtained with kala-azar disease.
Strip (١) : control serum .
Strip (٢) : kala – azaric serum .
Strip (٣) : kala –azaric serum after treatment .

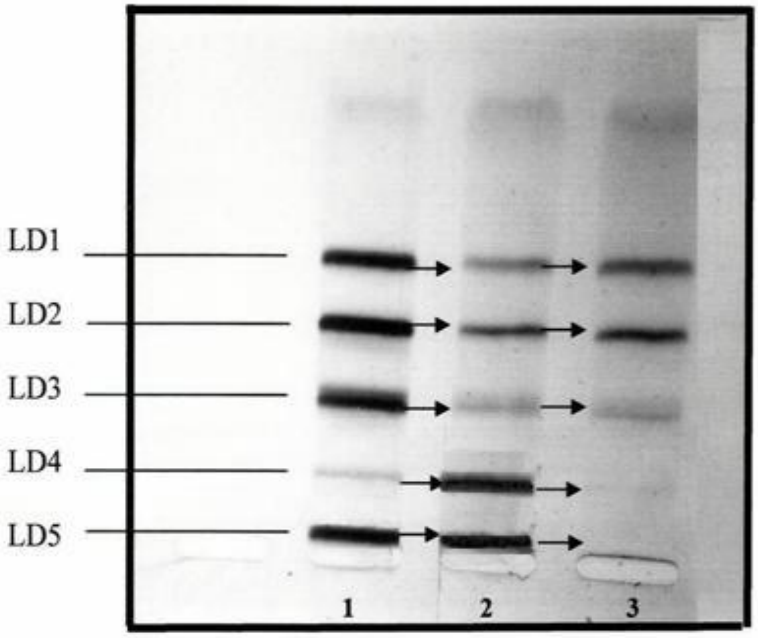


Fig. (4.12) : Photograph of the electrophoretic patterns obtained with kala-azar disease sera for lactate dehydrogenase (LD) isoenzymes .
strip (1) : sera from healthy children .
strip (2) : sera from infected children .
strip (3) : sera from children after treatment .

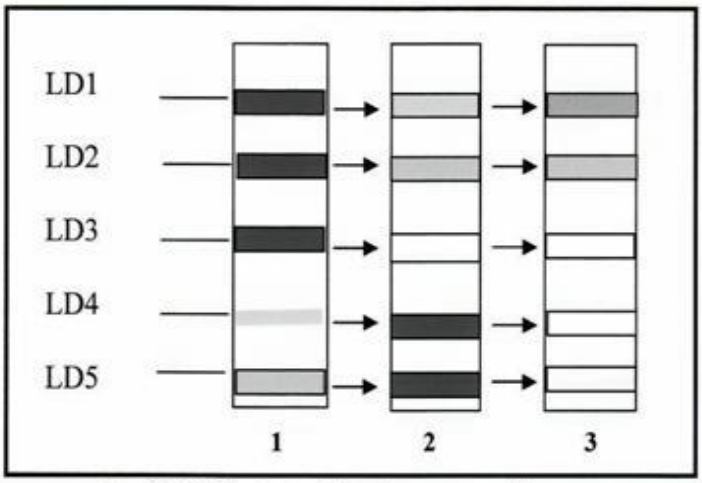


Fig . (4.13) : Zymograph of LD (lactate dehydrogenase) isoenzymes , obtained with kala – azar disease .
Strip (1) : control sera .
Strip (2) : kala – azaric sera .
Strip (3) : kala – azaric sera after treatment .

٤.١٢. Trace elements (fig ٤.١٤)

٤.١٢.١. Copper level in serum

There was an increase (p<٠.٠٥) in serum Cu levels of VL patients (٢٤.٩٩ ± ١.٣ µmol/l) compared to the normal control group (١٩.٣٣ ± ٠.٦ µmol/l) as shown in tab.(٤.١٨). There were no significant differences (p>٠.٠٥) observed between patients with some clinical parameters (sex and age) (tab. ٤.١٩).

٤.١٢.٢. Zinc levels in serum

A significant decrease (p<٠.٠٥) of serum Zn levels was shown in VL patients (٨.١٠ ± ١.٢ µmol/l) in comparison to that observed in the control group (١١.٠١ ± ٠.٩ µmol/l) (tab. ٤.١٨). There was no significant difference (p>٠.٠٥) seen when other clinical parameters studied such as sex and age (tab. ٤.١٩) .

٤.١٢.٣. Magnesium levels in serum

Serum Mg levels of VL patients (٠.٩٥ ± ١.٥ µmol/l) showed a significant decrease (p<٠.٠٥) in comparison with that observed in the control group (١.٣٣ ± ٠.٦ µmol/l) (tab. ٤.١٩). No significant statistical difference (p>٠.٠٥) was shown between VL patients in relation to sex and age (tab. ٤.١٩). There was an age dependence of serum copper (but not zinc or magnesium) in one age patients of more than one year age.

Tab. (٤.١٨): Mean concentration ±SE of copper, zinc and magnesium in the sera of ٥٢ patients infected with visceral leishmaniasis & healthy control.

Trace elements	Patients	Control	P- value
Cu µ mol / l	٢٤.٩٩ ± ١.٣	١٩.٣٣ ± ٠.٦	< ٠.٠٥
Zn µ mol / l	٨.١٠ ± ١.٢	١١.٠١ ± ٠.٩	< ٠.٠٥
Mg mmol / l	٠.٩٥ ± ١.٥	١.٣٣ ± ٠.٦	< ٠.٠٥

Tab. (٤.١٩): Mean concentration ± SE of copper, zinc and magnesium in the sera of ٥٢ patients infected with visceral leishmaniasis in relation to the sex & age.

Trace elements	Sex		P-value	Age (year)			P- value
	Male	Female		< year	year	> year	
Cu	٢٥.٦ ± ١.٥	٢٢.٥ ± ١.٦	> ٠.٠٥	٢٠.٢±١.٢٦	٢٥.٠±١.٩٠	٢١.٠±١.٨٧	< ٠.٠٥
Zn	١٤.٥ ± ٠.٧	١٣.٥ ± ٠.٤	> ٠.٠٥	٨.٠±٠.١٢	٨.٢±٠.١٢	١٠.٩±٠.١٢	> ٠.٠٥
Mg	٠.٥ ± ٠.٦	٠.٨٧ ± ٠.٥	> ٠.٠٥	٢.٠±٠.١٤	١.٩±٠.١٣	٢.٦±٠.١٣	> ٠.٠٥

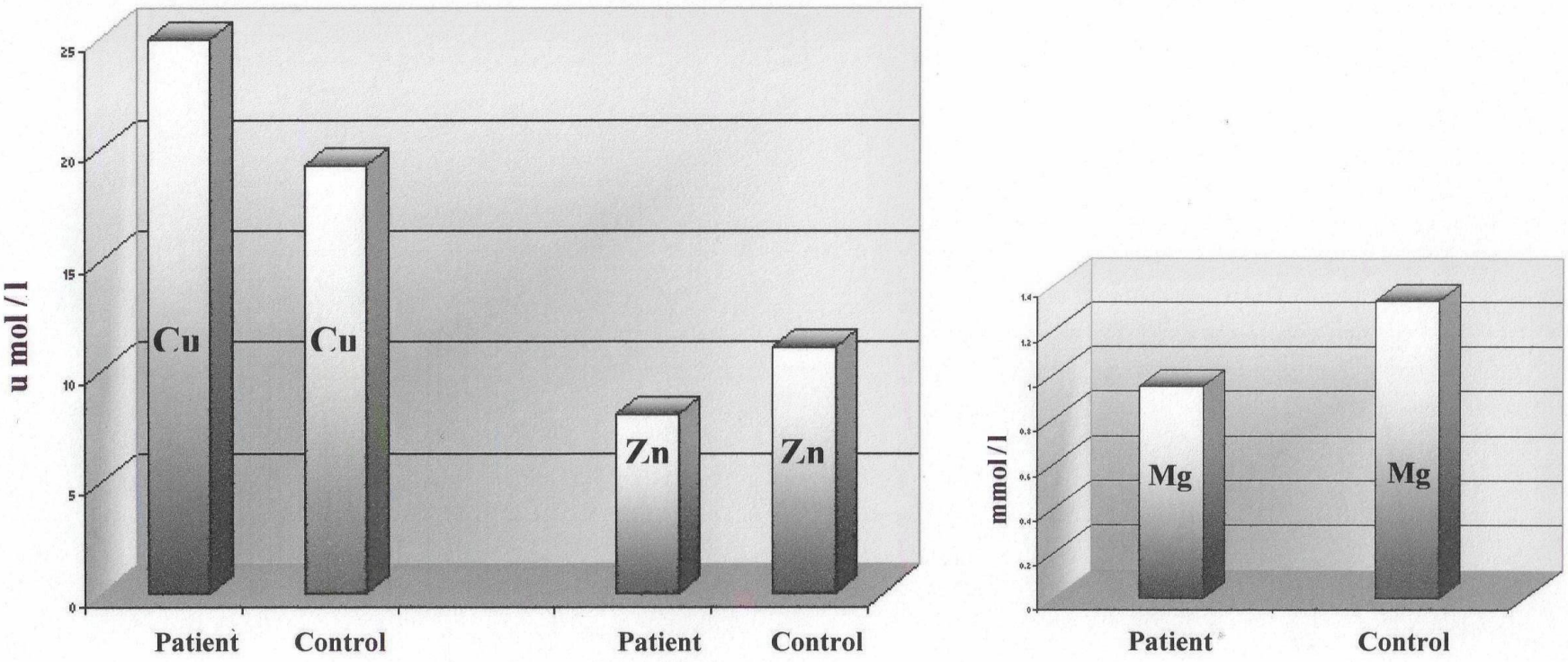


Fig.(4.14): Serum copper (Cu), zinc (Zn), & magnesium (Mg) of normal & Kala- azar patients.

Chapter Five

١٠٥

Discussion

Discussion

Visceral leishmaniasis is a fatal disease if left undiagnosed ; a definitive diagnosis of VL currently required demonstration of parasite by smear or culture from tissues , usually bone marrow or spleen , whereas an immunological diagnosis is based on indirect evidence (antibodies formed against parasite) . For this reason , immunological methods should be used in practice only when the techniques are highly sensitive , very specific and carefully evaluated . Over the last years , a number of tests were used for the detection of antileishmanial antibodies such as immunoflourescent antibody test (IFAT) , enzyme linked immunosorbent assay

(ELISA) with whole parasite or purified antigen , immunoblot analysis and direct agglutination test (DAT) . Even if IFAT and ELISA in general were more reliable than other serological techniques ; their application requires high level of skill , experience ; the need of equipments and expensive antisera will limit their use in epidemiological studies of visceral leishmaniasis (Sundar & Rai, ٢٠٠٢) .

Dot – blot immunobinding assay or Dot – ELISA a micro technique for the rapid diagnosis of VL utilizing promastigote antigen dotted onto nitrocellulose filter disc (Qu & Bao, ١٩٨٧). This rapid and inexpensive test should prove to be an important field diagnostic technique for VL patients (Papas *et al.*, ١٩٨٣).

It was a simple alternative to conventional ELISA and the intensity of dot staining can be read by eye without using photometer and considered as semiquantative readings of test results. The comparison between Dot – ELISA and conventional microtiter plate showed significant correlation between the two methods (Greiner, ١٩٩٣) . Dot – blot assay was proved to be more specific (٩٨.٩٪) , but similarly sensitive (٩٨.٥٪) and should be possible to produce a kit , suitable for large scale application at low cost in order to facilitate routine use of Dot – ELISA in diagnosis of VL (Senaldi *et al.*, ١٩٩٦) .

In the present work , dot – blot immunobinding assay (DIBA) and enzyme linked immunosorbent assay (ELISA) were evaluated for the diagnosis of visceral leishmaniasis .

Sensitivity

Sensitivity of ELISA and DIBA were tested with ٥٢ parasitologically proven cases (bone marrow smear positive). DIBA was slightly less sensitive (٩٦.٥٪) compared with ELISA (٩٧٪). Failure of the two methods to detect four parasitologically proven cases is possible due to the low level of the specific

antibodies in early course of the disease . This agrees with the study of AL-Bashir & Ali (٢٠٠٣) who mentioned that DIBA assay was sensitive to ٥٤ cases of VL from ٥٦ serum samples which were examined. The reason for failure of detection of low antibody level in ELISA assay is possibly due to lack of amplification effect of enzyme immunoassay whereby the conversion of many molecules of substrate by a single molecule of enzyme which increased the detectability of enzyme labeled antibody molecules (Jackson & Ekins, ١٩٨٦) .

The factors effecting the sensitivity of DIBA assay which play an important role in immunodetection were variable , possibly due to differences in antibody titer ; some times the reaction was so faint that a subject error is likely.

Specificity

Results of specificity of ELISA and DIBA were tested with sera from patients infected with diseases ; Brucellosis, Tuberculosis, Typhoid fever and Toxoplasmosis. DIBA assay was (٩٢٪) specific compared with ELISA (٩٩٪) . Specificity of the two tests depends upon preparation used in each test . In this study , the wells plate of commercial ELISA kit coated with *L. infantum* antigen. The results of commercial ELISA kit showed false positive reaction with sera from patients with toxoplasmosis and tuberculosis .The same results reported by Kar (١٩٩٥) that *Leishmania* antigens have common cross reactive epitopes shared with other microorganisms , particularly trypanosomiasis and tuberculosis . Different results were noted by Baqir, (١٩٩٩) who reported that ELISA used crude soluble antigen derived from locally strain of *L. donovani* , false positive reaction occured with sera from patients with tuberculosis , brucellosis , salmonellosis , schistosomiasis and hydatidosis . The results disagreed with EL-Amin *et al.* (١٩٨٦) which reported that no cross reaction occurred with sera from patients with diseases other than VL.

The whole parasite was used as antigen (promastigote) in locally prepared DIBA, the results showed that false positive reaction occurred with sera from patients with tuberculosis and typhoid fever, but not with brucellosis. Ibrahim (١٩٩٦) showed the cross reaction occurred with sera from patients with tuberculosis.

Finally, regarding the specificity and sensitivity of DIBA kit that were prepared in this study and used in the measurement of VL antibodies, it was found that DIBA kit is characterized by a good sensitivity and specificity in comparison with the commercial ELISA kit. The high specificity of DIBA test suggested that the test utilizes a good purified antigen which may lower the enhance for cross-reactivity , so it could be suggested for detection of VL antibodies in suspected cases as well as it could be used for survey purpose for this disease .

Immunoglobulin levels

The results indicated that the mean value of antibodies (IgG and IgM) were raised in the sera of patients with VL. This elevation may be due to polyclonal activation of B – cells by *Leishmania* antigens that induces proliferation and differentiation of B – cells into plasma cells that secreted antibodies . Also cytokine produced by T – helper cells had been implicated in the regulation of isotope switching of activated B – cells . Interferon-γ , preferentially secreted by Th١ subset , had been shown to stimulate the production of complement fixing $IgG_{٢}$ and $IgG_{٣}$ antibodies (Snapper & Paul, ١٩٨٧) .

Whereas cytokine of Th ٢ cells IL - ٤ and IL – ٥ are recognized as helpers for B–

lymphocyte and stimulate the production of high levels of IgE and IgM and non complement – fixing $IgG_{٤}$ in humans (Abbas *et al.*, ١٩٩٦) .

Treatment resulted in reduction of the mean value of IgM and IgG , wherease their value were remained higher than control . This result agrees with Hailu (١٩٩٠) who reported that the increase levels of antileishmanial antibodies may be present for along time after treatment.

The mean level of the third complement component C_3 was significantly increased in comparison with that observed in control group . This result agrees with Dominguez & Torano (١٩٩٩) who reported that infection with VL stimulates production of C_3 after the first weeks of infection. In contrast, Handman (٢٠٠١) reported that the C_3 level was reduced as a result to consumption by promastigote when enters the macrophage surface through the C_3 complement receptor and binds to the surface molecules (LPG and Gp ٣٦) of promastigote. Treatment resulted in reduction of mean value of C_3 than that observed in the control . This result was agreed with Ghose *et al.* (١٩٨٠) who explained the decreased level of C_3 than that observed in control. Increase and / or decrease in complement system depends on in situ tissue microenvironment need, nature of exposure to the first or second infection, extent of antibody antigen complex depends on rate in tissue and the balance between catabolism and anabolism of complex fractions (Roitt *et al.,* ٢٠٠١). The mean serum level of the fourth complement component C_4 was significantly increased in the infected group as compared with that of the control group, this was related to activation classical or alternative pathway in VL patients (Paredes *et al.*, ١٩٩٧). In addition there may be other cause of C_4 elevation which could be due to circulating immune complexes which were formed as a result of *Leishmania* infection leading to stimulate the classical pathway depending on the parasite species (Ravanbod, ٢٠٠٠) . Our results are not agreed with Ague *et al.* (١٩٨١) ; Dominguez &

Torano (١٩٩٩) who reported that decreased level of complement may be due to the activation of alternative pathway of complement in absence of $C_{٤}$.

SDS – PAGE and Western Blot

Several factors interferred with the performance of the immunoblot or Western Blot (WB), especially the need for trained personal , the adequate and correct identification of reactive bands , as well as electrophoretic separation and transfer (Pinto *et al.*, ٢٠٠١) . The results in the current study showed in SDS – PAGE , that antigens which were studied dissociated in at least ١٢ molecules which differ in their molecular weights . These ranged from ١٣ to at least ٧٤ k Da, while Goncharove *et al.* (١٩٩٠) reported that promastigote antigens dissociated in at least ٨ molecules range from ١٨ – ٢٨ k Da. Our result is in agreement with Aisa *et al.* (١٩٩٨) who showed that promastigote antigens dissociated in SDS – PAGE into ٩ discrete p ging from ١٢ – ٨٥ k Da.The banding patterns differ

to inherent strainal variation, geographic variants, antigenic variable and antigenic shed, shift and drift, possible antigen masking effect due to host microhabitats and extent of replenishing effect due to murent need (Rolland *et al.,* ١٩٩١ ; Aisa *et al.,* ١٩٩٨).

In WB for different types of *Leishmania* antigens were used, IgG recognized specially low to medium molecular bands between ١٣ – ٧٤ k Da . This results are in agreement with the Kumar *et al.* (٢٠٠٢) who showed that IgG recognized low to medium MW bands between ١٨ – ٨٠ k Da . Jeffrey *et al.* (٢٠٠٠) showed that the maximal specificity in diagnosis of VL was obtained by detecting antibodies against ١٨ – ٨٠ k Da . However, Marcy *et al.* (١٩٩٢) proved that the presence of antibodies against low subunits protein (٨–٢٥k Da), Aisa *et al.* (١٩٩٨) explained the presence of antibodies detecting low antigens fraction is a marker of the early phases of the infection .

The results showed that ١٨ k Da subunit of *Leishmania* promastigote was the most reactive one and ١٠٠٪ of our patients recognized this band . This polypeptide

fraction had been identified as nuclear proteins of the parasite (Suffia *et al.*, ١٩٩٥). The subunit ١٨ k Da was a powerful and good antigen for serology, because it contained a large spectrum of epitopes , which covered variations in the individual response among patients . However, it represents a complex structure, and there was always a balance between diagnostically relevant epitopes versus cross – reactive ones, which sometimes compromises the specificity of the system (Baqir, ١٩٩٩)

Finally, serodignostic tests based on antibody detection exhibited high sensitivity and reasonable specificity , and have played an important role in confirming clinical diagnosis and epidemiologic studies . The eluated selected bands the could be used in serodiagnostic procedure such as latex , ELISA and other techniques to asses their performance indices . The immunoblotting test was tried in the analysis of T – cell that could be a candidate for vaccine . The other possible application are detection of blotted enzymes , epitope mapping and raising antibodies against blotted immunogens . In addition to previous subjects , this test was excellent diagnostic tests , since many bands are reactive in active cases of VL , and did not react with other disease and more specific and sensitive than other diagnostic test (Baqir, ١٩٩٩ ; Kemeny & Challacombe, ١٩٨٦) .

Lymphocyte surface markers

Peripheral blood lymphocyte (PBL) phenotyping is a mirror image of immunity and can give an idea of the immunological status in patients with visceral leishmaniasis disease. So we intended to disclose the aspects of this important immunological feature for VL disease patients, in order to examine what differences were significantly present in comparison with control group.

Immunoperoxidase (IP) staining was compared with indirect fluorescent technique (IFAT) for the enumeration of lymphocytes. The percentage obtained for peripheral blood lymphocytes $CD_{٣}$, $CD_{٤}$, $CD_{٨}$ and $CD_{٢٢}$ using IP test was significantly lower than those obtained by IFAT . These differences are probably due to different steps which are required to prepare lymphocyte for analysis (Dhaliwal *et al.*, ١٩٩١). Moreover IFAT method a rapid and easy to perform while IP is a laborius method that a subjective error is likely needs to expensive antisera and experience to perform.

Total T-lymphocyte $CD_{٣}$ reduction in patients with VL was observed in comparison with control . This could be associated with reduction of T – lymphocyte proliferation . The same results reported by Ho *et al.* (١٩٨٣) who noted that the proliferative response of Kenyan patients with VL were severely depressed to heterogenous as well as *Leishmania* antigens . Suppression may be mediated by macrophage , either by defective antigen processing and presentation , or by elaboration of suppressive mediators like IL – ١٠ , TGF - β and prostaglandin $E_{٢}$ (Saha *et al.*, ١٩٩٥) .

The results of this study revealed that there was a significant increase of $CD_{٨}$ T – cells in patient with VL in comparison with control . These results were in agreement with results of Colmenares *et al.* (٢٠٠٣) who confirmed the increase of $CD_{٨}$ T – cells in VL cases, however, Rohtagi *et al.* (١٩٩٦) stated that peripheral blood $CD_{٨}$ cell count was normal in acute and uniformly low in chronic cases . This may be explained by the presence of IL - ٢ from macrophage and Th ١ which increases $CD_{٨}$ division . The role of $CD_{٨}$ T – cells against *Leishmania* infection was suggested by Stenger & Modlin (١٩٩٨) who reported that $CD_{٨}$ T – cell were shown to be responsible for the conversion of susceptible BALB/C mice into resistant phenotype after depletion of $CD_{٤}$ T – cells against *L. major* infection . Despite the fact that $CD_{٨}$ T- cells also produced IFN – γ on activation and can directly destroy the infected macrophage (Maash *et al.*, ١٩٩٨).

A different observation of CD_4 in patients with VL, it was significantly lower than control. These results are in agreement with Rohtagi *et al.* (١٩٩٦) who confirmed that acute and chronic VL cases are depressed in peripheral blood CD_4 count. This lowering of CD_4 may result from apoptosis as reported Das *et al.* (١٩٩٩) who suggested that CD_4 derived from susceptible mice undergo rapid apoptosis , produce less IL – ٢ and IFN – γ and fail to mediate DTH .

Results showed that CD_4 / CD_8 ratio was significantly lower than control group, this may be considered as an index of immune suppression in VL patients . These results agree with Ghosh *et al*. (١٩٩٦) who reported that at diagnosis CD_4 cells showed a significant decrease while CD_8 cells were significantly increases when compared with control and CD_4 / CD_8 ratio was inverted.

In the current study, CD_{22} (B – lymphocytes) showed relatively an increase but not significant in B – lymphocytes in comparison to the control group. The increased number of B – cells may result from polyclonal activation of these cells (Amit *et al.*, ١٩٧٦).

The antibody role of B – cells against *L. donovani* infection was noted by Smelt *et al.* (٢٠٠٠) who reported that B – cells deficient mice cleared parasite more rapidly from liver and infection failed to be established in the spleen.

Treatment resulted in a significant increase percentage of CD_3, CD_4 and CD_4/CD_8 ratio compared with pretreated group but still lower in comparison with control. In contrast the percentage of CD_8 and CD_{22} showed a significant decrease in comparison with pretreated group and still than control. These results confirmed the results of previous studies Ghosh *et al.* (١٩٩٦) who showed that CD_4 / CD_8 ratio returned to normal value three months after recovery. Neogy *et al*. (١٩٨٧) stated that follow up B – lymphocyte population during and after chemotherapy for the period of eight months showed that there was a relative decrease in CD_{22}.

Cytokine levels

In most parasitic diseases a predominantly cellular (Th١) or humoral (Th٢) immune response offers the best control over pathogens , the induction of an appropriate T – helper cell response is essential in determining a successful immune reaction . Th ١ cells are effective mediators for DTH and secrete interleukin – ٢ (IL – ٢) and interferon – gamma (IFN – γ) , the prime effectors of cell – mediated immunity (Perez *et al*., ١٩٩٥) . In contrast , Th ٢ cells do not transfer DTH , but they produce IL – IL – ١٠ and co-operate with B – cells to generate IgI and IgE responses (Finkeleman *et al*., ١٩٩٠) .

Results of IL – ١٠ confirmed the results showed by Margaret & David (٢٠٠١) who stated that VL patients have increased expression of IL – ١٠ mRNA as important strategy for down – regulating T – cell response. *L. donovani* infection is known to induce endogouns secretion of IL – ١٠ as a mechanism of parasitism because IL– ١٠ seems to be responsible for inhibition synthesis of IFN – γ the main macrophage – stimulating cytokine involved in the defense against *Leishmania* which facilitated the intracellular survival of parasite by down – regulating the oxidative and inflammatory response (Bhattachary *et al.,* ٢٠٠١) . In fact in human severity of VL has been closely associated with increased levels of IL-١٠ and the use of anti IL-١٠ antibody to block the IL-١٠ activity or IL-١٠ receptor blockade can be effective approach for the treatment of Leishmaniasis (Murrary *et al.,* ٢٠٠٢).

High levels of IFN-γ had been detected in serum of patients with VL in comparison with control. High levels of IFN-γ are necessary in the maintenance balance between Th١ and Th٢ responses. These results matched the results of previous researchers , Gomes & Dos (١٩٩٨) who were found a mixed Th١ / Th٢ response of parasite – specific T – cells from both acute and chronic murine visceral leishmaniasis , Kemp & Theander (١٩٩٩) stated that marked elevation of both IL-١٠ and IFN- γ mRNA found in patients with VL . A different observation showed that in

American VL, the IL-٢ and IFN-γ production were absent upon stimulation with *Leishmania* antigens. Also Bogdan & Rollinghoff (١٩٩٨) noted that IL-١٠ blocks Th١ activation and consequently a cytotoxic response by down – regulating IL-١٢ and IFN-γ production, IL-١٠ also inhibits macrophage activation and decrease the ability of these cells to kill *Leishmania*.

The data also supported the interesting finding of an imbalance in the ratio of Th١: Th٢ towards a predominantly Th٢ response in the group of VL patients.When IL-١٠ and IFN-γ levels were considered, a higher ratio of IL-١٠/ IFN-γ was observed. Decrease of IL-١٠ mRNA following therapy and detection of IFN-γ mRNA in a cute and treated patients was previously reported . Thus , it was suggested that , the presence of IL – ١٠ , rather than the absence of IFN – γ , is characteristic of a cute le

Circulating levels of TNF – α (which is mainly secreted by the activated macrophages) were detected with active kala – azar and during disease. TNF – α had raised levels. It is interesting to consider that the higher levels of TNF – α were detected simultaneously with the highest levels of IL-١٠, a known inhibitor of TNF – α secretion. We found that circulating levels of TNF – α declined as patients improved, but less than that of IL-١٠ and IFN-γ .In experimental visceral leishmaniasis, TNF- α had been shown to be a critical factor in disease control .In human VL circulating levels of TNF-α had been related to activity of disease (SalomÃo *et al.*, ١٩٩٦).

Finally the Th١ / Th٢ concept cannot entirely account for the true complexity of in *vivo*. Survival of the parasite depends on various mechanisms rather than a generic ability to ignore the Th١ or Th٢ type responses. The failure to control or resolve infectious disease often results from an inappropriate rather than an insufficient immune response (Powire & Coffman, ١٩٩٣).

Cationic protein from eosinophilic granulocytes .

The importance of this study concerns the indirect assessment of eosinophilia in serum of leishmaniasis children using eosinophil cationic protoein (ECP) which were determined by means of specific ELISA method. The level of ECP was significantly raised in serum from infected children compared to the control group . This elevation was due to the attachment of parasites to the eosinophils induced their degranulation with the release of granules contents onto parasite surface causing its destruction . Ingestion of parasite by eosinophils , induced drastic morphological changes , finally leading to leucocytes lysis with liberation of intact granules . This fact may increase the extracellular parasite killing (Pearson *et al.*, ١٩٨٧).

We found that the circulating levels of ECP were declined as patients improved. In contrast , the treated children infected with *Schistosoma haematobium* , the ECP levels persisted in a significant proportion indicated continuous eosinophil activity in the bladder wall (Reimert, ١٩٩٨) .

Superoxide dismutase (SOD)

The concentration of SOD in VL group was significantly higher than in the control group. Failure of macrophages which were infected by *Leishmania* parasites to produce high levels of respiratory burst as a mechanism defense of parasite against host led to persistence of leishmaniasis (Bogdan & Rollinghoff, ١٩٩٨).

The high level of the antioxidant SOD in the VL group protects host cells from the damaging effect of free radicals that are released in different infected host cells

especially in liver and spleen . The source of free radicals may be from the soluble agents like antibody antigen complexes (Roitt *et al*., ٢٠٠١) .

The increase of SOD as a host defence mechanism is associated with the immune response. WBCs membranes are composed of lipids, some of them unsaturated fatty acids which are highly susceptible to free radicals attacks that may be released from phagosomes that affect the integrity of the cell membrane and decrease its fluidity, consequently impairing their function. Also free radicals caused protein oxidation , affect DNA synthesis and may be involved in cell apoptosis (Dean *et al.*, ١٩٩٧) . All this effects cell growth and impairs the hosts immune response. This might have occurred in the study with VL patients, in the reduction of cell cycle parameters than in the control and CL group.

In this study, the control groups, which represent different ages did not differ in SOD concentration. Schacter *et al*. (١٩٨٥) found that there was no change in SOD activity with age.

Adenosine deaminase (ADA)

ADA enzyme is one of the most essential immune enzymes; its function gives a clear picture of the immune status of the body. It was found to play a critical role in the normal development of immune system, and for the proper development of T and B – lymphocytes in mammals.

The ADA activity in leishmaniasis was significantly higher in the VL group than in control. This may result from increased B – lymphocyte count or RBCs hemolysis by immune complexes and complement. ADA was localized within the cell membrane of RBCs and surrounding phagocytic vacuoles. These results are similar with those of Esteban *et al.* (١٩٩٤) who reported that patients with infectious or non infectious diseases in which high fever occurs showed slight or moderate increase in serum ADA activity like typhoid fever and Q fever pneumonia.

The results differ from previous studies with other parasitic infections. Shubber & AL-Khateeb (١٩٩٢) studied the activity of ADA enzyme in sheep infected with *Fasciola gigantica*, and Al-Shawk (١٩٩٩) in women chronically infected with *Trichomonas vaginalis* .All above found a relatively decreased activity in this enzyme which correlated with the immuno-suppression state. The decrease in ADA specific activity could cause a state of immuno-suppression, for ADA plays a critical role in the normal development of the immune system (Thompson & Seegmiller, ١٩٨٠). B-cell dysfunction was also found to be associated with ADA deficiency. This was found to be due to the alteration in antigen receptors (Gangi – Peterson *et al*., ١٩٩٩).

In our study ADA and SOD enzymes were found to be higher in VL group . This may be result from leak of infected tissue to the blood stream or from blood lysis . This agrees with Canbolac *et al*. (١٩٩٤) in their study with cancerous laryngeal tissue, that the source of elevated ADA and SOD results from leak to the blood stream and removal of the serum enzyme from the blood took long period (more than month) and the cancerous tissue is not the only source of the enzyme and also the significant increase of both of them could be explained as a healthy stimulated host immune response.

Glutathione reductase (GHR)

The antioxidant defense system is sophisticated and adaptive , GSH is a central constituent of this system (Cross *et al.*, ١٩٨٧) . GSH depletion may be the ultimate factor determining vulnerability to oxidant attacks , so intracellular GSH status appears to be a sensitive indicator of the cells overall health (Duke *et al.*, ١٩٩٦)

In this study there was a significant increase in glutathione reductase (GHR) enzyme activity in patients with leishmaniasis . The results differ from study of GHR activity in hydatidosis . Al-Qadhi (٢٠٠٥) reported that the mean of GHR of healthy controls was significantly higher than that of patients with hydatidosis . This enzyme is responsible for maintaining the reduced glutathione GSH in the cell by the glutathione

peroxidase – glutathione reductase system as previously mentioned in the literature review . So its depletion leads to depletion of GSH in the body . The consequence of sustained GSH depletion are grim . As cellular GSH is depleted , first individual cells d

st affected . The zones of tissue damage begin to ap

tissues with highest content of polyunsaturated lipids and / or the most meager antioxidant defenses are generally the most vulnerable (Kidd, ١٩٩٣) .

The liver is the organ most involved with the detoxification of xenobiotics (Substances foreign to the body) , and also is the main storage local for GSH (actually exporting GHS to other organs) (Deleve & Kaplowitz, ١٩٩٠) .

The increase in the activity of GHR indicated its formation in the liver with its damage cells causing a high rlease of enzyme in to the circulation .

GSH is considered the free radical scavenger and repairer of radical mediated biological damage (Kosower & Kosower, ١٩٧٨), so its deficiency led to accumulation of free radicals. Cross *et al*. (١٩٨٧) showed that the cumulative damaging effects of oxygen radicals and other oxidants are main contributors to degenerative disease .

Alkaline phosphatase (ALP)

The activity of ALP was found to be significantly higher in patients with leishmaniasis than in health control . Alkaline phosphatase enzyme was found in many tissues , but highest concentration were found in biliary tract epithelium and kupffer's cells of the liver (Pagana & Pagana, ١٩٩٨) . Some reports showed that any factor effecting the liver metabolism was found to cause an increase in ALP activity (Pincus & Schaffner, ١٩٩٦) . The infancy and childhood showed high ALP values as much as ٣ – ٤ times that of adult value (Smith *et al.*, ٢٠٠٠) . A damage or injury which was caused by VL liver tissue (principally parenchymal cells) led to releasing ALP into the blood circulation (Norbort & Tietz, ١٩٨٨) .

Lactate dehydrogenase (LD)

Similar results also were obtained with LD enzyme . There was an increase in its activity in VL patients in comparison with healthy controls . In general this enzyme is usually used for detection of hepatocellular damage , whenever liver cells are damaged or injured , the liver enzymes leak out into the circulation across the damaged cell membrane (Moss *et al*., ١٩٨٦) . As we mentioned previously ADA play a role in the liver function (Bollinger *et al*., ١٩٩٦) , so its deficiency lead to liver disturbance , hence , elevated levels of LD in VL disease patients will be attributed to h

by diseases (Kreisbergs, ١٩٧٨) , as well as the rise levels in blood may be due to tissue damage . This will lead us to understand the cause of LD elevation levels in liver VL patients .

Alanine aminotransferase (ALT) or GPT

Serum ALT activity was significantly increased in all cases of kala – azar studied in comparison to the normal controls . ALT was found predominantly in the liver , lesser quantities were found in kidneys , heart and skeletal muscle (Pagana & Pagana, ١٩٩٨) . The rise of serum ALT in children affected with VL was caused by *L. donovani* which damaged the liver and spleen cells , these injured cells released ALT into the circulation (Wilcocks & Manson, ١٩٩٥) . The increase in serum ALT activity could reflect the pathological picture of nonspecific granuloma formation in the liver of these patients (Ellis, ١٩٨٣) .

Aspartate aminotransferase (AST) or GOT

Serum AST was significantly increased in all cases of VL patients studied in comparison to the normal control . Serum AST levels were compared with ALT levels . The AST /ALT ratio is greater than one . This value may help in determining liver damage (Pagana & Pagana, ١٩٩٨) . The rise of serum AST in children affected with

VL which damage the liver , these injuried cells release AST into circulation (Wilcocks & Manson, ١٩٩٥) .

Isoenzymes

Alkaline Phosphatase (ALP)

Electrophoresis is considered the most widely useful technique for ALP and LD isoenzymes analysis . In the results obtained from the current study , in addition to the normal ALP and LD enzymes fractions ,there exists some variation in the amount of these fractions that may be associated with the periods of therapy which is represented in different electrophoretic mobility (Moss & Henderson, ١٩٨٦) .

It is quite obvious that there was a change in the isoenzymes profile of patients with VL compared to that of normal children . The changes were most stricking in the relative distribution of the three isoenzymes . Liver isonzyme band actively increased sharply and was apparently responsible for most of the serum ALP activity , while there was no appreciable change in the activity of intestinal isoenzyme band . The large increase in the activity of liver isoenzyme band indicates it [illegible]ver with its damaged cells causing a high release of [illegible] isoenzyme into the circulation (Wilcocks & Manson, ١٩٩٥) . The skeletal (bone) isoenzyme band also had appreciable activity ; this may be due to the bone marrow disorder as a result of parasitic invasion . Wooton (١٩٩٤) reported that specific activity of liver isoenzyme was increased ٣٤ times , while the specific activity of intestinal isoenzyme increased ١.٧٩ times in sera of patients with VL to that of normal children .

The effect of treatment with pentostam on the activity of ALP isoenzymes from sera of children affected by VL was studied . The activity of liver isoenzymes decreased gradually , reaching almost to normal level after treatment , while that of the intestinal isoenzyme was almost unaffected during the course of treatment . It

seems possible , therefore , that the pentostam has a direct influence on the liver , the organ responsible for the synthesis of liver isoenzymes (Norbort & Tietz, ١٩٨٨) . The result were agreed with Yousif (١٩٨١) .

Lactate dehydrogenase (LD)

The LD isoenzymes results were in disagreement with other investigators who showed that elevation of $LD_{٣}$ occurs most frequently with pulmonary involvement , and was also observed in patients with various carcinomas (Bishope *et al.*, ٢٠٠٠) .

The LD isoenzymes distribution in VL children were characterized by $LD_{٤}$ and $LD_{٥}$ predominating and electrophoretically slower fractions . $LD_{١}$ and $LD_{٢}$ bands are most marked but , slightly than $LD_{٤}$ and $LD_{٥}$. The appreciable activity of $LD_{٥}$ and $LD_{٤}$ were related to the damaged or injuried liver cells which were infected by *L. donovani* ; the intracellular enzyme released from the liver cells diffuse into the plasma and imposes its pattern over that normally present (Wilcocks & Manson, ١٩٩٥) .

After treatment , the activity of $LD_{٤}$ and $LD_{٥}$ isoenzymes decreased gradually reaching almost to normal level . It seems possible , therefore , that the drug had a direct influence on the liver , the organ responsible for the synthesis of liver isonzymes . The results were agreed with Akrawi (١٩٨٥) .

Trace elements

Copper

The results showed that there was a significant increase in serum copper in VL patients as compared with healthy controls . These results were due mainly to the body reaction against infection . Ceruloplasmin which is a copper containing protein is one of the acute phase reactant proteins that increased in inflammation and hence leading to increase serum copper. α–ceruloplasmin inhibits the oxidant injury of the cells by scavenging the superoxide radicals through dismutation reaction similar to superoxide dismutase enzyme (Goldstein, ١٩٧٩) or by reduction the copper ions within the protein (Bannester, ١٩٨٠) . Other workers Loustad (١٩٨١) had proposed that ceruloplasmin act by converting the reduced iron ($Fe^{+٢}$) to an oxidized from ($Fe^{+٣}$) because it acted as ferroxidase enzyme . Yet , the majority of the antioxidant activity in the serum is dependent on the level of this copper containing protein (Al-Timimi & Dramandy, ١٩٧٧) . There was also another cause explaining the increase of serum copper in those patients due to shed copper ions from damaged hepatocytes which was the late complication of the disease (Edwards, ١٩٩٥); this fact was confirmed by reports that recorded an elevation in the level of liver enzymes in the sera of kala – azar patients (Al-Saffar & Al-Mudhaffar, ١٩٧٩) . Copper levels were found to correlate with HAL-DR^{+} cells, including ß, T and NK activated lymphocytes, monocytes and IL-٢ (Chan *et al.,* ١٩٩٨).

Zinc

There was a significant decrease in serum zinc of VL patients as compared with healthy controls . It is known that the plasma zinc level may decrease with infections of many other diseases probably due to redistribution from plasma to tissues such as liver (Falchuk, ١٩٨٨) . Other cause may be due to in part to the hypoalbuminaemia developed in those patients after a period of incubation of the disease (Herwaldt, ١٩٩٩), zinc is transported mainly bound to albumin molecules and the decrased in albumin concentration in serum leads to decrease zinc level in serum . Other reason is associated with the immune changes in VL patients . Those patients impaired immunity (Baqir *et al.,* ٢٠٠١) and because there were good evidences

about the relationship between the zinc level and immunity due to the indirect effect appears to be immunomo dulatory, resulting in increased phagocytosis and killing the parasites by the host macrophages (Ripa & Ripa, ١٩٩٥; Yasser *et al.,* ٢٠٠٥). These r ment with other previous work regarding serum trac
levels in cutaneous leishmaniasis patients (Kocyigit, ١٩٩٨) , and visceral leishmaniasis (Al-Basheer *et al.*, ٢٠٠٥) .

Magnesium

Serum magnesium of patients with kala – azar were significantly decreased in comparison with healthy controls . Magnesium levels are normally kept within narrow limits , which implies close hemostatic control and about ٣٥٪ of the magnesium in plasma is protein – bound mainly to albumin (Whitby *et al.,* ١٩٨٨) .This reduction in Mg levels might be one of the causes of immunosuppression recorded in these patients , since magnesium is an essential factor for the preservation of DNA , RNA and ribosome and it is also needed in binding of RNA to polysomes during protein synthesis (Teitz *et al.*, ١٩٩٤) . Therefore , the decrease in magnesium levels might lead to the destruction of the immune cells responsible for the host defenses against leishmaniasis , as well as this deficiency might be due to the consumption of Mg by the parasite metabolism . Mg is essential cofactor for ٣٠٠ enzymes and for protein synthesis (Smith *et al.*, ٢٠٠٠) .

There was an age dependence of serum copper (but not zinc or magnesium) in one – year age patients , and had a higher serum copper than the patient of more than one year age . There were no significant differences between the serum metals and sexes in patients of visceral leishmaniasis in our study . This subjects requires more details to predict the accurate explanation for sexual variance levels of metals .

Finally , the body reaction against parasite infection was associated with different changes in serum level of trace elements . The results were agreed with

result of (Al-Basheer *et al.*, ٢٠٠٥) .

Conclusions and Recommendation

Conclusions

١. Visceral leishmaniasis is a puplic health problem in our country that cause a considerable health and economical impact . This problem needs more attention , and cooperation of responsible personals in different fields of the disease .

٢. The technique , Dot – blot immunobinding assay (DIBA) or Dot – ELISA is a sensitive and specific test for diagnosis of VL and could be used as a kit suitable for large scale application at low cost .

٣. Immunoblot proved to be a good confiramatory test especially when most IgG antibodies recognized small and most reactive subunit ١٨ k Da of VL and CL

promastigote antigens and there are several factors that interfere with its performance .

٤. High levels of specific IgG and IgM against *Leishmania* antigens estimated in sera of patients with VL then reduced after treatment whereas , they were slightly higher than the normal level . There was no change in IgA level in infected children before , during and after therapy .

٥. The third and fourth complements components ($C_٣$ and $C_٤$) were elevated in sera with VL patients then reduced after treatment .

٦. The ratio $CD_٤/CD_٨$ is reversed in VL infection then increased after treatment but it was less than its normal range .

٧. High levels of IL-١٠, IFN-γ and TNF-α measured in sera of patients with VL then decrease after treatment and reach to their normal levels .

٨. The ECP activity was raised in serum of VL children compared to the control then declined as patients improved reflecting its role in killing extracellular parasite.

٩. The activity of ALP , LD , ALT and AST enzymes were found to be significantly higher in patients with VL.

١٠. The enzymes , SOD , ADA and GHR showed an elevation over the normal controls reflecting its significant in the immune response.

١١. Electrophoresis is considered the most widely useful technique for ALP and LD isoenzymes analysis.

١٢. The decreased levels of the trace elements (Zn and Mg) may reflect a decrease in the immune response and it may also play a role in the disease development. The increased level of copper may reflect the late complication of the disease and its shedding from damaged hepatocells.

Recommendations

١. DIBA assay is recommended to use as a reference technique in public health laboratory in less equipped laboratories.

٢. New techniques as polymerase chain (PCR) and hybridization are essential for any reasonably accepted studies in future.

٣. A better standardization of the Dot-ELISA may increase its sensitivity compared with existing diagnostic methods.

٤. Immunoblotting needs to be further modified to be applied in laboratories and not for research purpose only.

٥. Assessing the microbiocidal activity of eosinophils such as major basic protein (MBP), eosinophils peroxidase (EPO) and eosinophil-derived neurotoxin (EDN) in addition to eosinophil cationic protein (ECP). They are important to investigate the role of eosinophil in the evolution of leishmaniasis.

٦. Studying the murine model of VL to provide a mean to characterize the operative protective immune mechanisms as well as identifying parasite antigens with suitable adjuvants which have potential use for human vaccination.

٧. Extraction from our results of ideas and conclusion that can be of value in vaccine design.

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Appendix

Appendix

Appendix : ١

Study Data Sheet

DATE : **SEX :** **AGE :** **NAME :**

Address :

Governorate : District : Sector :

Symptoms :

Splenomegaly: Hepatomegaly : Hepatomegaly :

Hb. : Pcv % : Total WBCs count :

Is the child a Yes **ng pe** When **apy ?**

No

Method used in diagnosis: **esults**

Positive

Negative

History of the disease:

Appendix: ٢

Solution used in electrophoresis :

Reagents and solution for gel electrophoresis :

a. ٣٠٪ acrylamide / ٠.٨٪ bisacrylamide

٣٠ gm acrylamide

٠.٨ gm N , N-methylene – bisacrylamide , $H_٢O$ to ١٠٠ml .

Filter through a ٠.٤٥-µm filter and store at ٤ ^{o}C in dark bottle,discard after ٣٠ days.

b. ٥ x SDS / electrophoresis buffer , pH ٨.٣ :

١٥.١ gm Tris base

٧٢.٠ gm glycine

٥.٠ gm SDS

١٠٠٠ ml D.W.

c. ٢ x SDS / sample buffer

To ٤٠ ml $H_٢O$:

١.٥٢ gm Tris base

٢٠ ml glycerol

٢.٠ gm SDS

١.٠ mg Bromophenol blue

٢.٠ ml ٢-mercaptoethanol

Adjust to pH ٦.٨ with ١N HCL

Add D.W. to ١٠٠ ml .

d. ٤ x Tris. (١/SDS , pH ٦.٨ (٠.٥ M Tris . Cl containing ٠.٤٪ SDS)

To ٤٠ ml $H_٢O$:

٦.٠٥ gm Tris base

٠.٤ gm SDS

Adjust to pH ٦.٨ with ١ N HCl

Add water to ١٠٠ ml .

e. ٤ x Tris – Cl / SDS , pH ٨.٨ (١.٥ Tris . Cl containing ٠.٤٪ SDS) .

To ٣٠٠ ml $H_٢O$

٩١ gm Tris base

٢ gm SDS

Adjust to pH ٨.٨ with ١ N HCl

Add water to ٥٠٠ ml .

Reagents and solutions for Western blotting :

a. Electroplotting buffer (٢٠mM Tris/١٥٠mM glycine , pH ٨)

١.٢ gm tris base

٥.٥٨ gm glycine

٣٣٣.٣ ml D.W.

١٠٠ ml methanol

bring to ٥٠٠ ml with D.W.

b. Ponceau-S solution (٠.٥٪ Ponceau-S / ١٪ acetic acid) :

٠.٥ gm Ponceau-S

١.٠ ml glacial acetic acid

bring to ١٠٠ ml with $H_٢O$

c. Blocking buffer , pH ٧.٢ :

٢٠ mM Tris base

١٣٧ mM NaCl

٠.١٪ Tween ٢٠

٣ gm skim milk / ١٠٠ ml PBS.

d. DAB substrate solution :

٥٠ mg ٣.٣ – diaminobenzidine

٢.٠ ml ١٪ COCl in $H_٢O$

٩٨ ml PBS

٠.١ ml ٣٠٪ $H_٢O_٢$, added immediately prior to use .

e. Coomassie blue solution:

٥٠ % methanol

٠.٠٥ % Coomassie Brilliant Blue R

١٠ % acetic acid

f. Destaining solution: ٤٠ % D.W.

٥ % methanol

٧ % acetic acid

٨٨ % D.W.

g. Fixing solution:

٥٠ % methanol

١٠ % acetic acid

٤٠ % D.W.

Appendix : ٣

Solution used in phenotyping analysis (Indirect method)

***** Diamino benzidine tetra hydrochloride (DAB) / H_2O_2 substrate :

Dissolve DAB at a concentration of ٠.٦ mg / ml (TBS) .

Immediately before use , add hydrogen peroxide to give a final concentration of ٠.٠١٪ .

***** (TBS) Tris – buffered saline :

Dissolve tris – base in D.W. at concentration of ٠.٥ M .(٦٠.٦ g / l), adjust the pH to ٧.٦ with concentrated HCl . Store at ٤ °C .

Appendix : ٤

Buffers used in determination of cytokine standard

***** For recombinant human IFN – γ standard (٢ µg) , dissolve standard vial in ٢٠ µl pure water to a concentration of ٠.١ mg / ml . Dilute in PBS with

٠.١٪ BSA to make up a stock solution of ١٠ mg / ml .

***** For recombinant human IL – ١٠ standard (٢ µg) , dissolve standard vial in ٢٠ µl ٥ mM tris , pH ٨.٢ , to a concentration of ٠.١ mg / ml . Dilute in PBS with ٠.١٪ BSA to make up a stock solution of ١٠ mg / ml .

Appendix : ٥

Reagents for Glutathione reductase activity :

Potassium phosphate buffer ($KH_٢PO_٤$) ٠.١mol / l , pH ٧.٤ :

Solution A : ١.٣٦١ gm of $KH_٢PO_٤$ dissolved in ١٠٠ ml D.W.

Solution B : ١.٧٨ gm of $Na_٢HPO_٤$ dissolved in ١٠٠ ml of D.W.

Mix ٢٠ ml of solution A with ٨٠ ml of solution B , then complete the volume to ١٠٠ ml with D.W.

Appendix : ٦

Reagents for protein measurement :

Reagent A :

Dissolve ١٠٠ gm of $Na_٢CO_٣$ in ١ liter final volume of ٠.٥ N NaOH (٢ gm $Na_٢CO_٣$ in ١٠٠ ml of ٠.١ NHCl).

Reagent B :

١ gm $CuSO_٤$.٥ $H_٢O$ in ١٠٠ ml final volume of D.W.

Reagent C :

٢ gm of Na or K tartarate in ١٠٠ ml final volume D.W.

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