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Identification of Immunoreactive Leishmanial Epitopes Selected by High Positive Human Sera

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DECLARATION

I Certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or Institution.

Signed: _____

Nisreen Saed Alqadi

Date: 16 /05/2012

DEDICATION

I dedicate this work to my Father and my mother, I hope you know how much your tenderness and thoughtfulness mean to me.

Nisreen saed Al-qadi

ACKNOLEDGMENT

It is difficult to mention all those to whom I am grateful at many levels, I apologize to many people that I can't mention here. I would like to express my deep and honest esteem to my supervisor; Dr. Ibrahim Abbasi for his support, directing, encouragement, and technical training in presenting this work. I thank him for his patience and kindness; all words can't express my appreciation and happiness.

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ABSTRACT

Leishmaniasis is a world wide spread disease, it is an endimic health problem in more than 88 countries and threatens over than 350 million people in the world. *Leishmania* parasite is transmitted to vertebrates by the bite of infected female sand fly. The disease exists in three different forms: 1) Visceral Leishmaniasis (VL) or Kala-azar, 2) Cutaneous Leishmaniasis (CL), 3) Mucocutaneous Leishmaniasis (ML).

The main aim of the current study is to identify immunoreactive leishmanial epitopes using M13 phage display library for identifying peptides that mimic some naturally existing leishmanial antigens. Phage display was done by plaque assay in 12 amino acid peptide phage library. For phage screening purposes sera were collected from individuals infected with VL or CL, high antibody titer sera from different patients were pooled after screening for anti-Leishmania crude antigens by Enzyme Linked Immuno Sorbent Assay (ELISA).

By the aid of the high antibody titer anti- VL pooled sera, two screening cycles of M13 phage display library (12 amino acids peptide) were achieved. In the first screening cycle 41 plaques were obtained and 54 plaques were obtained after the second screening cycle. From the 54 plaques, 35 plaques that gave the strongest signals with VL pooled sera were selected for further analysis. The DNA coding the 12 amino acid peptide fused with the PIII M13 phage gene was amplified using specific primers flanking the peptide designed based on the PIII gene. DNA sequence determination was performed for 15 different clones, followed by bioinformatics DNA analysis by means of DNA alignment and BLAST search analysis. From the 15 sequenced peptides 4 clones (namely Ph3, Ph23, Ph5, and Ph19) showed high similarity with *Leishmania infantum* and other *Leishmaina* species

together with other flagellated protozoan parasites mainly *Trypanosoma* and *Trichomonus* species. Since the peptides are inserted in known location in the PIII gene of the M13 phage; with known starting and final amino acids, the peptide sequence for the four mentioned clones was translated; and two clones (Ph3 and Ph23) showed high similarity with *Leishmania infantum* epitopic regions. These findings strongly recommend to conduct further experimental analysis including synthesis of the two identified peptides and to evaluate their prospective use for diagnostic purposes or vaccines for VL.

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LIST OF ABBREVIATIONS

2-ME	2-Mercaotoethanol
Ab(s)	Antibodies
Ag(s)	Antigen(s)
AIDS	Acquired Immune Deficiency Syndrome
BSA	Bovine Serum Albumin
CD 4\8	Cluster of differentiation of 4\8
CL	Cutaneous Leishmaniasis
Con A	Concanavalin A
CP	Cystine Proteinases
CTL	Cytotoxic T-Lymphocyte
D.W	Distilled Water
DAB	Diaminobenzidine
DAT	Direct Agglutination Test
ELISA	Enzyme-Linked Immunosorbent Assay
FCS	Fetal Calf Serum
GBP	Gene B Protein
Gp	Glycoprotein
GRP 94	Glucose-Regulated Protein
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IFA	Indirect Fluorescence Antibody test
IFA	Immunofluoresence Assay
IFN-γ	Interferon-γ
IL	Interleukin
KDa	Kilo Dalton
KMP II	Kinetoplasid Membrane Protein

LeIF	Leishmania elongation Initiation Factor
LmST II	Leishmania major Stress inducible I
LPG	Lipophosphoglycan
LZ	Leishmaniazation
mbA	Monoclonal antibody
ML	Mucocutaneous Leishmaniasis
øD	Phage display
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PKLDL	Post- Kala-Azar Dermal Leishmaniasis
PMOH	Palestinian Ministry of Health
PSA-2	Parasite Surface Antigen 2
rK 39, 26, 9	Recombinant K39,26,9
RT	Room temperature
SDS-PAGE	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
Th T	T helper cell
TNF-α	Tumor necrosis factor
TPBS	PBS containing 0.05% Tween-20
TSA	Thiol-Specific Antioxidant protein
V	Viannia
VL	Visceral Leishmaniasis
WHO	World Health Organization
YPY	Tyr-Pro-Tyr

Chapter One: Introduction

1.1 Background

Leishmania, members of the protozoan family *Trypanosomatidae*, are obligate intracellular parasites that cause disease in human and animals. Leishmaniasis affects 12 million men, women and children in 88 countries around the world, over 350 million people are at risk, and over 2 million cases emerge every year (1). Leishmaniasis occurs in many parts of the world including parts of Europe, Asia, Africa, and the Americas. *Leishmania* parasites infect macrophage of verify of humans and mammalian reservoirs and it is transmitted by the bite of different species of infected female sand fly (Fig.1) *leishmania* parasites are exist during life cycle in two basic forms, promastigotes (flagellated extracellular stage, which are easy to grow in culture) that develops and transmitted by the sand fly, and amastigote (intracellular stage) that reside and multiplies in phagolysosomal vesicles of the macrophages in mammalian hosts(1, 2, 3).

1.2 Life cycle

Leishmania life cycle of the parasite by inoculation of the promastigote form into the skin by female *Phlebotomine* sand flies that transmit the disease, the parasites are internalized by dendritic cells and macrophages in the dermis and transform into amastigotes by losing their flagella. They multiply and survive in phagolysosomes through a complex parasite–host interaction. The parasites in VL disseminate through the lymphatic and vascular systems and infect other monocytes and macrophages in the reticulo-endothelial system, resulting in infiltration of the bone marrow, hepatosplenomegaly and sometimes enlarged

lymph nodes (lymphadenopathy), whereas; in case of CL the parasite remained close to the inoculation site (3,4) (Figure. 2).

igure 1: Female *Phlebotomus* (Adapted from CDC)

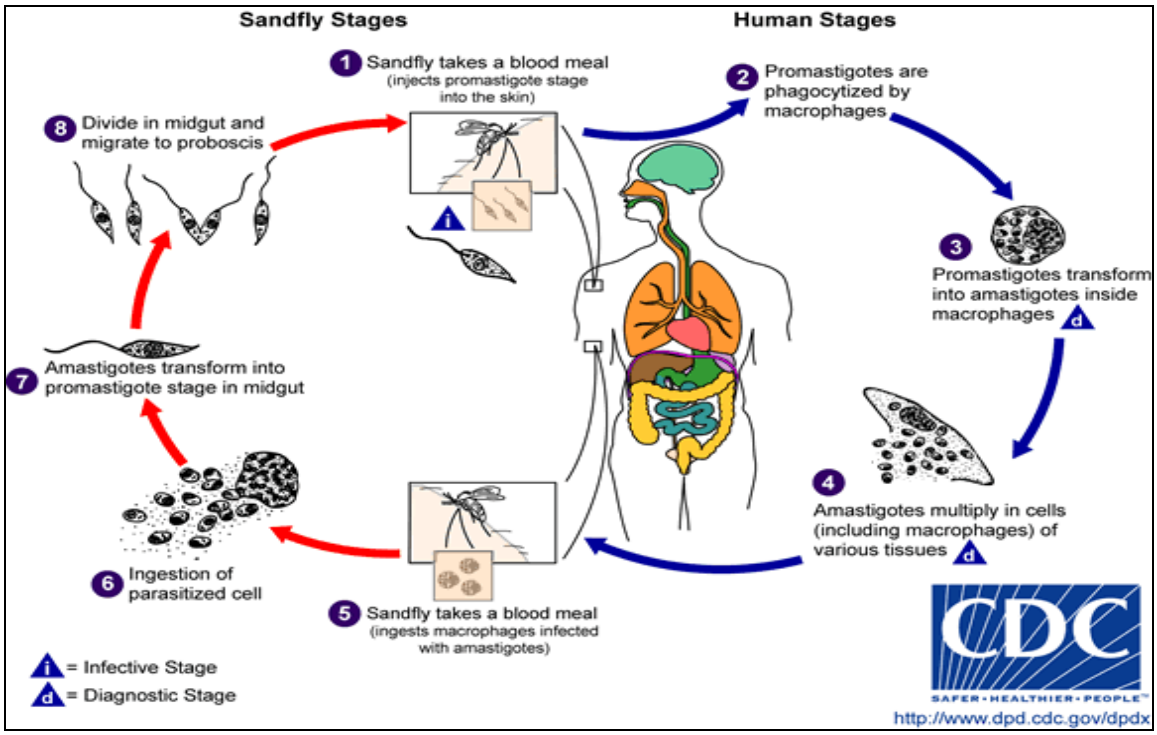

Figure 2: Life cycle of *leishmania* parasite, adapted from CDC.

Infection with leishmaniasis species can result in three main types of disease depending on the species, geography region and host immune response, the three main forms of disease are visceral leishmaniasis(VL) or Kala-Azar caused by *Leishmania infantum* and *Leishmania donovani*, Coetaneous leishmaniasis(CL) caused by *Leishmania major, Leishmania tropica*, *Leishmania aethiopic*a in addition to *Leishmania braziliensis* and *Leishmania mexicana* in new world, Mucocutaneous leishmaniasis(ML) primarily caused by *Leishmania braziliensis braziliensis* does not heal.(3,4,5,6). Most infections in *Leishmania* exist as zoonoses amongst wild animals, such as rodents and dogs, and are prevalent in rural of forest areas and new world, while man is usually an incident host (3). Globally, there are an estimated 1.5–2 million new cases; and 70 000 deaths each year, and 350 million people are at risk of infection in 88 different countries (1,4). Around twenty different species of *Leishmania* parasite are capable of infecting humans(1)

1.3 Cutaneous leishmaniasis

Is considered as a public health problem in many developing countries worldwide (1,3) and is endemic in the tropics and subtropics areas (1,7), is generally a mild disease in man with incubation period of one to 12 weeks (3,7,8), has extensively studied in both humans and mouse model systems (3). the parasite is confined to the skin(8) so it is usually called self – limiting skin disease, usually on exposed parts of the body such as the face, arms or legs (fig. 3) (3,8,9), appears as either single or multiple lesions varying in size (3,9,10). The lesion is limited to a single part of the skin (localized cutaneous leishmaniasis) or may produce a large number of lesions (diffuse cutaneous leishmaniasis) (3,10). The appearance of these lesions is like volcano, with (a raised edge and central crater) (Fig.3), some of them are covered by a scab (8,11). Cutaneous leishmaniasis can be divided into:

New World disease, primarily caused by *Leishmania braziliensis* complex and *L. mexicana* complex, and Old World disease (Table.1), primarily caused by *L. major*, *L. tropica*, and *L. aethiopica (3,7,8,11),* Some Leishmania species are closely linked to humans so it found in cities (e.g., *L. tropica*), whereas some associated with animal species (zoonoses) so it more traditionally (e.g., *L. major*).

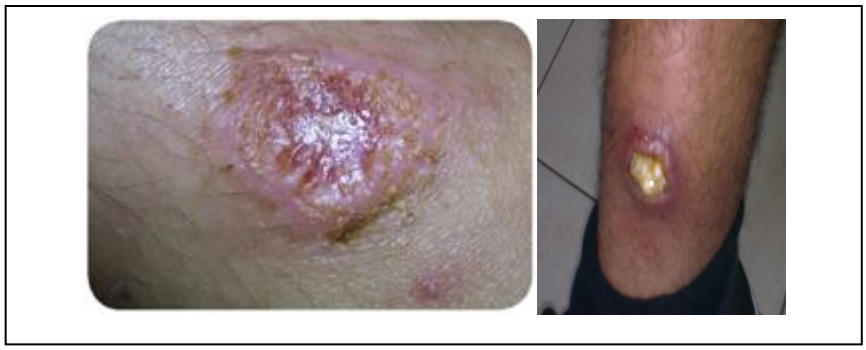

Figure 3: Cutaneous Leishmaniasis

Table. 1: Geographic distribution of Leishmaniasis species in the old world.

***Leishmania* Species**	**Geographic distribution**
L. major	Middle East, Indian Subcontinent, northwestern China, Africa
L. tropica	Middle East, Indian Subcontinent, Western Asiatic areas.
L. aethiopica	East Africa, Yemen
L. infantum	Mediterranean basin
L. donovani	Sudan, East Africa, Asia

1.4 Mucocutaneous leishmaniasis

Is life threatening in contrast of CL where it causes a lesions at site of infection that can lead to partial or total destruction of the mucous membranes of the nose, mouth and throat cavities and surrounding tissues(fig. 4) with incubation period about 2-6 months or longer. Relapsing may act within 10 years, it caused by Leishmania species of the Viannia subgenus (*Leishmania*. (V) *braziliensis*, *Leishmania* (V) *amazonensis*, *Leishmania*. (V) *panamensis*, and *Leishmania* (V) *guyanensis* (9,12,13) but the ML that caused by *L. braziliensis braziliensis* is not heal (9)

Figure 4: Mucocutaneous leishmaniasis

1.5 Visceral leishmaniasis (kala-azar)

Is a systemic disease and may be causes death if not treated, so it is considered as the most dangerous of the three manifestations of disease (1,5,14,15), whereas; The parasites invade bone marrow causing the immune system to weaken and increasing a person's vulnerability to infection and disease. The risk for co-infection with AIDS is also rising.

VL caused by two *Leishmanial* species, *L. donovani* or *L. infantum*, depending on the geographical area, where *L. infantum* infects mostly children and immunosuppressed individuals, whereas *L. donovani* infects all age groups. VL causing chronic fever, weight loss, splenomegaly, hepatomegaly, and anemia (fig. 5). (3,6,16,17,18) it endemic in 65 countries about 90% of cases found in poorest areas as Bangladesh, Brazil, Ethiopia, India, Nepal and Sudan with 100,000 death case each year (1,17). VL classified into two types according to transmission way: zoonotic VL found in area of *L. infantum* it transmit from animal to vector to human and anthroponotic. VL found in area of *L. donovani* it transmit from human to vector to human (6,18,19), the major reservoir for *L. infantum*(*chagasi*) are dogs, some types of this species of VL may cause CL in humans (21). In some studies the Leishmaniasisi classify into four types instead three, whereas in some patients of VL after recovery the disease may develop into Post-Kala-Azar dermal leishmaniasis(PKLDL) that requires prolong and expensive treatment(1,5,6), PKLDL is syndrome that develop after VL relapsing, whereas it appears as skin lesions that prominent on the face, may be the patients with chronic PKLDL could be a considered as reservoir host so they play a role in VL transmission(1,5,6).

Figure 5: patients with visceral leishmaniasis adapted by WHO

1.6 Diagnosis and identifications

The first Diagnosis of *leishmania* classically by symptoms and direct microscopy through detection of amastigote stages by Giemsa-stained smears. Also it is possible to use culturing methods of promastigote originated from *Leishmania* amastigote stage isolated from bone-marrow, lymph nodes or spleen aspirate (15,19), all these methods are considered as a classical methods and have disadvantages as in microscopic, it lack sensitivity, in cultures technique is time consuming and have contamination risk (19). Also, serological and molecular diagnostics approaches were followed, serological test based on direct agglutination test (DAT) with sensitivity of 94% but specificity only 72%, or immuofluorescence (IFA) and ELISA that using a recombinant antigen of parasite lysate (18,19). The use of whole parasites or its lysate in diagnosis did not showed high specificity to the disease. Western blotting techniques have been used to identify antigens for the specific and sensitive serodiagnosis of VL. One antigen, rk39, is a 39 amino acid repeat that is part of a kinesin-like protein expressed by *L. chagasi* tissue amastigotes (19). Recombinant k39 (rk39) was used in an ELISA as well as in a rapid immunochromatographic dipstick test (15,18,19) for the serodiagnosis of VL patients with active disease, with HIV co-infection, and after successful treatment (15,17,18).

Developing rapid, inexpensive, sensitive and specific diagnostic tests approach based on molecular diagnosis was adopted by many researchers (15,18). A number of PCR assays have also been developed over the years for the detection of *Leishmania* DNA in a variety of clinical samples such as skin biopsies and smears, bone marrow and lymph node aspirates and peripheral blood. Nevertheless, there is no “unified” *Leishmania* PCR

available yet and the application of PCR as a routine diagnostic method is still limited by the mandatory technical expertise and equipment required.

1.7 Treatment

The main goal of Treatment of (CL) is inhibition of amastigote growth and the reduction of lesion size. In general; new world leishmaniasis is more sever with long lasting compared to old world that is heal spontaneously. Leishmanial lesions can be removed by traditional skin treatments including: cryotherapy, thermotherapy, electrotherapy, and surgery (7,8,11). Of the most famous chemical treatments is the use of paromomycin, pentavalent antimony, and amphoteracin B. The most common treatment is pentavalent antimonial derivatives sodium Stibgluconate (Pentostam) developed with meglumine antimoniate in the 1940s, their mode of action is inhibit the glycolysis and fatty acid oxidation of leishmania. Amphotericin B is a second line treatment using when pentostam is ineffective (WHO/ 2002,7,8,9,11,13).

1.8 Epidemiology of leishmaniasis:

1.8.1. Globally:

The disease is endemic in 88 countries (66 are found in old world and 22 in new world), in southern Europe, central and south America, Africa, the middle east and south Asia (WHO 1991, 1996, 1998, 2003,3,4,7,9). Today, an estimated 10-15 million individual have leishmaniasis all over the world, approximately 350 million at the risk of infection, and there is a new 1.5- 2 million cases each year (1,7,9). In case of CL an Estimation of 1-1.5

million of CL each year, 90% occurs in seven countries: Afghanistan, Algeria, Brazil, Iran, Peuro, Saudi Arabia and Syria in old world and Brazil and Peuro in the new world, around (7,9,16,). There are about 500,000 new cases of VL with 70,000 death each year, 90% occur in India, Bangladesh, Nepal (form 1-3%), Sudan, Kenya (form 50%) and Brazil (WHO 1991, 1996,4,6). 25-33% of new cases of CL comprised with VL. (1,5,7,9).

1.8.2. Locally:

Although and before many decades Cutaneous leishmaniasis clinical features were called "Jericho sore" but till now there is no comprehensive study that definitely indicate the *Leishmanial* causative species, the transmitting *Phlebotomine* vector, and the incriminated reservoir host in Jericho city. Only two epidemiological studies were performed on Jericho city, the former of them showed that 26.3% out of 190 individuals were sero-positive for Lesihmaniasis by ELISA test (20). The authors related the positive cases of CL to *L. major* without further parasitological studies that confirm the causative *Leishmanial* species. One year later and in another study; CL cases in Jericho were related to *L. major* and *L. tropica* species (20). In this study *Leishmanial* species were analyzed by ITS-PCR technique followed by endonuclease HaeIII digestion and showed out of the 156 CL examined samples 81cases(51.9%) were due to *L. major* infection, while 72 cases (46.1%) were related to *L. tropica*, and two samples were not identified (21). Since that time there is no studies and there are no evidence that indicating the species of the transmitting sand fly or the reservoir host in Jericho city. The clear transmission of *Leishmanial* parasite by *Phlebotomus papatasi* among their animal reservoir hosts and then its anthropophilic transmission from animals to humans is yet not studied in this area.

Regarding the prevalence of *L. infantum* the causative agent of VL in Palestine, reports of Palestinian Ministry of Health (PMOH) indicated that VL is located in southern and northern parts of the West Bank with high prevalence rate in the Hebron and Jenin districts and in the Bal'a village in north western Tulkarem city, all cases in children less than 9 years old with annual incidence of 3.02/100,000 child (22,23).

1.9 Immunity

Leishmania parasite transmitted to the person through the bite of female sand fly, the effect of it on victim can be influenced by immunity status of the host so it depending on both innate and adaptive immunological response. The first target cells faced *leishmania* parasite are macrophage cells in skin or in visceral organs depends on *Leishmanial* species. So with regard to the fact that macrophages are the major host immune defense mechanism against Leishmania, infected macrophages are activated by Interferon-γ produced by T-cells that mediate acquired resistance to leishmaniasis (24, 25, 26, 27).

IL-12 required for initiation of the infection by development of Th1 cells and maintain resistance to leishmania, and this because IL-12 enhances IFN-γ production by Th1 cells; in absence of IL-12 the IL-4 produced by Th2 cells, this called Th1/Th2 paradigm (24,25,27,28,29,30,31,32,33). Previous studies(1994,1997) described that the activation of Th1 cells are associated with cellular immune response that develop protection through production of IFN-γ-activated infected macrophages to produce nitric oxide to kill the intracellular replicating amastigotes. IL-12 considered as the important cytokine between innate immune response and acquiring protective Th1 cellular response specially in CL, whereas in VL and ML appeared insufficient production of Th1 cytokines to control the

infection (33,34,35,36,37,38,39,40). $CD^{+}8$ cells have two mechanisms in protection against *Leishmainia*: producing cytokines as (INF-γ and TNF-α) or by killing the infected cells directly by Cytotoxic T-lymphocyte (CTL) – mediated mechanism (40,41,42,43). In case of B cells and antibody response considered not have a critical role against leishmania that hide inside the cells but have correlation with pathology since IgG induce IL-10 that promote the infection, humoral immune response appears in patient with VL, where they have a predominant IgG antibodies (39).

1.10 Antigens of *Leishmania*

Detecting of specific anti-*Leishmanial* antibodies is needed to know the antigen that evoke the immune response whereas the correct diagnosis of Leishmaniais essential to select the specific therapeutic approach (37,38,39,40). Several antigens of *Leishmania* species were identified and were used for diagnostic purposes, Parasite expressed antigens play important role in mediating *Leishmania* infection, rehearses Detecting specific anti-*Leishmanial* antibodies need know the antigen that evoke the immune response. So in the last decade, several *Leishmania* antigens characterized to diagnose of *Leishmania* and develop vaccine and advanced methods of diagnostic with high sensitivity and specificity, whereas the correct diagnosis of Leishmaniais essential to select the specific therapeutic approach(40,44,45,46,47). several antigens were discovered and characterized genetically and antigenically in *Leishmania* species that used in diagnosis, whereas immunodiagnostic methods use whole promastigotes as antigen source.

rK39 is a recombinant antigen used to diagnose VL caused by *L. donovani*, *L. infantum* and *L. chagasi*, it is predominant in *L. infantum* and *L. donovani* tissue amastigotes so

rK39 used to diagnose *L. infantum, L. donovani* and *L. chagasi* although it firstly derived from *L. chagasi* as a super family of motor proteins(19,43,44), derived from a 39-amino acid repeatitive immunodomenant B-cell epitope of the 230KDa LcKin protein (19,43,48,49), but the seroreactivity of rK39 relates with active disease, whereas it is highly sensitive and specific for acute VL diagnosis so used for the development of strips test, but it's sensitivity limited in endemic area(19,43,49). There is no reactivity with other pathogens or to one *Leishmania* species as Cutaneous leishmaniasis.(49). Recently, new related hydrophilic antigens of recombinant antigens of *L. chagasi* cloned and characterized to use in serodiagnosis of VL. rK9 and rK26, rK26 differ in presence of 11 copies of a 14- amino- acid repeat in the open reading frame(49,50,51). rK26 also specific to VL but it not tested as rK39(no other recombinant *leishmanial* protein has been tested(19,44,52). rK39 dipstick is only available rabid test for diagnostic *Leishmania* (WHO, 2009), according to several studies; the rK39 is the most suitable antigen for serological diagnosis followed by rK9 and rK26.

Leishmania. major also as most *leishmanial* species express surface protein that play important roles in mediating *leishmania* infection. Two major classes of membrane antigens identified on the surface of the *leishmania* parasite; a family of glycolipids (LPG) and a family of glycoprotein gp63 and PSA-2 were well characterized in *L. major*, LPG, gp63 and gp46 expressed in promastigote stage and contribute in host-parasite interaction and disease pathogenesis (53,54,55,56).

LPG consisting of lipophosphglycan (LPG) involved in parasite attachment to host macrophages and initiation of infection.

Gp36 is major surface glycoprotein, it is zinc-requiring actoprotease which anchored on the surface of Kinetoplastid of Protozal parasites of the genus of *leishmania* consist of 63 KDa surface protein expressed with more than 500.000 copies it distributed over promastigote, some highly expressed in long-phase promastigote or in stationary phase promastigotes, so it described as major protease (leishmanolysin) because it mediate the entry mechanism into macrophages, inducing phagocytosis and survival in macrophage, Gp63 is also present in amastigote (53,54). This protease active molecule play a main role in resistance of complement-mediated lysis, so it is a candidate molecule for vaccination (53, 54). The role of gp63 in *L. amoazonesis* was examined experimentally and was identified to play role in binding and survival of promastigotes within host macrophages. Macrophage binding could be mediated in two ways: (i) indirectly, through the complement component C3bi, which, after being generated from C3b by gp63 protease, attaches to the complement receptor on macrophage; here C3bi acts as a bridge between gp63-bearing promastigotes and the macrophage; and (ii) gp63 binds directly to an integrin receptor on the macrophage (24, 53).

PSA-2: (Parasite Surface Antigen 2 family of proteins), which is another surface protein, belongs to a family of genes, which are highly polymorphic and produce multiple mRNA transcripts (56). These complex gene loci and its protein products have been detected in all *Leishmania* species examined except *L. braziliensis.* The function of PSA-2 play role in prophylactic immunization by encoding into plasmid DNA, also PSA-2 is effective in vaccination (11,56). However, areas of PSA-2 show similarity to *Trypanosome cruzi* and human mucins and to *Drosophila* glu protein, suggesting a possible role in protein- protein interaction. *L. major* PSA-2 has been shown to protect vaccinated mice subsequent to challenge with parasite by a Th1 type immune response (24, 56)

In addition there are other several proteins present in VL and CL evoke antibodies response; such as proteoglycolipid complex expressed by amastigotes P8 PGLC (55) *Leishmania. major* stress inducible1 (LmSTI1) protein (57), *Leishmania* thiol-specific antioxidant (TSA) protein (57), specific amastigote *L. donovani* A2 proteins, repetitive sequence of *L. major* gene B protein (GBP, for infective stage of leishmania). LmSTI1, GBP and HASPB1 can evoke antibodies in Post-Kala-Azar Dermal Leishmaniasis (PKDL) patients. While in ML the antibodies react with *leishmania* elongation initiation factor (LeIF) and KMPI1. The presence of IgM considered as early diagnostic marker (30) whereas the predominant antibodies in patients are IgG (55,57).

1.11 Vaccination

Leishmaniasis as any other disease needs to inducing immunity against re-infection. Development of vaccines need to understand the different immunological mechanisms against Leishmania and its expressed antigens. Nevertheless vaccination by the amastigote antigens are the most important antigens candidates since their antigens are exposed for longer time to host immune system (58,59,60,61,62).

1.11.1. Leishmanization:

Leishmanization is the oldest form of vaccination against Cutaneous leishmaniasis and has been practiced for over a century (58,61,62). Exudates from active lesions were inoculated into a covered part of the body of healthy children to induce a self-healing lesion and protection against multiple lesions on the face and other exposed parts of the body. This

approach later became known as “Leishmanization” (LZ) and live virulent promastigotes of *Leishmania major* harvested from cell free cultures of the parasite replaced inoculation from exudates lesions (62, 60, 63, 64, 61). Leishmanization use was later on limited due to:1) long-lasting skin lesions, 2) the spread of HIV (58,61,62,64).

1.11.2. First generation vaccine (killed or fractions parasite):

The first approach to developing a leishmaniasis vaccine was to use attenuated organisms. The ease of growing *Leishmania* in culture media made it possible to use promastigotes grown in vitro. Most vaccines used the *L. amazonensis* autoclaved lysate, while studies in the old world used autoclaved *L. major* antigen (58,59,60,61,63).

1.11.3. Candidates for second generation vaccines:

Live vaccines, genetically modified *Leishmania* species, and recombinant antigens are of the most important candidate second generation vaccines (58,60,61). These vaccines were started by having purified *Leishmania* sub-fractions such as the *Li*ESAp-MDP, which is composed of the 54 kDa excreted protein of *L. infantum* plus MDP, protected dogs in a kennel assay against *L. infantum* infection, *Li*ESAp vaccine induced protection against infection, but not against severe disease or death by VL.

The second approach in second-generation vaccines is the use of recombinant proteins that were intensively tested since the 1990s. Of these antigens: the LeIF and HASPB1 proteins (58,61). Also, the TSA (thiol-specific antioxidant) and LmSTI1 (*L. major* stress inducible

protein 1), and LeIF (*Leishmania* elongation initiation factor) which are protective for mice and monkeys against CL (58,59,65)

1.12 Random Peptide Phage Display Libraries

An alternative approach for vaccine discovery is the utilization of phage display libraries. Epitope discovery by the aid of phage display technique is a new approach for identifying peptides or peptides that mimic protein and non-protein epitopes (66,67,68). The phage display describes a selection technique in which a peptide or protein is expressed as a fusion protein on the surface of filamentous phage resulting in display of the fused protein on the surface of the phage, while the DNA encoding the fusion resides within the phage (69). Phage display involves the production and screening of large numbers of random peptide sequences of a specific length (8-20 amino acids) expressed on the surface of phage particles (70). Phage display libraries have been used to study the binding specificity of numerous peptides and protein domains. Practical applications include the identification of peptide sequences that bind with high affinity to antibodies, enzymes or receptors, and may serve as diagnostics and vaccine or drug candidates (69,70.). Most phage display libraries consist of short peptides or protein fragments expressed on the surface of filamentous phage as N-terminal fusions to the pIII or pVIII coat proteins(71). There are four or five copies of PIII protruding from one end of the phage particle, and up to several thousand copies of pVIII, the major phage coat protein. Both proteins are translocated through the bacterial membrane, where phage particles are assembled before exported into the periplasmic space. (72)

Several groups were able to produce different phage libraries, with random inserts of six or 15 (70,73) amino acids. Using well-characterized monoclonal antibodies, it was shown that specific phage bearing specific sequence motifs could be isolated from these libraries. These libraries have been used for a variety of purposes, such as the mapping of antibody epitopes (73), development of novel enzyme substrates (74) and inhibitors (75), identification of recognition sequences on cell adhesion molecules (74), selection of high-affinity DNA-binding zinc fingers (76), cloning of receptor ligand-binding domains (77). Phagotopes displaying the desired peptide can be isolated by affinity chromatography or biopanning on immobilized selector molecules. Selectors that have been used include antibodies, membrane receptors, enzymes, cultured cells, serum samples, and even the whole animal (77).

Since peptide libraries almost provide an infinite source of molecular shapes, theoretically they include peptides that mimic epitopes of any antigen. Therefore, it becomes reasonable to investigate whether such small peptides can mimic carbohydrate epitopes. Using such libraries, small peptides have been identified that interact with the carbohydrate binding site of the lectin Concanavalin A (Con A) (78, 79). It was demonstrated that peptides containing the consensus sequence (YPY) bind Con A (78,80). Also, a peptide mimotope of the Lewis Y antigen was selected by screening a phage library using a specific mAb (79). Four residues (PWLY) were found to be critical for peptide binding to the antibody against the Lewis Y antigen, (79).

So, one major application of random phage display peptide libraries is to determine the epitope of an antibody. Antibodies recognize small peptide motifs based on only three or four conserved residues (81). Based on the epitope motif identified by phage display, it is

possible to define the region of a protein recognized by the antibody. An antibody can either be directed against a linear amino acid sequence in a protein or a discontinuous epitope, which has a distinct conformation formed by the protein folding. Phage library screening with such antibodies has yielded peptides that mimic the structure of a folded protein. These ligands are called mimotopes (81).

The pIII phage display library that was used in this study have peptides expressed at the N-terminus of pIII coat protein of M13. The phage library was the Ph.D-12 random peptide phage library (New England Biolabs, Beverly, MA). Each phage expresses 12 different amino acids and in total the library contains 1.5×10^{13} pfu/ ml with a complexity of 2.17×10^{9} transformants.

1.13 Objectives

The main aim of this research is to identify immunoreactive leishmanial epitope selected according to the reactivity of high antibody positive sera (obtained mainly from visceral leishmaniasis patients and cutaneous leishmanisis patients) with different plaques assay of phage displayed library that contain millions of antigenic epitopes mimics some naturally existing leishmanial antigens. Bioinformatic analysis will be performed to determine order the DNA sequence of the selected epitope. The newly identified epitopes will serve as potential diagnostic markers of infection or they can be used in future leishmanial vaccination development.

Chapter Two: Methods And Material

2.1 *Leishmania* stocks

Several *Leishmanaia* species were used in this study (*L. major, L. tropica*, and *L. infantum*). The cultures were obtained from the WHO *Leishmania* references office from the Hebrew University. The *Leishmanial* species were brought in culture flasks containing promastigotes stages in M199 medium with (HEPS,10% FCS, Gentamycin and penicillin 100mg\ml). Flasks were kept at 26˚C until antigen preparation.

2.2 Antigen Preparation

Leishmanial antigen was prepared for ELISA and Western Blot analysis according to the following procedure: Promastigotes of the *L. major, L. tropica* or *L. infantum* were collected from 50 ml M199 culture containing at least 10^6 parasites/ml by centrifugation at 3,000 rpm for 10 minutes. The supernatant was completely aspirated and the parasite pellets were resuspended in 3 ml of lysis buffer (10 mM EDTA, 50 mM Tris, 50 mM NaCl, 0.01% SDS and PMSF Saint Louis protease inhibitors (Sigma, USA)) incubated at 4˚C for 30 minutes. Protein concentration in the crude lysate was determined as indicated below. Parasite lysates containing crude antigen were frozen at -20˚C; crude antigen was thawed prior to its use in ELISA or Western blot.

2.3 Protein Determination

Protein concentration for the crude lysate antigen preparations was determined by Bradford macroassay using Coomassie Brilliant Blue G-250 dye according to standard method and to the manufacturer instructions (BioRad, Hercules, CA, USA).

2.4 Enzyme Linked Immunosorbent Assay (ELISA)

96-well microtiter plates were coated with leishmanial antigen (50 ul/ well) and incubated for 24 hours at 4˚C, plates were washed 3 times with phosphate buffered saline with 0.05% Tween-20 (PBS-T), blocked with 200 ul/well of PBS-T + 5% FCS or BSA and incubated for 30 min at room temperature, washed 3 times with PBS-T, 100 ul of serially diluted serum samples (starting from 1:100) were added as first Abs and incubated for 2 hours at 37˚C, washed 3 times with PBS-T, 100 ul diluted protein A or HRP- conjugate (1:10000) was added as second Abs and incubated for 1 hour at room temperature, washed 3 times with PBS-T. color development was accomplished by adding 200 ul of the substrate-chromogen solution (1mg of the O-phenyldiamine to each 1ml of citrate buffer (PH 4.5) then 4 ul of the substrate (H_2O_2) was added to each 10 ml of the citrate buffer containing the chromogen and incubated for 30 minutes at room temperature, color development was measured at 360 nm by using ELISA autoreader. According to ELISA reader, the cutoff values and later the antibody titers were calculated. Negative controls were included on each microtiter plate.

2.5 SDS-PAGE Gel Electrophoresis

Lysates of *L. major*, *L. donovani*, *L. infantum* and *L. tropica* were seperated on 8% SDS-polyacrylamide gels (SDS-PAGE) according to Garfin 2003 (82). Antigens lysates (10 µl of 50µg total protein) were mixed with 5 µl loading buffer (10% glycerol, 5% 2-ME (disulfide reducing agent), 5% SDS and 0.5M Tris PH 6.8), the mixture was boiled for 5 min and loaded on the SDS-PAGE gel. The gel was run at 50v in stacking and 100v in seperating gel. Bio-Rad molecular weight markers (10-250 KDa) of low range were used.

2.5.1. Coomassie Blue:

Polyacrylamide gels were stained with comassie blue (1.25g coomassie blue R 250, 125ml methanol, 50ml acetic acid and 325ml D.W) for 15 min then destained (400ml D.W, 50ml methanol and 50ml acetic acid).

2.5.2. Silver stain:

The Polyacrylamide gel was fixed in 50% methanol minimum for one houre, washed 3X with D.W for 5 minute each, stained with A-silver nitrate (A- 0.8g nitrate stain was dissolved in 4ml D.W; B- 0.036g NaOH mixed in 10ml with 0.7ml ammonium hydroxide completed to 48ml with D.W, mixture A was titrated to mixture B, gel was stained with A-B mixture for 15 minutes, the gel was washed 3X with D.W for 15 min each, the gel was developed for 1-10 min in (1.25ml of 1% acetic acid mixed with 0.125ml of 38% formaldehyde made to 250ml with D.W) until the bands observed, developer was removed by D.W.

2.6 Western Blot Technique

Western blot analysis was done according to Towbin, 1979 (83). Following SDS- PAGE, described above, the gel was equilibrated for 30 minutes in transfer buffer (0.025M Tris-HCl, 0.192M glycin and 20% methanol). Proteins from polyacrylamide gel were transferred to nitrocellulose membranes (Whatman, USA). After transfer, the membrane was cut into strips and blocked with 5% skimed milk prepared in phosphate buffer saline with 0.1% Tween-20 (PBS-T) for 30 min at RT. After that; stripes were incubated for 2 hours at room temperature with diluted 1:500 VL infected pooled sera in PBS-T solution. Unbound antibodies were washed 3X for 5 minutes each with PBS-T and then seconding Abs (Protien A-HRP or anti-Human IgM-HRP) diluted in PBS-T (1:4,000) for 1 hour at room temperature. Excess secondary antibodies were washed off 3X for 5 min each with PBS-T. Bound antibodies on gel strips were detected with DAB stain (25 mg DAB in 25 ml PBS, 0.05% H_2O_2), Strips were incubated for 15-30 minutes at room temperature until the color was developed.

2.7 Phage Display Peptide Library:

The øD. 12 peptide ø - display library Kit E8110S (New England Biolabs, Ipswich, MA, USA) was used, mainly according to the recommendation of the manufacturer with slight modifications; the specific used materials and protocols are described below.

- Bacterial cells: *E. coli* ER2738 host strain

- Lauriabroth (LB) Medium: was prepared by dissolving 10 g Bacto-Tryptone, 5g yeast extract, 5g NaCl in double distilled water, autoclaved and stored at room temperature.
- LB Plates: To 1 liter LB medium, 15 g bacto-agr was added, dissolved and autoclaved, and then poured in Petri dishes, plates were stored upside-down at 4°C until used (within 3 weeks).
- LB top agar: To 100ml LB medium, 0.7g bacto-agr was added, the solution was prepared in 200ml bottle, autoclaved, and stored at 4°C until used. The molten top agar was equilibrated to incubated 50°C before use.

2.7.1. M13 bacteriophage titration:

M13 phage was obtained as part of the kit (phage display library NE, Biolabs, USA):

1. 5ml of LB broth was inoculated with E. coli ER2738 from a freshly grown plate culture and incubated overnight with shaking 200 rpm. In the next day, a dilution of 1:50 was made in 50 ml LB broth and cells were grown at 37^{o}C with shaking 200 rpm until reached mid-log phase, (OD600 ≈ 0.5).
2. While cells are growing, top Agar was melted in microwave and dispensed into 3 ml in 15ml sterile culture tubes, one tube per each phage dilution. Tubes were maintained at 45°C.
3. LB agar plates were pre-warmed, for at least one hour at 37°C until ready for use.
4. Serial dilutions of M13 bacteriophage in LB (10^4 to 10^8-fold) were prepared in 1 ml LB final volumes.
5. When the culture in Step 1 reached mid-log phase, 200 µl aliq-uots were dispensed into Eppendorf tubes, one for each phage dilution.

6. To carry out infection, 10 µl of each phage dilution was added to each Eppendorf tube containing the bacteria, mixed, and incubated at room temperature for 1–5 minutes.
7. Infected cells were transferred one infection at a time to culture tubes containing 45°C top agar. Tubes were vortexed briefly and immediately poured onto a pre-warmed LB plate. Plates were gently tilt and rotated to spread top agar evenly.
8. Plates were incubated overnight at 37°C.
9. Phage titer that allows growth of about 500 plaques per plates was used.

2.7.2. M13 phage plaque assay:

The M13 phage titer that gave about 400 to 500 plaques per plate was used along all the peptide screening assays. In each screening cycle about 10 plates 500 plaques/ plate were prepared as mentioned above in M13 phage titration, the obtained plaques were transferred onto nitrocellulose membranes as described below.

2.7.3. Plaque lifts onto nitrocellulose membranes:

The prepared M13 phage plates were chilled for 2 hours at 4°C to prevent the top agar from sticking to the nitrocellulose membrane. To each LB agar plate containing plaques a nitrocellulose membrane was placed over the plaques for 2 to 5 minutes to allow the transfer of the phage particles to the membrane. A needle soaked with waterproof ink was used in making prick through the membrane and agar for orientation. The membranes were removed from the plates and kept at room temperature for 1 hour to dry and to allow the binding of the phage particles to the membrane. The dried membranes were submerged in

phosphate buffered saline for few minutes then blocked with 5% BSA in PBS with 0.5% tween-20 for 30 min, then screened by VL high antibody titer sera as mentioned in Western blotting (see 2.6).

2.7.4. Screening by normal human sera:

All selected plaques that showed reactivity with VL collected sera were screened by normal human sera. All the analyzed clones of high potential as VL epitopic regions did not show any reactivity with normal human sera.

2.8 Polymerase Chain Reaction (PCR)

PCR reaction was carried out in a final volume of 25µl Ready Mix PCR tubes (Syntezza, Jerusalem). For each tube a 20 µl of sterile water containing 15 pmoles of each primer (reverse and forward) was added followed by the addition of 5 µl from each extracted DNA sample. The used PCR amplification protocol involving 5 min at 95^{o}C, followed by 35 cycles each of 30 seconds at 95^{o}C, 30 seconds at 55^{o}C (depends on the used primers), followed by 1 min at 72^{o}C, and a final elongation step at 72 ^{o}C for 10 min.

2.9 Agarose Gel Electrophoreses

The PCR products were separated on a 1.5 % agarose gel in TAE buffer (Tris base, acetic acid and EDTA). The pUC 8 Mix Marker (Fermentase, Lithuania) was used for sizing PCR amplifications.

2.10 DNA Purification

The PCR products were cleaned by Qiaquick PCR purification Kit (Qiagen, Hilden, Germany), according to manufacturers' instructions. In a few cases, where multiple bands appeared, these were excluded from this study.

2.11 DNA sequencing

Purified PCR products were sequenced according to dye terminator method, using Automated DNA Sequencer machine (AB477). Using the primers which were used for PCR amplification, for each PCR product forward primers were used in DNA sequences acquaint. Obtained sequences were then aligned by using BLAST online service on Pub- med to determine homology.

Chapter Three: Results

3.1 Enzyme Linked Immunosorbent Assay (ELISA)

The collected serum samples from known Visceral and Cutaneous leishmaniasis infected individuals were examined by ELISA method for their reactivity against *Leishmania* parasite crude antigen. The antibody titers for the different tested serua samples were identified (Table 2), taking into consideration a cut-off value calculated based on local normal human serum samples. From the obtained results it is clearly seen that most of the examined VL cases have a higher antibody titer (1/1600) compared to CL cases that ranged from 1/100-1/800.

Table 2: Antibody titers of the different tested sera based on ELISA test against *L. donovani* crude antigen**.**

Sample No.	Location	Leishmaniasis Type	Antibody Titer
1	Jenin	VL	1/1600
2	Hebron	VL	1/1600
3	Jenin	VL	1/1600
4	Jenin	VL	1/1600
5	Jenin	VL	1/1600
6	Hebron	CL	1/100
7	Jericho	CL	1/100
8	Jericho	CL	1/100

9	Hebron	CL	1/100
10	Hebron	CL	1/100
11	Jericho	CL	1/100
12	Jericho	CL	1/100
13	Hebron	CL	1/100
14	Hebron	CL	1/100
15	Jericho	CL	1/200
16	Jericho	CL	1/200
17	Jericho	CL	1/200
18	Jericho	CL	1/200
19	Jericho	CL	1/600
20	Hebron	CL	1/800

Serum samples of high antibody titers were pooled together, 5 samples of VL cases and 6 samples of CL cases with antibody titers ranged from 1/200 to 1/800, the CL cases with low antibody titers (1/100) were excluded from the study. The main aim of the serum pooling strategy is to have larger volumes of starting anti-*Leishmania* antibodies that is sufficient for western blot and ø - display analysis to be conducted in this study.

3.2 SDS-Polyacrylamid gel electrophoresis (SDS-PAGE) and Western blotting

3.2.1. SDS-PAGE:

Different *Leishmanial* crude extracts were analyzed by SDS-PAGE and Western blotting techniques. The extracts were prepared from whole cultured *Leishmania* parasite cells; extracts from *L. major and L. tropica* (the causative agents of CL) and *L. infantum* (the causative agent of VL). From the prepared three extracts about 10µg total proteins in 15µl total volume were separated on SDS-PAGE. Four different gels were prepared; each containing the three different parasites' crude extracts and MW marker. Two of the gels were stained with 1- Commasie blue satin (Figure 6), and 2- Silver stain (Figure 7). As seen in figure 6 and 7, that represent comparison between the proteins banding patterns from the three different crude extracts; relatively all the extracts have the same protein quantities.

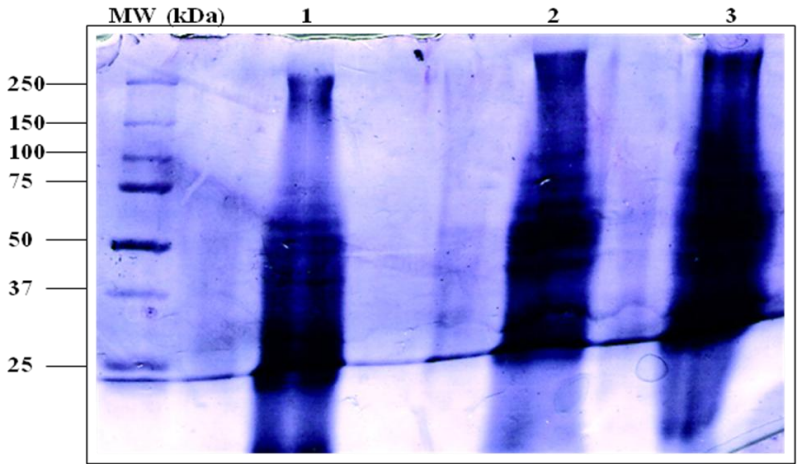

Figure 6: Commassie Blue-Stain of SDS-PAGE gel representing crude extract protein banding patterns from 1- *L. major*, 2 - *L. tropica*, 3- *L. infantum*. MW: molecular weight markers are indicated in KDa.

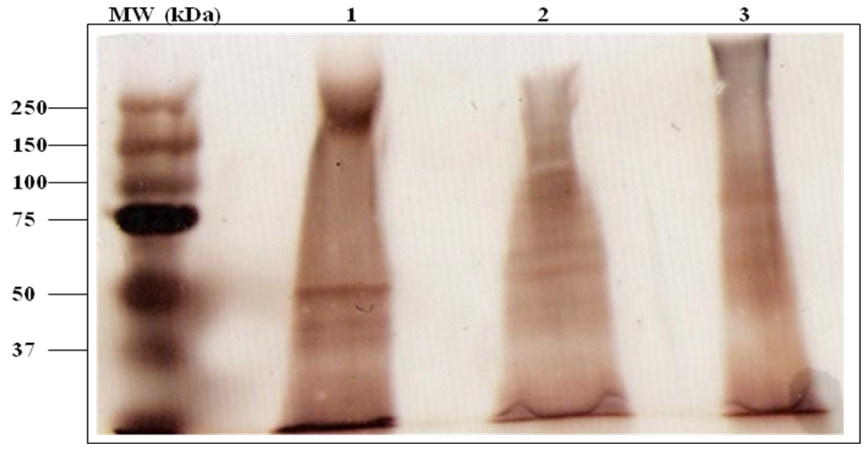

Figure 7: Silver-Stain of SDS-PAGE gel representing crude extract protein banding patterns from 1- *L. major*, 2 - *L. tropica*, 3- *L. infantum.* MW: molecular weight marker.

3.2.2. Western Blotting:

The reactivity of the three *Leishmanial* extracts was done by the aid of two high antibody titer serum pools, one was collected from VL infected individuals and the second from CL infected individuals as indicated in (3.1). For this purpose four SDS-PAGE gels were prepared, each gel contains extracts from *L. major*, *L. tropica*, and *L. donovani* species. Antibody reactivity from each tested serum pool was examined by anti-human IgM and anti-human IgG, this was carried out in order to determine the type of most reactive antibodies produced in infected individuals and later to determine the second antibody to be used in phage display screening protocol. Western-blot analysis of CL pooled sera did not showed any reactive antibody against any of the tested lesinmanial extracts (data not shown), while there was different reactivity pattern upon applying the pooled serum from VL infected individuals (Figure 8). Also, it is clearly seen that the major antibody class of the provoked antibodies against leishmanial antigens in human sear were of the IgG istotype (Figure 8-A), since using anti-human IgM as secondary antibody could not detect

any antibodies reactive against any of the leishmanial extracts (Figure 8-B), but using *Staphylococcus*/*Streptococcus* protein A (that has a high affinity only for IgG antibodies) identified several reactive bands from the three analyzed leishmnial species (Figure 8-A). The results indicate that there are specific antigenic determinants that can be detected by the collected VL pooled sera and this evoked antibody response was detected by the aid of secondary IgG antibodies or compatible molecules (i.e.: protein A). This step was important to enable planning for the screening of the phage library and to prove presence of a reactivity with VL or CL pooled sear against the different ø-D epitopes.

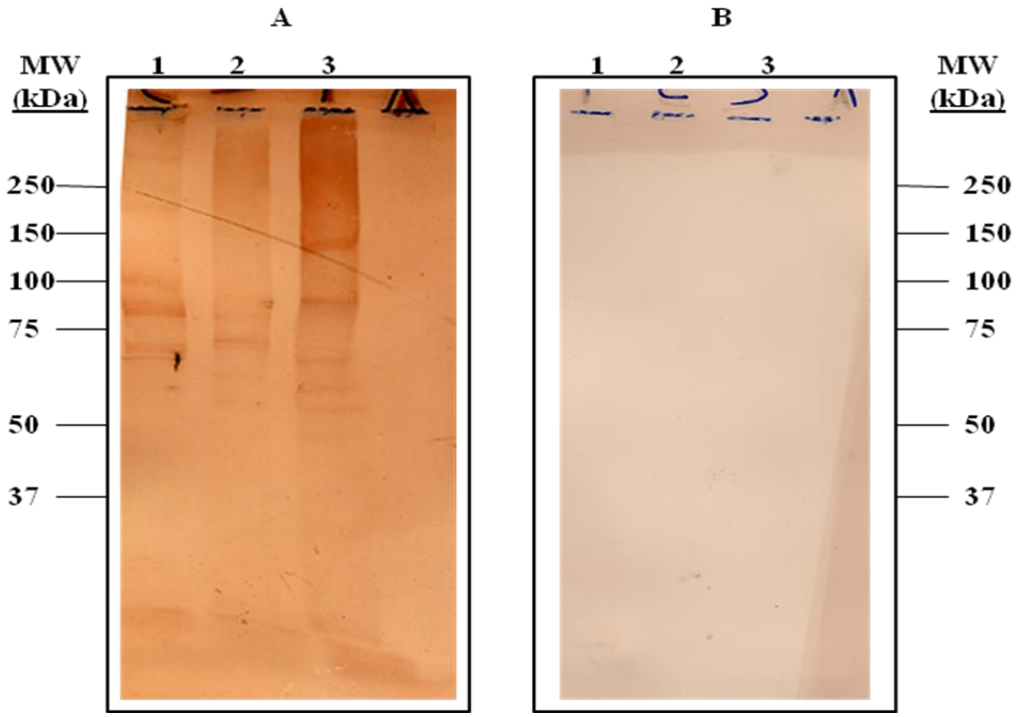

Figure 8: Western-blot analysis representing the reactivity of VL collected pool serum against different leishmanial crude extract, 1- *L. major*, 2 - *L. tropica*, 3- *L. infantum*. In A: protein A-HRP was used as secondary antibody, and in B: anti-human IgM-HRP was used as secondary antibody. MW: molecular weight marker.

3.3 Phage display

Phage library titration: The phage library titer was estimated to be 10^{13} plaque forming units (pfu)/ml, and this was in agreement with number supplies by the phage library manufacturer (New England Biolabs, Ipswich, MA, USA). The titer of the phage library decreased along storage time, for this reason and for obtaining a workable number of plaques in each plate; the phage library was titrated using different dilutions of phages starting from 10^{-4} down to 10^{-10}. Each dilution was used to infect 0.1ml of freshly grown *E. coli* bacteria followed by plating on LB agar plate with minimal media components as indicated in the materials and methods. After performing this test it was found that diluting the phage up to $1:10^9$ is the titer that produces about 500 separated plaques per plate, this phage dilution was used all over the screening cycles in this study.

3.3.1. Screening of Phage library with VL serum:

Screening of phage library using the serum pooled from VL infected individuals was done at two cycles. In the first cycle, 10 plates containing M13 phage plaques were screened with 1:1000 diluted VL serum as first antibody and protein A-HRP as a secondary antibody, only the plaques that gave strong color signals were isolated. The obtained results of the first screening cycle were as shown in (Table 3):

1. Plaques were only detected in 5 plates out of 10.
2. Assuming the total number of plaques in each plate is 500, then only 41 out of 5000 M13 phage plaques showed reactivity with VL pooled sera, which is less than 1% of the plaques.

Table 3: A summary of obtained M13 plaques after first and second screening with VL pooled sera of high antibody titer against leishmanial crude antigen.

Plate number	Screening cycle	Used serum	Number of obtained plaques	Totals
1	**First**	**VL**	**22**	**41**
2	**First**	**VL**	**3**	
3	**First**	**VL**	**4**	
4	**First**	**VL**	**9**	
5	**First**	**VL**	**3**	
6	**Second**	**VL**	**11**	**54**
7	**Second**	**VL**	**27**	
8	**Second**	**VL**	**10**	
9	**Second**	**VL**	**6**	

The reactive plaques were marked and picked up from the agar plates and incubated in LB media with hst bacteria for further analysis.

In the second screening cycle we supposed to grow of each the obtained plaque in a separate plate and to rescreen with VL sera, but because the limited amount of the VL pooled sera sample it was suggested to rescreen each five plaques together. So, bacterial cells were infected with a mixture of five plaques and this was followed by plating the phages on agar plates as indicated in materials and methods. Theoretically this approach aims to enrich the plate with many plaques of the same type, and it is recommended at this stage to grow the phages in a titer that gives a limited number of plaques per plate in order

to enable easier isolation of independent plaques without overlapping each other. As seen in table 3, another 54 plaques were selected to be used for further analysis in this study. None of the selected plaques were reactive with normal human sera when they screened with 1:1000 diluted serum pooled from normal human.

3.4 M13 phage epitope identification

From a total of 54 selected and purified M13 phage plaques that were chosen after two screening cycles with VL pooled sera, 35 different plaques were selected for sequence analysis and epitope mapping. The 35 plaques were selected based on their strong signals, obtained after reactivity of the bound protein-A-HRP to the human α-VL antibodies. Signal were judged strong based on the color intensity relative to other plaques. These plaques represent antibodies with a high affinity to M13 phage epitopes that mimics leishmanial epitopes. This part of the results shows the similarity of the obtained M13 phage epitopes to already known leishmanial proteins or sequences in the Gene Bank data base and identification of some potential clones to express specific leishmanial epitopes.

3.4.1. DNA amplification of the fused peptide/pIII M13 phage from the selected clones:

It is known that the insertion of the random peptides in the pIII M13 phage gene to form a sequence of random peptide library pIII fusion peptides (Figure 9) (New England Biolabs, Ipswich, MA, USA). Based on this information of M13 phage library construction, and on the DNA sequence information of the pIII gene; a pair of primers were designed to enable a specific amplification of the fusion peptide (Figure 9). At the beginning, the PCR primers

were designed based on the provided information from the manufacturer company of the M13 phage library. At this stage; DNA was amplified using two primers that are flanking the peptide region and amplifying a DNA segment of 120 bp (Table 4).

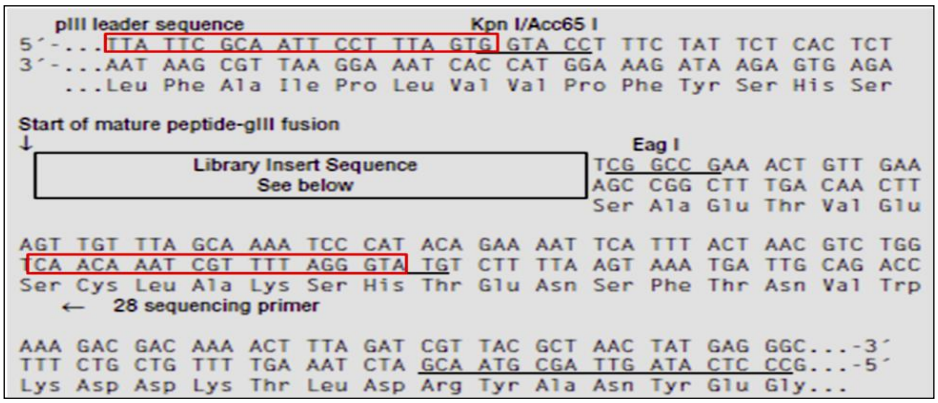

Figure 9: DNA and amino acid sequence information of the pIII-peptide fusion in M13 phage. The sequence in the rectangular represents primers location (ø D1: at 5'end and ø R1: at 3'end).

Table 4: Primers and their nucleotide sequence information that were used in amplifying the pIII-peptide fusion DNA fragment from selected ø-D library M13 phage.

Phage primers	**Nucleotide sequence of primers**	**Size of the amplified DNA band**
ø D1	TTA TTC GCA ATT CCT TTA GTG	
ø R1	ATG GGA TTT CGT AAT ACA AC	Using PhD1 and PhR1 **(120bp)**
ø R2	TCC CTC ATA GTT AGC GTA ACG	Using PhD1 and PhR2 **(245bp)**

Using the primer combination ø D1 and ø R1 in PCR amplification of the fused peptides; could only amplify DNA segments from 10 clones out of the 35 selected clones (Figure 9).

The amplified DNA segments were not suitable for DNA sequence analysis because they were very low in concentration and because their small size (120 bp) it was not possible to purify them out of the PCR primers. To solve this problem, another primers was designed based on the full DNA sequence information of pIII gene of the M13 phage that was retrieved from the Gene Bank (accession number gp AF031088) (Figure 10). The previously used forward primer was kept and a new reverse primer was designed that is located an additional 120 bp don in strum at 5'-end of the previous reverse primer. These PCR primer combinations (Ph D1 and Ph R2) were able to amplify a 245bp DNA segment that include the inserted peptide and the flanking region from the pIII gene. This PCR system was able to amplify the expected DNA segment from all the 35 selected clones (Figure 11). These selected clones were numbered from Ph1 to Ph35 according to their arrangement in figure 10.

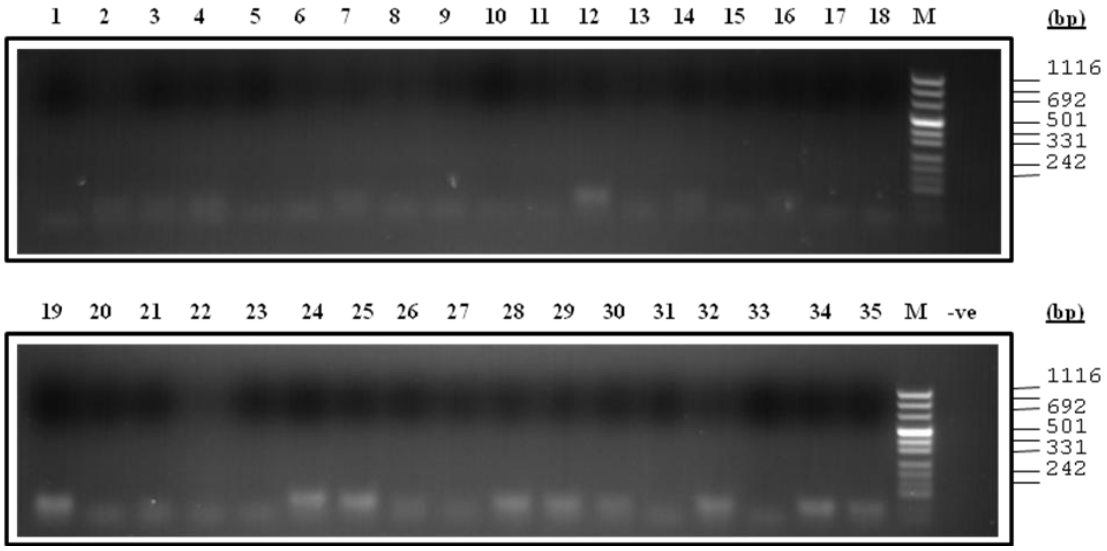

Figure 10: Agarose gel electrophoresis analysis of the amplified PCR products using Ph D1 and Ph R1 primers targeting the fused peptide in pIII M13 phage gene in the 35 different selected clones.

5'-ggagcctttt ttttggagat tttcaacgtg aaaaaattat

tattcgcaat tcctttagtg gtacctttct attctcactc

T **Site of inserted peptide**

gctacttcg catctgcatg ttcggttgcc gtcgaagggt

ggaggttcgg ccgaaactgt tgaaagttgt ttagcaaaat

cccatacaga aaattcattt actaacgtct ggaaagacga

caaaacttta gatcgttacg ctaactatga ggga -3'

Figure 11: DNA sequence of the pIII M13 gene with the site of the inserted DNA sequences. Based on this sequence forward primer (ø D2) and a reverse primer (ø R2) are indicated.

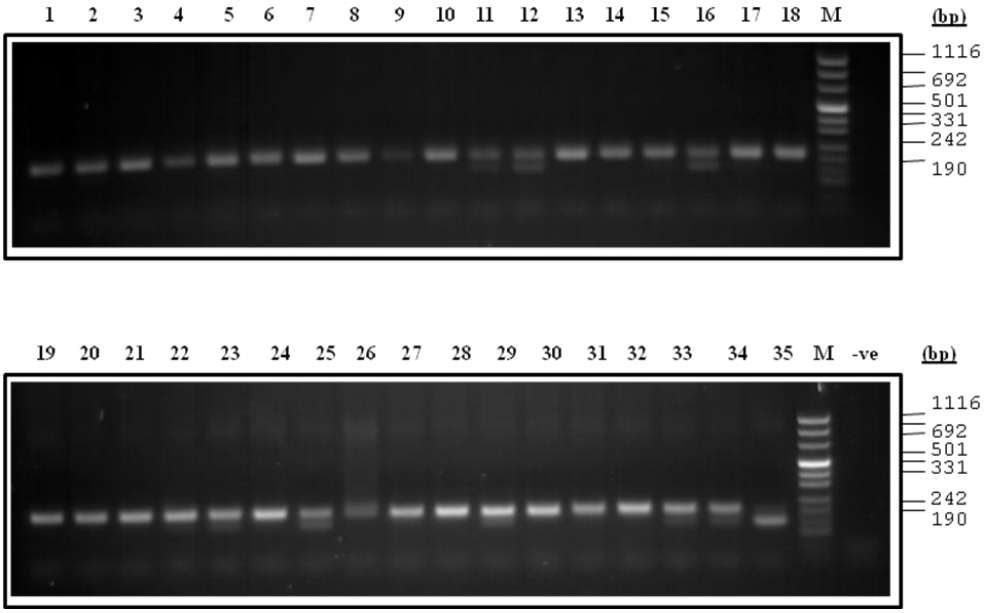

Figure 12: Agarose gel electrophoresis analysis of the amplified PCR products using Ph D1 and Ph R2 primers targeting the fused peptide in pIII M13 phage gene in the 35 different selected clones.

3.4.2. DNA sequencing of the fused peptides pIII gene:

From the 35 selected clones based on a second screening cycle with VL high titer pooled sera, 18 clones were chosen for further sequence analysis and later for peptide identification. Namely these clones were (Ph3, Ph5, Ph12, Ph19, Ph20, Ph22, Ph23, Ph24, Ph25, Ph27, Ph28, Ph29, Ph30, Ph31, Ph32, Ph33, Ph34, and Ph35). The PCR product amplified by the second primers combinations (Figure 12) for these indicated clones was purified and sent for DNA sequence analysis service to where. the nucleotide sequences poor Ph12 was not successfully sequenced, and this seems for to be caused by mixed PCR amplification products (Figure 12), also clones 31, 33, and 35 showed sequences with many unidentified nucleotides reflecting contamination of more than one clone in these PCR amplified products. So DNA sequence analysis was only performed for the remaining 14 clones.

Duplication PCR band amplification, as seen in gel, lanes 12,23,25,29 and 33 represent a mixed plaques due to presence of more than one plaques, or co-infection when performing library

Figure 13 shows the DNA alignment of the obtained sequences using BLAST, identified nucleotides belonging to the inserted peptide. The homology between all clones in the flanking regions that belongs to the pIII gene of the M13 phage is evident. For accurate similarity analysis among DNA sequences of the 14 different peptides; a second alignment was performed without the flanking regions and this in order to remove any extrinsic factors affecting the alignments (Figure 13). Based on the second DNA alignment; it was concluded that there is no similarity at the DNA level among all the obtained fused peptid ; each of these clones represented different epitope that needs more analysis.

DNA sequence information of the 14 inserted peptide-pIII M13 gene without the flanking region of the known pIII gene (Figure 14) is shown in appendix A.

```
ph3     TTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCT------ACTCGTCAGAG 54
ph19    TTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCT------GATCATCATGG 54
ph28    TTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCT------CATATTACTAC 54
ph5     TTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCT------AGTGTTCATAG 54
ph22    TTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCT---CATGGTATNTATGN 57
ph24    TTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCTGGTCAGGGTAGTT---C 57
ph25    TTATTCGCAATTCCTTTAGNGGNACCTTTCTATTCTCACTCT---CCGGATNNTTTTNC 57
ph32    TTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCT---AC------------ 45
ph23    TTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCT---AAGTATATGCATGC 57
ph27    TTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCT---G-GTGTGGTTACGC 56
ph33    TTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCT---AATATTCTTTATGC 57
ph34    TTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCT---TAGGATCTTTTGGC 57
ph29    TTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCT------ACGAATGAGAC 54
ph30    TTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCT----TTGCCTCCTATGT 56
ph20    TTATTCGCAATTCCNTTAGTGGTACCTTTCTATTCTCACTCT-------CTNCCTGNAT 53
        *************** **** ** ******************

ph3    TCGTAATTCTCATGA-TATGCGGGTG---GGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 110
ph19   G-ATGGTT--CATGC-TGATCGGATGATTGGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 110
ph28   GAGGACTAGTACTGT-TGAGCGGAAT---GGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 110
ph5    TGAGAGGAGTAAGTT-TCTGCGGAAT---GGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 110
ph22   --GNATAATNAGAC--TCAGAAGCCT---GGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 110
ph24   --GTTTGGGTAGAA--TATGAAGCTT---GGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 110
ph25   A-AANCCNANAGA---GGNACCGCCN---GGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 110
ph32   --ANCNNNAGAGG---CAGNNNCCNG---GGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 97
ph23   --ACCTCT-TACGCC-GGCGCCTTCG---GGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 110
ph27   --ATCTTTATACGCC-TTTGCCGGCT---GGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 110
ph33   --AAATCATTAAAT--TGTTCCTAAT---GGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 110
ph34   --AATTCCTTTAGT--TGATCCTTTG---GGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 110
ph29   TCGTACTGTGCGGCA-TATTCGGTTT---GGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 110
ph30   --AGTTTAAGGTGGG-GGCGCAGTTT---GGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 110
ph20   NGNAATCAGNAGACNAGGCNTAGTNT--TGGTGGAGGTTCGGCCGAAACTGTTGAAAGTT 111
                                   ******************************

ph3    GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAAC-TCG-GAAAGACGA-NAAAAA 166
ph19   GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAAC-TTG-GAAAGNNGA-CAAA-- 164
ph28   GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAAC-TNT-GAAAGACGA-CAAA-- 164
ph5    GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAAC-TNT-GAAAGACGA-CAAA-- 164
ph22   GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCGTGAAAGACGA-CAAA-- 166
ph24   GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAAC-TCG-GAAAGACGA-CAAA-- 164
ph25   GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCGTGAAAGACGA-CAAA-- 166
ph32   GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCGTGAAAGACGA-CAAA-- 153
ph23   GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAAC-TCG-GAAAGACGA-CAAA-- 164
ph27   GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCG-GAAAGACGAACAAA-- 166
ph33   GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAAC-TCG-GAAAGACGA-CAAA-- 164
ph34   GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAAC-TCT-GAAAGACGA-CAAAA- 165
ph29   GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCGTGAAAGACGA-CAAA-- 166
ph30   GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAAC-TCG-GAAAGACGA-CAAA-- 164
ph20   GTTTAGCAAAATCCCATACAAANAATTCATTTACTAAN-TCN--GAAGAGGA-CAAA-- 164
       ******************* * **************  *     ***  **  ***
```

Figure 13: DNA alignment of the obtained sequence information of the 14 different analyzed clones including the flanking regions of the pIII gene of M13 phage. Indicated with striks.

```
ph22   CACTCT---CATGGTATNTATGNGNATAATNAGACTCAGAAGCCT------GGTGGAGGT 51
ph24   CACTCTGGTCAGGGTAGTT---CGTTTGGGTAGAATATGAAGCTT------GGTGGAGGT 51
ph20   CACTCT---CTCTCTNCCTGNATN-GNAATCAGNAGACNAGGCNTAGTNTTGGTGGAGGT 56
ph30   CACTCT---TTGCCTCC---TATG--TAGTTTAAGGTGGGGGCGCAGTTT-GGTGGAGGT 51
ph3    CACTCT---TAC--TCGTCAGAGTCGTAATTCTCATGATATGCGGGTG---GGTGGAGGT 52
ph19   CACTCT---GA---TCATCATGGG-ATGGTTC--ATGCTGATCGGATGATTGGTGGAGGT 51
ph28   CACTCT---CATATTACTACGAGGACTAGTAC---TGTTGAGCGGAAT---GGTGGAGGT 51
ph5    CACTCT---AGTGTTCATAGTGAGAGGAGTAA---GTTTCTGCGGAAT---GGTGGAGGT 51
ph29   CACTCT---ACG---AATGAGACTCGTACTGTGCGGCATATTCGGTTT---GGTGGAGGT 51
ph23   CACTCT---AAGTATATGCATGCACCTCT-TA--CGCCGGCGCCTTCG---GGTGGAGGT 51
ph27   CACTCT---TGGTGTGGTTACGCATCTTTATA--CGCCTTTGCCGGCT---GGTGGAGGT 52
ph33   CACTCT---AATATTCTTTATGCAAATCATTA--AATTGTT-CCTAAT---GGTGGAGGT 51
ph34   CACTCT---TAGGATCTTTTGGCAATTCCTTT--AGTTGAT-CCTTTG---GGTGGAGGT 51
ph25   CACTCT---CCGGATNNTTTTNCAAANCCNANAGAGGNACCGCCN------GGTGGAGGT 51
        ******                                              *********
```

Figure 14: DNA sequence alignment of the 14 different peptides, similarity sequences at 5'-end represents histidin and serine amino acids and those at the 3'-end represents three glycine molecules.

3.4.3. Bioinformatics and DNA sequence analysis:

This part of the work involved similarity search to the obtained peptide sequences, this was done using BLAST online service provided through the PubMed /US National Institute of Health (http://www.ncbi.nlm.nih.gov/pubmed/).

From the 14 examined peptide DNA sequences, only two clones showed similarity to *Leishmania* parasite DNA genomic sequences, clone Ph3 anPh23. Table 5 shows a summary of the most similar clones to *Leishmania* or other related protozoan parasites (such as *Trypansosma* and *Trichomonus*). Blast analysis of clone Ph3 showed a 34% of

this sequence has 100% similarity with *Leishmania donovani* and *Leishmania infantum* species, which are the causative agents of VL (Table 5). Also, and in another region of the peptide sequence that represents 34% coverage of the sequence, it showed 100% similarity with both *Trypansosma* and *Trichomonus* parasites. These parasites are protozoan flagellates and are closely related to leishmania family (*Kinetoplastidae*). This fact supports the potential identity of the Ph3 peptide as a Leishmanial epitope. An analogous result was also obtained for peptide Ph23, but it showed similarity to *L. major* and *L. braziliensis*. Other peptides (Ph5 and Ph9) showed similarity to related parasites with coverage between 51%-64%, and a minimum similarity of 95%.

Table 5: Peptide DNA sequence similarity and coverage percentages with other related species.

Clone	Similarity description	Sequence coverage	Similarity percent
Ph3	- *Leishmania donovani* BPK282A1 complete genome	34%	100%
	- *Leishmania infantum* JPCM5 conserved hypothetical protein	34%	100%
	- *Trypanosoma congolense* IL3000 annotated genomic contig	34%	100%
	- *Trichomonas vaginalis*	32%	100%
Ph23	- *Leishmania major* strain Friedlin complete genome	40%	100%
	- *Leishmania braziliensis* MHOM/BR/75/M2904	37%	100%
Ph5	- *Trichomonas vaginalis* G3 hypothetical protein	64%	95%
Ph19	- *Schistosoma mansoni*	51%	100%

The DNA sequence similarity data analysis for Ph3 and Ph23 peptides is shown below.

Species and DNA sequence similarity to clone Ph3:

1- emb|FR799619.2| Leishmania donovani BPK282A1 complete genome, chromosome 32: hypothetical protein, conserved

```
 Score = 32.2 bits (16),  Expect =   212
 Identities = 16/16 (100%), Gaps = 0/16 (0%)
 Strand=Plus/Minus

Query  31       GCGGGTGGGTGGAGGT  46
                ||||||||||||||||
Sbjct  1102812  GCGGGTGGGTGGAGGT  1102797
```

2- emb|HE575316.1| Trypanosoma congolense IL3000 annotated genomic contig, chromosome 3: conserved hypothetical protein

```
 Score = 32.2 bits (16),  Expect =   212
 Identities = 16/16 (100%), Gaps = 0/16 (0%)
 Strand=Plus/Plus

Query  8       CAGAGTCGTAATTCTC  23
               ||||||||||||||||
Sbjct  616033  CAGAGTCGTAATTCTC  616048
```

3- ref|XM_001467931.2| Leishmania infantum JPCM5 conserved hypothetical protein (LINJ_32_2920) mRNA, complete cds

GENE ID: 5072032 LINJ_32_2920 | hypothetical protein [Leishmania infantum JPCM5]

```
 Score = 32.2 bits (16),  Expect =   212
 Identities = 16/16 (100%), Gaps = 0/16 (0%)
 Strand=Plus/Plus

Query  31    GCGGGTGGGTGGAGGT  46
             ||||||||||||||||
Sbjct  2588  GCGGGTGGGTGGAGGT  2603
```

4- ref|XM_001308024.1| Trichomonas vaginalis G3 hypothetical protein (TVAG_204370) partial mRNA
GENE ID: 4752839 TVAG_204370 | hypothetical protein [Trichomonas vaginalis G3]

```
 Score = 34.2 bits (17),  Expect =    54
 Identities = 17/17 (100%), Gaps = 0/17 (0%)
 Strand=Plus/Minus
```

```
Query  11   AGTCGTAATTCTCATGA  27
            |||||||||||||||||
Sbjct  637  AGTCGTAATTCTCATGA  621
```

Species and DNA sequence similarity to clone Ph23:

1- emb|FR796414.1| Leishmania major strain Friedlin complete genome, chromosome 18

```
 Score = 36.2 bits (18),  Expect =    13
 Identities = 18/18 (100%), Gaps = 0/18 (0%)
 Strand=Plus/Plus

Query  19      CTTACGCCGGCGCCTTCG  36
               ||||||||||||||||||
Sbjct  432976  CTTACGCCGGCGCCTTCG  432993
```

2- emb|FR799007.1| Leishmania braziliensis MHOM/BR/75/M2904 complete genome, chromosome 32: conserved hypothetical protein

```
 Score = 34.2 bits (17),  Expect =    52
 Identities = 17/17 (100%), Gaps = 0/17 (0%)
 Strand=Plus/Plus

Query  19      CTTACGCCGGCGCCTTC  35
               |||||||||||||||||
Sbjct  230149  CTTACGCCGGCGCCTTC  230165
```

In order to provide more evidence for the relevance of Ph3 and Ph23 peptides to *Leishmania* parasites, the DNA nucleotide sequences was translated into amino acids according to protozoan codon translation standards and this was followed by protein BLAST similarity search.

Table 6: Amino acid translation of Ph3 and Ph23 peptides based on their DNA sequence.

Peptide	**Translated amino acid sequence**
Ph3	TRQSRNSHDMRV
Ph23	KYMHAPLTPAPS

The BLAST similarity analysis shown below, sowed homology of the Ph3 peptides and Ph23 peptides to some leishmanial antigenic epitopes, at least six amino acids from each peptides was showed to be similar to known leishmnial proteins.

Amino acid similarity to Ph3 peptide:

1- XP_001469700.1| conserved hypothetical protein [Leishmania infantum
emb|CBZ38746.1| hypothetical protein, conserved [Leishmania donovani]

GENE ID: 5073804 LINJ_36_3080 | predicted zinc finger protein [Leishmania infantum JPCM5]

Identities = 11/17 (65%), Positives = 11/17 (65%), Gaps = 3/17 (18%)

```
Query  2    RQSRNSHDMRV---  15
            RQ R S DMRV
Sbjct  366  RQPRVSLDMRVPAA  382
```

2- emb|CAM43204.2| conserved hypothetical protein [*Leishmania braziliensi*
emb|CBZ37129.1| hypothetical protein, conserved [*Leishmania donovani*]
emb|CBZ29781.1| conserved hypothetical protein [*Leishmania Mexican*
ref|XP_001468073.1| conserved hypothetical protein [*Leishmania infantum*
ref|XP_001685712.1| conserved protein [*Leishmania major*]

Identities = 7/9 (78%), Positives = 7/9 (78%), Gaps = 0/9 (0%)

```
Query  2    RQSRNSHDM  10
            RQ RN HDM
Sbjct  654  RQVRNPHDM  662
```

Amino acid similarity to Ph23 peptide:

1- ref|XP_001469372.1| hypothetical protein, [*Leishmania infantum* JPCM5]
emb|CAM72479.1| hypothetical protein, [*Leishmania infantum* JPCM5]

```
 Identities = 11/23 (48%), Positives = 11/23 (48%), Gaps = 11/23 (48%)

Query  4    HAPLTPAP-----------S  15
            H PLTPAP           S
Sbjct  185  HVPLTPAPPTSPPCSSRRAS  207
```

2- emb|CBZ29642.1| conserved hypothetical protein [*Leishmania mexicana* MHOM/GT/2001/U1103]

```
Identities = 10/15 (67%), Positives = 10/15 (67%), Gaps = 3/15 (20%)

Query  4    HAPLTPAPS---  15
            H PLTP PS
Sbjct  462  HTPLTPSPSSAA  476
```

Chapter Four: Discussion

Infectioon caused by *leishmania* parsite in human is characterized by the appearance of anti-leishmnial antibodies in the sera of the patients with great variation in humoral immune response in VL and CL cases. In CL, antibodies are present at low level during the active phase of the disease (84). In contrast, high anti-leishmanial antibody titers are well detected in VL (85,86). The role of elevated anti-leishmanial antibodies in VL patients towards protection or disease is not clear. Analysis of *leishmania* antigen specific immunoglobulin isotypes revealed elevated levels of IgG, IgM, and IgE antibodies (87,88). IgG antibodies do not protect against intracellular pathogens (specifically against *leishmania* parasite) but it contributes to disease prognosis. Since, passive administration of anti-*leishmania* IgG results in larger lesions in BALB/c mice with greater amount of IL-10 production. And this can be linked to high elevation of antibody titer during active phase of infection, and fall in antibody titer after cure (89).

Studies in human, dogs and mouse models, have demonstrate that protective immunity against *leishmania* is T cell mediated, while disease production is associated with the production of antibodies and absence of cell mediated immunity. In dogs leishmaniasis develops a humoral response but non-protective immune response. The increase in serum immunoglubulins appears to be due to polyclonal B cell activation (90). The dogs mainly produce IgG antibodies that can be detected in 1-4 months after parasite challenge. The relative level of IgG1 and IgG2 are prognostic indicators of cure and disease, associating IgG1 to the development of the disease and IgG2 to an asymptomatic infection (91).

To emphasize the type of immune response against *leishmania* in mammalian hosts; the murine model using *L. major* and *L. donovani* are excellent studied experimental models to

demonstrate immunity against leishmaniasis. BALB/c mice are highly susceptible to *L. major* infection and undergo progressive and fatal disease while *L. major* infection in humans is usually self-healing. On the other hand, C57/bl6 and CBA/n mice are resistant to *L. major* infection, and develop small lesions that resolve(33,36). Mice are generally resistant to *L. donovani* infection and do not die of the disease, even though this parasite can cause fatal VL in humans (28,33,35). The differences between infection in the experimental murine model and human infection are not completely related to host genetics but may also reflect differences in the size and nature of the infected parasite and route of infection (92).

The progression of CL in the murine system, to disease or cure, is mainly regulated by T-cell responses. Infection of experimental mice with *L. major* promastigotes involves activation of CD4+ cells. Upon challenge with *L. major* different mouse strains evoke varying immune responses, CD4+ T-helper type 1 (Th1) and type 2 (Th2), which are associated with different clinical outcomes. The nature of the immune response, Th1/Th2, has been shown to determine the resistance or susceptibility of mice to *Leishmania* infection (93,94,95). The CD4+ T-cell subsets can be distinguished by the cytokines they secrete. Th1 cells secrete IL-2, IFN-γ and TNF-α which activate cell-mediated immunity, while Th2 cells secrete cytokines such as IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and TGF-β which promote antibody responses (96). Susceptible BALB/c mice characterized by Th2 response and disease development. IL-4 secreted by Th2 cells down-regulates the expression of IL-12 receptors on Th1 cells making them unresponsive to IL-12 inhibiting the production of IFN-γ and nitrogen oxide (97).

In contrast to CL, less is known about the immunological mechanisms controlling the development of VL. Immunity to *L. donovani* is dependent upon the effective generation

of cell-mediated immune mechanisms causing host macrophage activation to kill the intracellular parasites. T cell depletion decreases the resistance in mice infected with *L. donovani* and the transfer of T cells from recovered mice protects naive mice from infection (98). Patients with acquired immunodeficiency syndrome (AIDS) and other functional T-cell defects are highly susceptible to *L. donovani* infections.

Epidemiological studies on human VL suggest that up to 85% of the infected individuals may spontaneously control infection. Those individuals either remain asymptomatic or develop minor symptoms of infections that eventually resolve without treatment. This response is associated with skin test reactivity and appears to correlate with intact antigen-specific production of IFN-γ (99). The presence of IL-4 and IL-10, Th2 cell associated cytokines, appears important since both can suppress the secretion of activating cytokines, including IFN-γ and IL-2, which are important in host defense. This suggests the possibility that Th2 activation may be involved in the progression of the human VL.

There is no data about the epidemiology of visceral leishmaniasis in Palestine, except an old report from the Palestinian Ministry of Health indicating the presence of 53 VL cases in Hebron between 1994 to 2004. In another abstract the epidemiology of VL was reported to be 7.2 % in Beit Ola near Hebron based on ELISA serodiagnosis. Until now there is a missing accurate data about the epidemiology of VL cases in Palestine and in many cases it seems they are misdiagnosed for lack of experience. In this study all the obtained sera of VL cases showed high antibody titers, four out of the five cases were from Jenine. This indirectly indicates about the presence of active foci of VL in Jenine, and most probably all these cases are active infections that plays a major role in disease transmission. The results

of Western blot analysis are consistent with the known findings of VL and CL immune response. The pooled VL sera was able to detect high molecular bands of the crude leishmanial extracts, and as indicated the VL humoral response is a polyclonal in nature and it has the ability to detect many circulating or cellular lesihmaial antigens. The main antibody isotype in VL pooled sera is of IgG, and we could not detect an IgM antibody classes against Leishmanial crude antigens which is normally decline after a time of the infection.

Phage display technology is a powerful method of epitope mapping that has been used to select peptides capable to mimic linear epitopes, nonlinear epitopes, and even non-protein molecules (100). Different studies have been performed to determine whether mimotopes selected from phage display libraries by protective antibodies or antibodies against protective antigens could serve as the basis for a peptide vaccine or a diagnostic tool. Two different phage display libraries were used to map the epitopes of a mAb against the ring-infected erythrocyte surface antigen (RESA) of *Plasmodium falciparum* which is a vaccine candidate for malaria (101).

In this study, we screened pIII random peptide phage display library (a 12-mer) with the VL pooled serum. From a total of 35 reactive peptides with VL sera only 18 were sequenced and from those only four peptides showed similarity with known antigenic epitopes related to *L . donovani* the causative agent of VL. This finding does not exclude the importance of the other obtained peptides; sequenced or not. This is because these peptides were bound specifically to antibodies from VL infected pool sera. In general most of the selected and sequenced peptides (13 out of 18) had no complete consensus linear

motif in common and no linear sequence identities with any currently known proteins or DNA sequences. This fact may eliminate the role of these peptides as sequential epitopes but never as conformational epitopes or as mimitopes that mimic different antigenic proteins of similar amino acid composition, or carbohydrates that have similar conformational structure.

The amino acid sequence composition of the obtained epitopes was determined by translating the nucleotide sequence based on protozoan standard codons. A comparison of the amino acid composition of these peptides in terms of exact sequence and or the type of the amino acids (acidic or basic amino acids, polar or non-polar amino acids) that form the epitope did not show any similarity among each other. This is is mainly related to the polyclonal antibody nature of the VL immune response against the *leishmania* parasite. On the other hand, 2 out of 4 peptides that showed similarity with known *leishmania* DNA sequences, also they showed high similarity at the protein level of these peptides with many antigenic determinants of leishmaial origins.

Conclusion

Phagotopes selected using one mAb may be specifically recognized by other antibodies, such as infected human sera, against the same determinant (102). Such peptides can act as diagnostic markers for the detection of disease related antibodies in patient sera. In certain endemic regions total leishmanial crude antigen cannot be used for VL serodiagnosis because sera from other diseases, such as Chagas' disease, cross-reacts with this antigen. The search for specific and sensitive diagnostic epitopes for use in VL assays has focused on pure and recombinant proteins (102). In this sense the potential use of the identified peptides in serodiagnosis is one the important recommendations for the future work. This

will involved peptide synthesis, binding to large protein carrier and validation by testing these peptides with different VL sera in ELISA test.

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Appendix A

Peptides coding sequences of the 14 different clones identified by high antibody titer VL pooled sera.

>Ph3
ACTCGTCAGA GTCGTAATTC TCATGATATG CGGGTGGGTG GAGGT

>Ph5
AGTGTTCATA GTGAGAGGAG TAAGTTTCTG CGGAATGGTG GAGGT

>Ph19
GATCATCATG GGATGGTTCA TGCTGATCGG ATGATTGGTG GAGGT

>ph20
CTNCCTGNAT NGNAATCAGN AGACNAGGCN TAGTTTGGT GGAGGT

>Ph22
CATGGTATNT ATGNGNATAA TNAGACTCAG AAGCCTGGTG GAGGT

>Ph23
AAGTATATGC ATGCACCTCT TACGCCGGCG CCTTCGGGTG GAGGT

>Ph24
GGTCAGGGTA GTTCGTTTGG GTAGAATATG AAGCTTGGTG GAGGT

>Ph25
CCGGATNNTT TTNCAAANCC NANAGAGGNA CCGCCNGGTG GAGGT

>Ph27
TGGTGTGGTT ACGCATCTTT ATACGCCTTT GCCGCTGGT GGAGGT

>Ph28
CATATTACTA CGAGGACTAG TACTGTTGAG CGGAATGGTG GAGGT

>Ph29
ACGAATGAGA CTCGTACTGT GCGGCATATT CGGTTTGGTG GAGGT

>Ph30
TTGCCTCCTA TGTAGTTTAA GGTGGGGGCG CAGTTTGGTG GAGGT

>Ph33
AATATTCTTT ATGCAAATCA TTAAATTGTT CCTAATGGTG GAGGT

>Ph34
TAGGATCTTT TGGCAATTCC TTTAGTTGAT CCTTTGGGTG GAGGT

التعرف على المعلمات السطحية لطفيل الليشمانيا من خلال استخدام مصل المصابين ذو التركيز العالي بالمضادات

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ملخص

يعتبر داء الليشمانيا من الأمراض واسعة الانتشار ويعد من المشاكل الصحية التي تعنى بها منظمة الصحة العالمية، يعتبر مرض الليشمانيا من الأمراض المتفشية في 88 دولة حول العالم و يهدد ما يقارب 350 مليون شخصا بالإصابة وفقا لتقارير منظمة الصحة العالمية. ينتقل طفيلي الليشمانيا إلى العائل من خلال لسع أنثى ذبابة الرمل المصابة لضحيتها من الكائنات الفقارية. يوجد مرض الليشمانيا بثلاثة أشكال وهي: الليشمانيا الحشوية و الليشمانيا الجلدية و الليشمانيا الجلدية المخاطية.

تهدف هذه الدراسة إلى التعرف على المعلمات السطحية لليشمانيا باستخدام تقنية العاثيات phage باستخدام النوع الخيطي منها M13 وذلك للكشف و التعرف على البيبتيدات و التي تحاكي أنواع المعلمات السطحية لطفيل الليشمانيا من خلال ما يسمى البلاكس plaques أو المناطق الناتجة من العاثيات والمكونة من 12 حمض أميني في ما يعرف مكتبة العاثيات. لذا تم عمل مسح كامل لجميع العينات التي تم جمعها من كل من نوعي الليشمانيا الحشوية و الجلدية والتي تم فحص

الأضداد النوعية لليشمانيا فيها و تحديد العينات ذات الأعلى تركيز من مصول المرضى المصابين من خلال تطبيق اختبار ELISA ومن ثم تم تجميعها وعمل مجمعات pools من هذه العينات ذات الأعلى تركيز.

من خلال استخدام التجمعات pools ذات الأعلى تركيز من عينات الليشمانيا الحشوية تم عمل دورتي مسح لهذه العينات باستخدام العثيات phage المكونة من 12 حمض أميني وتم الحصول في دورة المسح الأول على 41 plaques أما من المسح الثاني تم الحصول على 54 plaques منها ما يقارب 35 plaquesأعطت تفاعل ايجابي قوي مع مجمعات العينات من الليشمانيا الحشوية. وللتعرف على الشيفرة المكونة لهذه البيبتيدات المكونة من 12 حمض أميني تم استخدام جين 3 المكون لبروتين 3 من phage M13 و العمل على تكثيره باستخدام البارئاتprimers والتي تم تصميمها اعتمادا على الأطراف المحيطة لجين 3 ومن ثم تطبيق فحص PCR. تم تحديد الترتيب القواعدي ل 15 clones مختلفة ومن ثم تم تحديد التشابه لهذا الترتيب من خلال عمل بحث في بنك المورثات ومن ضمن 15 تم تحديد أربعة سميت كما يلي Ph3,Ph5,Ph19,Ph23 لإظهارها تشابها عاليا مع *Leishmania donovani* وأصناف أخرى من الليشمانيا وغيرها من الطفيليات ذات السوط تحديدا(*Trypanosoma* and *Trichomonus)* . تم إدخال هذه البيبتيدات التي تم التعرف عليها وتحديدها على موقعها في جين3 والمعروف بدايته ونهايته من النيوكليوتيدات. وهذه المستنسخات الأربعة clones أنفة الذكر تم ترجمتها وقد تبين أن اثنين منها وهي Ph3 , Ph23 أظهرت تشابها كبيرا مع المعلمات المميزة ل *Leishmania donovani* وهذا يمهد مستقبلا إلى عمل التجارب ألتحليله لتصنيع هذه المعلمات epitopes واستخدامها في التشخيص بهدف إيجاد لقاح لليشمانيا الحشوية.