Studies on Generation and Characterization of Live Attenuated *Leishmania* Parasites as Vaccine Candidates of Visceral Leishmaniasis

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CERTIFICATE

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Abstract

Leishmania parasite is the causative agent of leishmaniasis, a group of diseases affecting more than 12 million people globally. Visceral leishmaniasis (VL) is often fatal if not treated and responsible for causing second highest number of deaths by any parasitic disease after malaria. Currently, no licensed vaccine is available against any form of human leishmaniasis. *Leishmania* parasite has a dimorphic life cycle, flagellated promastigotes form in sand-fly (*Phlebotomus argentipes*) vector and non-flagellate amastigotes form in the mammalian host. The morphological and biochemical alteration between the two life stages of *Leishmania* parasites are regulated by stage specific expression of several genes. Amastigote specific genes are believed to be responsible for the survival and replication of the parasite in the hostile environment of mammalian hosts and could be targeted to block new infection. Further, they can also serve as a target to generate live parasite vaccine candidates, specifically attenuated at the mammalian infecting amastigote stage. In the present study, we have characterized amastigote stage over-expressing *Ld*A1 gene to explore its potential as a target gene for generation of live attenuated *Leishmania* parasite vaccine candidate. LdA1 single allele deleted parasites ($LdA1^{+/-}$) were generated using homologous recombination method, however, its double allele deleted mutants ($LdA1^{-/-}$) failed to survive. $LdA1^{+/-}$ mutants showed reduced motility, size and growth rate at both the life stages compared to the wild type parasite. However, their infectivity to the macrophages was similar to the wild type, suggesting their ability to infect macrophages was not altered. Additionally, to gain insight into relevance of LdA1 in the *Leishmania* life cycle, *in silico* analysis and over-expression studies were also performed. Sequence homology search revealed that LdA1 is unique to *Leishmania* genus as no homologs are present in any other organism. Sequence and structure level

functional annotations predicted that LdA1 might be involved in the biological processes critical for the survival of parasites. Over-expression of LdA1 in *Leishmania* parasite did not affect its growth, phenotype and infectivity. In conclusion, LdA1 is an amastiogte specific gene and it may have an essential role in *Leishmania* life cycle, as reflected by *in silico* function prediction and inability to generate null mutants.

In other part of the study, we evaluated vaccine potential of centrin1 and p27 gene deleted live attenuated *Leishmania* parasites (*Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$) reported as vaccine candidates. Both *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$parasites specifically get attenuated at the mammalian infecting amastigote stage and are safe, immunogenic and protective in animal models. Here, to assess vaccine potential of *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ for humans, we evaluated immune responses induced by them in human blood samples obtained from healthy, healed VL (HVL), post kala-azar dermal leishmaniasis (PKDL) and VL subjects. Both parasites infected human macrophages, as effectively as the wild type parasites. Further, *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$strongly stimulated production of pro-inflammatory cytokines including, IL-12, IFN-γ, TNF-α, IL-2, IL-6 and IL-17 in the PBMCs obtained from individuals pre-exposed to *Leishmania* parasites (HVL and PKDL). There was no significant stimulation of anti-inflammatory cytokines (IL-4 and IL-10). Induction of Th1 biased immune responses was supported by a remarkable increase in IFN-γ secreting CD4+ and CD8+ T cells and IL-17 secreting CD4+ cells in PBMCs from HVL cases with no increase in IL-10 secreting T cells. Hence, *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ are promising as live vaccine candidates against VL since they elicit strong protective immune response in human PBMCs from HVL, similar to the wild type parasite infection, mimicking a naturally acquired protection following cure.

Contents

Chapters	Page nos.

1. **Chapter-1. Introduction** **1-4**
2. **Chapter -2. Review of literature** **5-49**

2.1 History 5

2.2 Systemic Position 6

2.3 Morphology and Life cycle 7

2.4 Epidemiology 10

2.5 Types of Leishmaniasis 13

2.5.1 Visceral Leishmaniasis (VL) 14

2.5.2 Post-Kala-Azar Dermal Leishmaniasis (PKDL) 15

2.5.3 Cutaneous Leishmaniasis (CL) 16

2.6 Diagnosis 19

2.6.1 Microscopic Examination 19

2.6.2 Immunodiagnostic Methods 20

2.6.3 Molecular Tests 23

2.7 Drug and Treatment 25

2.7.1 Pentavalent Antimonials (Sb^V) 26

2.7.2 Amphotericin B (AmB) 27

2.7.3 Paromomycin (PMM) (Aminosidine) 28

2.7.4 Miltefosine (MIL) 29

2.7.5 Pentamidine 30

2.7.6 Sitamaquine 31

2.7.7 Combination Therapy 31

2.8 Preventive Methods 32

2.9 *Leishmania* Macrophage Interaction 33

2.9.1 Entry into the Macrophages 33

2.9.2 Intracellular Survival Strategies of *Leishmania* Parasite 35

2.10 Immune Responses 37

2.11 Vaccines for Leishmaniasis 41

2.11.1 Killed *Leishmania* Vaccine 42

2.11.2 Subunit Vaccines 42

2.11.3 Live Attenuated Vaccine 44

2.11.4 DNA Vaccine 47

Gaps in the Existing Research 48

3. Chapter- 3. Aims & Objectives **50-51**

4. Chapter-4. Characterization of *Leishmania* Amastigote Specific Gene (LdA1) by Gene Deletion/Episomal Expression Study for Vaccine Development **52-83**

4.1 Introduction 52

4.2 Material & Methods 54

4.2.1 *In silico* analysis of putative LdA1 protein 54

4.2.2 Parasites cultures and growth kinetics 55

4.2.3 Cloning, sequencing, expression and purification of LdA1 protein 56

4.2.4 Plasmid construct for Over-expression/Episomal expression of LdA1 In *Leishmania* parasite 57
4.2.5 Electroporation 57
4.2.6 Western blot analysis 58
4.2.7 Immunolocalization of LdA1 58
4.2.8 Construction of DNA for LdA1 gene disruption by homologous recombination 59
4.2.9 DNA transfection and selection of LdA1 gene deleted mutants by PCR 60
4.2.10 *In Vitro* macrophage infection 61
4.3 Results 62
4.3.1 Putative LdA1 protein: sequence and structure based annotations 62
4.3.2 Recombinant LdA1 protein preparation, antibody generation 67
4.3.3 Confirmation of up regulated expression of LdA1 at amastigote stage 67
4.3.4 Immunolocalization of LdA1 in *Leishmania* parasite 68
4.3.5 Episomal expression of LdA1 and its effect on parasite growth and phenotype 69
4.3.6 Formation of constructs for deletion of LdA1 by homologous recombination 72
4.3.7 Generation of LdA1 single deleted mutant parasites by 75

transfection with knockout constructs

4.3.8 Effect of LdA1 single allele deletion on parasites phenotype and growth 76

4.3.9 Evaluation of macrophage infectivity of mutant parasites 77

4.3.10 Evaluation of survival of mutant parasites inside macrophages 79

4.4 Discussion 80

4.5 Conclusion 83

5. Chapter - 5. Evaluation of Immune Responses in Human PBMCs Elicited by Live Attenuated *Leishmania* Parasites as Vaccine Candidates 84-116

5.1 Introduction 84

5.2 Material &Methods 86

5.2.1 Study subjects and ethical consideration 86

5.2.2 Parasite culture and molecular analysis to confirm the absence of *Leptomonas* in all cultures 88

5.2.3 PBMCs isolation and stimulation with parasites 89

5.2.4 Multiplex ELISA for cytokine estimation 89

5.2.5 Preparation of human peripheral blood macrophages and *in vitro* macrophage infections 90

5.2.6 Intracellular staining and flow cytometry 90

5.2.7 Statistical analysis 91

5.3 Results 92

5.3.1 Confirmation of pure cultures of *Leismania* parasites 92

5.3.2 Determination of infectivity of *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites in human PBMCs differentiated macrophages 93

5.3.3 Evaluation of pro-inflammatory cytokines response induced by *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ in PBMCs 95

5.3.4 Correlation between Th1 cytokines 100

5.3.5 Evaluation of anti- inflammatory cytokines response induced by *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ in PBMCs 101

5.3.6 Flow cytometry analysis for the determination of cellular source of IFN-γ, IL-10 and IL-17 cytokines upon stimulation with parasites 104

5.3.7 Ratio of IFN-γ/IL-10 producing cells 107

5.4 Discussion 108

5.5 Conclusion 116

6. Conclusions and Future Scope of Work **117-119**

7. Bibliography **120-145**

8. Appendix **I -XI**

9. List of Publications and Presentations

10. Brief Biography of the Candidate

11. Brief Biography of the Supervisor

12. Brief Biography of the Co-Supervisor

List of Tables

Content		Page no.
2.1	Summary of clinical manifestations and geographical distribution of *Leishmania* species	13
2.2	Genetically defined live attenuated *Leishmania* vaccine candidates	46
4.1	Sequence level functional annotations of putative LdA1 protein	63

List of Figures

Content		Page no.
2.1	Schematic representation of cell organelles in *Leishmania* parasites.	8
2.2	Life cycle of *Leishmania* parasite in two different hosts	9
2.3	Maps of the geographic distribution of cutaneous leishmaniasis (CL)	11
2.4	Maps of the geographic distribution of visceral leishmaniasis (VL)	12
2.5	Patients with clinical manifestation of VL	15
2.6	Clinical presentation in PKDL	16
2.7	Clinical presentation in CL	17
2.8	Patients with clinical manifestation of MCL	18
2.9	Clinical presentation in DCL	18
2.10	Demonstration of amastigotes by Microscopy in bone marrow sample of VL Patient.	20
2.11	Serological tests for visceral leishmaniasis	22
2.12	Chemical structures of currently used antilesihmanial drugs	30
2.13	Receptor mediated phagocytosis of *Leishmania* parasite by phagocytic cells	34
2.14	Transfer of *Leishmania* infection to the macrophages by neutrophils	35
2.15	Th1 and Th2 dichotomy in leishmaniasis	38
2.16	Different cytokines involved in activation/suppression of macrophage function during *Leishmania* infection	41
4.1	Phylogenetic analysis showing evolutionary patterns of putative LdA1 protein in *Leishmania*	63

4.2 *In silico* characterization of putative LdA1 protein 65

4.3 Preparation of recombinant LdA1 protein 67

4.4 Comparison of expression of LdA1 between promastigote and amastigote stage using western blot. 68

4.5 Immunolocalization of LdA1 in promastigotes and axenic amastigotes 69

4.6 Over-expression of LdA1 in *Leishmania* parasite by using *Leishmania* specific expression vector pKSNEO 70

4.7 Growth curve of mock transfected (Ld1S) and LdA1overexpressing (LdA1-OE) mutants. 71

4.8 PCR amplification and cloning of 5’ and 3’ flanking regions of LdA1 gene 72

4.9 Cloning of 3’ and 5’ flanking regions of knockout targeting plasmid. 73

4.10 Schematic diagram showing design of constructs for LdA1 gene disruption from *Leishmania* genome. 74

4.11 Generation of knockout constructs for homologous recombination 74

4.12 Confirmation of single allele deletion of LdA1 75

4.13 Effect of LdA1 single allele deletion on phenotype of parasites 76

4.14 Effect of LdA1 single allele deletion on growth of parasites 77

4.15 *In vitro* infectivity of mutant parasites 78

4.16 Effects of LdA1 deletion on the replication of *L. donovani* amastigotes inside macrophages 79

5.1 Diagnostic confirmation of cases for the recruitment in the study 88

5.2 PCR-RFLP analysis of HSP-70 gene from different parasite cultures 92

5.3 *In vitro* macrophage infectivity of live attenuated *Leishmania* parasites 94

5.4 Level of pro-inflammatory cytokines stimulated by wild type (*Ld*1S), *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites in culture supernatant of PBMCs obtained from healthy, VL, HVL and PKDL patients 96

5.5 Level of pro-inflammatory cytokines stimulated in culture supernatant of PBMC obtained from healthy, HVL, VL and PKDL groups by wild type (*Ld*1S), *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites. 98

5.6 Pairwise correlation of IL-12 and IFN-γ cytokines stimulated in response to parasites in culture supernatant of PBMCs obtained from HVL 101

5.7 Level of anti-inflammatory cytokines stimulated by wild type (*Ld*1S), *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites in culture supernatant of PBMCs obtained from Healthy, HVL, VL and PKDL patients 102

5.8 Level of anti-inflammatory cytokines stimulated in culture supernatant of PBMCs obtained from healthy, HVL, VL and PKDL groups by wild type (*Ld*1S), *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites 103

5.9 Flow cytometry analysis showing the gating strategy used to identify the phenotype of cytokine (ex:- here IFN-γ) producing cells 104

5.10 Percentage of IFN-γand IL-17 -producing cells after stimulation of blood PBMCs obtained from healthy and HVL with wild type (Ld1S), *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites 105

5.11 Percentage of IL-10 producing cells after stimulation of blood PBMCs obtained from healthy and HVL with wild type(Ld1S), *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/}$ 106

5.12 Ratio of IFN-γ/IL-10 producing cells after stimulation of blood PBMCs obtained from HVL with wild type (Ld1S), *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites. 107

Abbreviations

Å	Angstrom
AmB	Amphotericin B
BSA	Bovine Serum Albumin
BLAST	Basic Local Alignment and Sequence Tool
bp	Base pair
CD	Cluster of differentiation
cDNA	Complementary DNA
CL	Cutaneous leishmaniasis
cm	Centimeter
CMI	Cell mediated immunity
CRP	C-reactive protein
CTLA	Cytotoxic T lymphocyte associated antigen
cGLP	Current good laboratory practice
DAPI	4',6-diamidino-2-phenylindole
DCL	Diffuse cutaneous leishmaniasis
ddH2O	Double Distilled water
dNTP	Deoxyribose nucleotide triphosphate
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbant assay
FCS/FBS	Fetal Calf/Bovine Serum
FITC	Fluresceine isothyanate
Foxp3	Forkhead box protein 3
HEPES	N-2-hydroxyethgylpiperazine-N-2 ethane sulfonic acid
HIV	Human Immune Deficiency Virus
HRP	Horseradish peroxidase
IFA	Inflorescence assay
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ITS	Internal transcribed spacer

IV	Intravenous
KA	Kala-azar
Kb	Kilo base
kDa	Kilo dalton
KO	Knock-out
LB	Luria Bertani medium
M	Molarity
MCL	Mucocutaneous leishmaniasis
MCP	Monocyte chemoattractant protein
MD	Molecular dynamics
MHC	Major histcompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metello-proteinase
N	Normailty
$NaHCO_3$	Sodium bicarbonate
NO	Nitric oxide
O/N	Over night
OD	Optical Density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PBS-T	Phosphate buffered Saline with Tween-20
PCR	Polymerase chain reaction
Pg	Picogram
PHA	Phytohemagglutinin
PKDL	Post Kala-azar Dermal Leishmaniasis
RFLP	Restriction fragment length polymorphism
RPMI	Roswell Park Memorial Institute medium
RNase	Ribonuclease
RORγt	Retenoic acid related orphan receptor gamma
rpm	Revolution per minute
SDS	Sodium Dodecyl Sulphate
SEM	Standard error mean
SSG	Sodium stibogluconate

STAT	Signal transducer and activator of transcription
TAE	Tris acetate EDTA
TGF	Transforming growth factor
Th1	T helper-1 cell
Th2	T helper-2 cell
TNF	Tumor necrosis factor
Tris	Tris (hydroxymethyl) aminomethane
U	Unit
UTR	Untranslated region
UV	Ultraviolet
VL	Visceral leishmaniasis
WHO	World Health Organization

Chapter 1

Introduction

Leishmaniasis is a group of diseases caused by obligate intracellular protozoan parasites belonging to the genus *Leishmania.* More than 20 species of *Leishmania* are capable of causing disease in humans and they are transmitted by more than 30 different species of phlebotomine sandflies (Akhoundi *et al*., 2016). Manifestations of leishmaniasis depend on host responses, parasite species and poorly understood host-parasite-vector interations. Different *Leishmania* species have different tissue tropism. Some of them (i.e. *Leishmania major*) mainly infect the skin resident macrophages causing lesion at the bite site, which is known as cutaneous leishmaniasis (CL) and it may further result in the involvement and destruction of mucosa in case of *Leishmania amazonensis* and giving rise to the muco-cutaneous leishmaniasis (MCL). Species such as *Leishmania donovani* and *Leishmania infantum* infect liver, spleen and bone marrow causing visceral leishmaniasis (VL), which is the most aggressive form of leishmaniasis and potentially fatal if left untreated. Post kala-azar dermal leishmaniasis (PKDL) is a dermal sequel of VL, characterized by the appearance of hypopigmented macules, red papules or lesions like nodules on any part of the body, especially on the face. The features that control different tropism of the parasite (viscerotropism or dermatotropism) have not yet been elucidated but are thought to be the result of host and parasite genetics as well as the status of immunity. Leishmaniasis is endemic in 98 countries with more than 1 billion people living in endemic areas are at risk of getting infected (Over 616 million for VL and 431 for CL). An estimated 0.2–0.4 million new cases of VL causing 20,000- 40,000 deaths and 0.7–1.3 million new cases of CL occurs each year worldwide (Borghi *et al*., 2016, WHO 2016).

At present there is no effective vaccine available against any clinical form of leishmaniasis. Currently, the only means to treat and control leishmaniasis are

chemotherapy and vector control. The number of available anti-leishmanial drugs is limited, having toxic effect, require long duration of treatment and may become ineffective due to the emergence of resistant strains (Ramesh *et al*., 2015; Savoia *et al*., 2015; Sundar *et al*., 2014). It is estimated that a prophylactic vaccine with relatively short duration of protection ~ 5 years and efficacy as little as 50% would remain cost–effective as compared to the chemotherapy (Bacon *et al*., 2013). Studies have shown that development of vaccine against leishmaniasis is a feasible goal as the patients recovered from *Leishmania* infection develop life long immunity, making them resistant to re-infection (Gannavaram *et al*., 2014; Kumar *et al*., 2014). Although several experimental vaccines against leishmaniasis have been developed in recent years, which include heat killed or live attenuated parasites, recombinant proteins or DNA encoding *Leishmania* proteins (Srineevas *et al*., 2014; Okwar *et al*., 2009); however, only a few vaccine candidates to date have entered clinical trials. Live attenuated parasites developed by genetically defined irreversible mutations are safer as vaccine, compared to the parasite lines developed by other means including long term culture, irradiation or chemical treatment because in such conditions nature of attenuation is unknown that might cause reversion of parasites to the virulent type and result in disease development especially in the immune-compromised condition. Recent advances in the molecular techniques has facilitated the discovery of new genes, which are essential for parasite survival and has also increased the possibility of manipulation of the *Leishmania* genome for the development of genetically defined/modified live attenuated parasite vaccine. The characteristics of genetically modified live attenuated parasite as a whole parasite antigen vaccine can mimic the natural immunity similar to that observed in subjects recovered from a natural VL infection. Such a whole parasite vaccine has distinct advantage over other vaccine candidates that do not fully mimic a natural

infection (Srineevas *et al*., 2014).

Leishmania parasite has dimorphic life cycle with flagellated, extracellular promastigote form in the alimentary canal of sand fly vector and non-flagellated intracellular amastigote form in the macrophages of mammalian host. Dramatic morphological and biochemical changes occur in the parasite during the process of differentiation, which is correlated with the change in expression of several genes (Flinn *et al*., 1992; Charest *et al*., 1994; Wu *et al*., 2000; Selvapandiyan *et al*., Vaccine 2014). To understand the mechanism of parasitic infection, characterization of the genes, which might be involved in parasite differentiation, is of fundamental importance. Further the genes, which are highly, or exclusively expressed at the amastigote stage, are considered to play some essential role in the survival of parasites inside the phagolysosomal compartment of macrophages. Since specifically the amastigote stage causes the pathology in mammalian hosts, these genes have great potential as targets to block the infection process and development of live attenuated parasites as vaccine candidate (Selvapandiyan *et al*., 2014).

In the present study, amastigote stage over-expressing A1 gene (LdA1) has been targeted to generate knock–out mutant parasites by using homologous recombination method. Furthermore, to understand role of LdA1 in *Leishmania* life cycle, *in silico* analysis, episomal expression, subcellular localization and macrophage infectivity studies were carried out. Besides, the other goal of the study was to evaluate the vaccine potential of two live attenuated *Leishmania donovani* parasites for human use: i) lacking centrin1gene (*Ld*cen1$^{-/-}$), which is an amastigote growth regulating gene (Selvapandiyan *et al*., 2004) and ii) lacking p27 gene (*Ld*p27$^{-/-}$), which is an essential component of cytochrome c oxidase complex, involved in oxidative phosphorylation (Dey *et al*., 2010). Both parasites specifically get attenuated at the mammalian infecting amastigote stage

and are safe, immunogenic and protective in animal models (Selvapandiyan *et al*., 2004, 2009; Dey *et al*., 2010, 2013; Fiuza *et al*., 2013, 2015). These mutant parasites have intrinsic defects in replication that do not allow them to replicate as amastigotes. Hence, their clearance does not depend on host immune reactions or in presence of bystander immunity arising due to a previous infection. In the present study, to evaluate vaccine potential for humans, we have determined the infectivity and immune responses induced by *Ld*Cen1$^{-/-}$and *Ld*p27$^{-/-}$in human peripheral blood mononuclear cells (PBMCs) obtained from different clinical groups including healthy, healed VL (HVL) and active cases of VL and PKDL.

Review of Literature

Leishmaniasis is one of the most neglected tropical diseases in the world, affecting largely the poorest of poor, mainly in the developing countries. It is caused by protozoan parasite *Leishmania* that shuttle between sandfly vector and the mammalian host. Transmission of parasite may be zoonotic (from animal to human) or anthroponotic (from one human to another). In India, the disease is completely anthroponotic where as in certain parts of the world, there are one or more reservoirs or zoonotic hosts such as, dogs in the Mediterranean region and rodents in South Africa. Among 600 known sand flies species, 30 are proven *Leishmania* vector. Old World (Europe, Asia, and Africa) forms of *Leishmania* are transmitted by sandflies of the genus *Phlebotomus,* while New World (America) forms mainly by the genus *Lutzomyia.* In humans, leishmaniasis infection is caused by about 21 of 30 species of *Leishmania* that infect mammals (Akhoundi *et al*., 2016). The different species of *Leishmania* are morphologically indistinguishable but they can be differentiated by DNA sequence analysis, isoenzyme analysis or monoclonal antibodies. The number of cases of leishmaniasis is increasing as a result of widespread migration from rural to urban areas and fast urbanization causing increased exposure to the sandfly, climate changes expanding the geographic range of the vectors and increase in the overlapping of acquired immunodeficiency syndrome (AIDS) and VL (Desjeux *et al*., 2004).

2.1 **History**

Leishmaniasis has a long history, dating back as far as the 2,500 B.C., with several primitive descriptions of the disease in ancient writings and recent molecular findings from ancient archeological material (Akhoundi *et al*., 2016). There are detailed descriptions of oriental sore by Arab physicians, who described it as Balkh sore from Northern Afghanistan, and there are later records from various places in the Middle East including Baghdad and Jericho, where many of the conditions were

given local names by which they are still known. In India and Africa, reports in the middle-18th century describe the disease now known as visceral leishmaniasis as "kala-azar" or "black fever". In India it was first noticed in Jessore (presently in Bangladesh) in 1824, when quinine resistant fever received serious attention, which was initially thought to be malaria (Kumar, 2013). In 1901, Scottish army doctor William Leishman identified certain organisms in smears taken from the spleen of a patient who had died from fever and proposed them to a new organism morphologically related to trypanosomes (Leishman, 1901). After few months Charles Donovan confirmed the findings separately. Major Ronald Ross discovered the link between these organisms and kala-azar and named them as *Leishmania donovani* (Ross, 1903). Experimental proof of transmission of *Leishmania* parasite to humans by sandflies belonging to the genus *Phlebotomus* was demonstrated in 1921 (Cox *et al*., 2002; Swaminath *et al*., 2006).

2.2 **Systemic Position**

Leishmania belongs to the family Trypanosomatidae and is characterized by the possession of kinetoplast, which is a disk-shaped structure within a single mitochondrion. Kinetoplast contains mitochondrial DNA, known as kinetoplast DNA (kDNA). kDNA structure is different to that of any other DNA in nature. It consists of two types of circular DNA molecules, which are present in the form of maxi and mini circles and they are topologically interlocked. In brief, systemic position of *Leishmania* is as follows (Levine *et al*., 1980):

Kingdom: Protista,
Subkingdom: Protozoa
Phylum: Sarcomastigiphora
Subphylum: Mastigophora
Class: Zoomastigophora

Order: Kinetoplastida
Suborder: Trypanosomatina
Family: Trypanosomatidae
Genus: *Leishmania*

2.3 Morphology and Life Cycle

Leishmania species are dimorphic and unicellular parasites having a digenetic life cycle that involves promastigote stage in the female sand fly and amastigote stage in the mammalian hosts. Parasites at both life stages are morphologically distinct from each other. Promastigote is extracellular and flagellated form, which is found in the lumen of sandfly. Fully developed promastigotes have highly elongated, spindle shaped body measuring 15-20 µm in length and 1.5-3.5 µm in width. Flagellum is present at the anterior end of the parasite and it is made of paraxial rods with lattice and tubular structure measuring 15-28 µm in length. The nucleus is present at the center and kinetoplast lies transversely in front of it **(Figure 2.1A).** Procyclic promastigotes migrate toward the stomodeal valve located in the anterior midgut and transformed into infective metacyclic promastigote form. Both procyclic and metacyclic promastigotes form can be differentiated morphologically. The amastigote form resides and multiplies within the macrophages of reticuloendothelial system of the vertebrate host. The parasite is round or oval shaped, 2-4 µm in length along longitudinal axis and 1-3 µm in width. Although the cellular organelles of amastigotes are similar to the promastigotes, in place of flagellum, there is a delicate filament extending the basal body to the margin of the body, which represents root of the flagellum **(Figure 2.1 B)**. The aflagellar amastigote stage of the parasite transform into flagellar procyclic promastigote form in posterior mid gut of sandfly within few hours

of ingestion with blood meal.

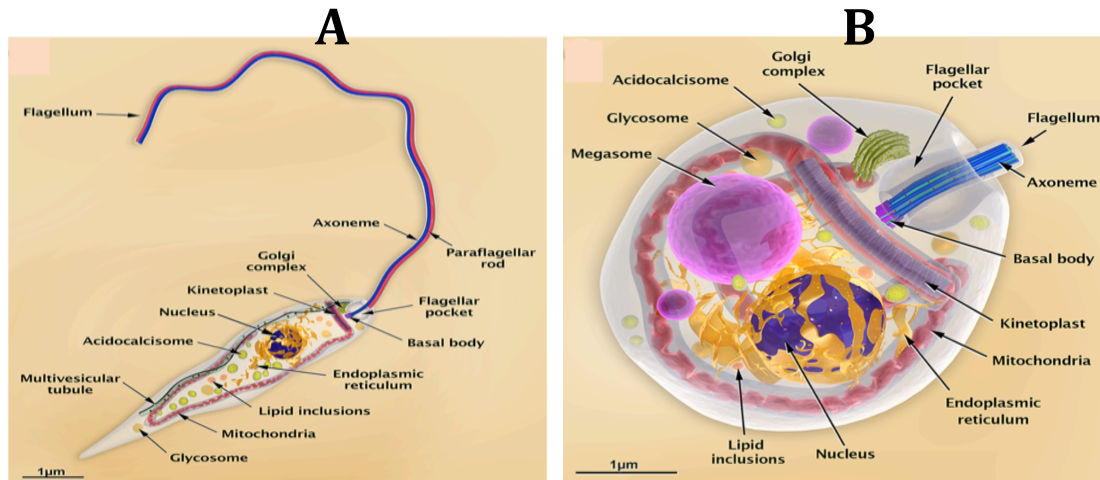


Figure 2.1: Schematic representation of cell organelles in *Leishmania* parasites. (A) Promastigote form and (B) amastigotes form. (Source:- Teixeira *et al*., 2013; PLoS Pathog. 9: e1003594.)

Leishmaniasis infection begins when a phlebotomine sandfly harboring *Leishmania* parasites bites a mammalian host for blood feeding. During the bite, it also injects saliva that prevents blood clotting (Teixeira *et al*., 2013). Following the ingestion of blood, sand fly regurgitates metacyclic promastigotes together with many substances into the vertebrate hosts. These substances induce infiltration of neutrophils and increased recruitment of macrophages at the bite site (Peters *et al*., 2008). The parasites first infect neutrophils and macrophages, which are rapidly recruited to the bite site. However, many other cell types, such as dendritic cells, fibroblasts and langerhans cells, can also be infected (Peters *et al*., 2008). Neutrophils are believed to play an important role as ‘‘Trojan horse’’ as they transfer infection to the macrophages, which serve as the main host cell for *Leishmania* parasites. After engulfment by macrophages, *Leishmania* parasites either remain at the same site, as in the species that cause CL, or they are carried by macrophages to the mucocutaneous

junctions or the reticulo-endothelial tissues as in MCL and VL respectively. After establishing intracellular residence in macrophages, promastigotes rapidly transform into the amastigote stage and start division by binary fission inside the host cells. Intense amastigote multiplication results in rupture of host cells and release of many amastigotes into the surrounding tissues, where they infect new macrophages and cycle continues **(Figure 2.2**).

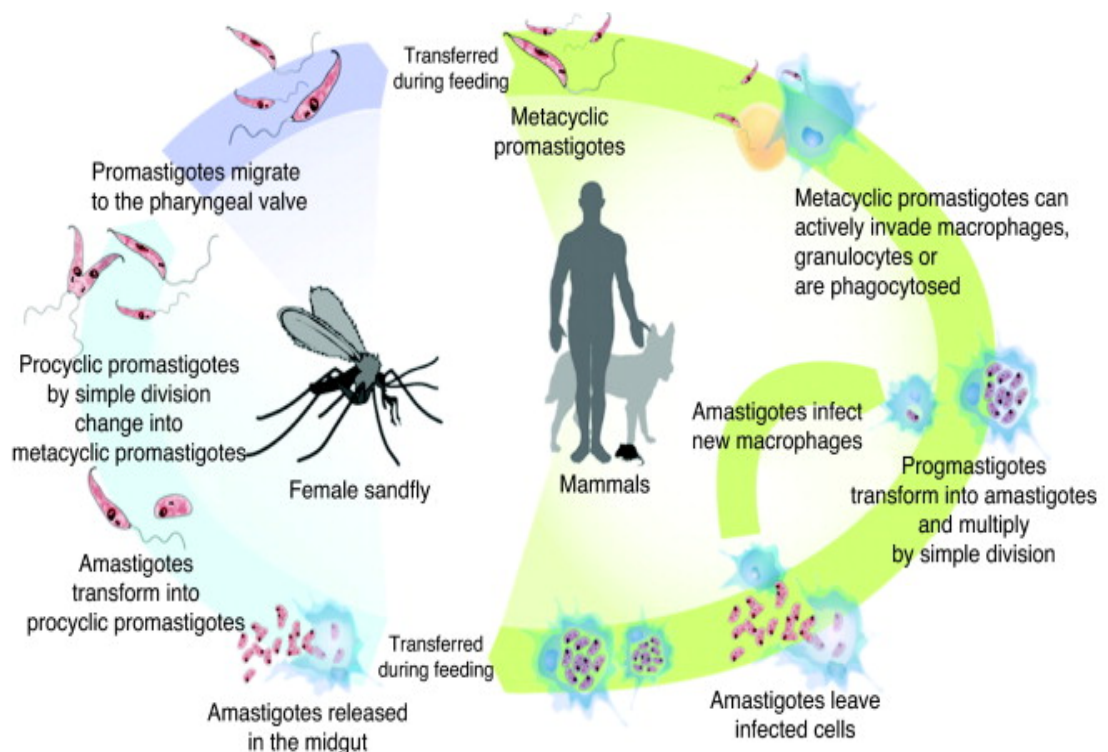


Figure 2.2: Life cycle of *Leishmania* parasite in two different hosts. (Source:- Haray *et al*., 2011; Trends Parasitol. 27:403-9)

In VL, infection eventually spread to all organs containing macrophages and phagocytic cells, especially the visceral organs such as liver, spleen and bone marrow. The infection of sandfly begins when it bites an infected mammal during its blood meal. In sandfly these amastigotes transform into promastigotes, which continue dividing by binary fission and then transform into procyclic promastigotes in posterior mid gut. Procyclic promastigotes keeps on dividing and finally transform into the

infective metacyclic promastigote in the anterior midgut or foregut or both. During the next blood meal, sandfly transfers these infective metacyclic promastigote to the mammalian hosts via regurgitation **(Figure 2.2).**

2.4 **Epidemiology**

Leishmaniasis is amongst the most significant health problems in developing countries, endemic in tropical and sub-tropical regions of the world, as well as southern Europe. According to the World Health Organization (WHO), it is endemic in 98 countries and 3 territories of 5 continents, with more than 350 million people at risk (WHO 2013). It is estimated that 0.7–1.3 million new cases of CL and more than 0.2–0.4 million new cases of VL are diagnosed each year worldwide (Borgie *et al*., 2016; WHO 2016). However, it is likely that true incidence is underestimated due to misdiagnosis and poor reporting guidelines, as reporting is compulsory only in 33 of 98 affected countries (WHO 2013). The cases of leishmaniasis are mainly reported from countries such as Algeria, Bangladesh, Brazil, Colombia, Costa Rica, Ethiopia, India, Iran, Nepal, Peru, Sudan, South Sudan, and Syria **(Figure. 2.3 and 2.4)** (Borgie *et al*., 2016; Karimkhani *et al*., 2016; WHO 2016). Amongst these, over 90% cases of VL occur in just six countries: Bangladesh, Brazil, India, Ethiopia, Nepal and Sudan whereas 70% of global estimate of CL cases occurs in 10 countries: Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, Iran, Peru, Saudi Arabia and Syria (Pigott *et al*., 2014). India remains most important foci of VL, where it is endemic in Bihar, Jharkhand, West Bengal and eastern Uttar Pradesh (Katiyar JC *et al*., 1992). The state of Bihar bears highest disease burden with more than 90% cases reported from here.

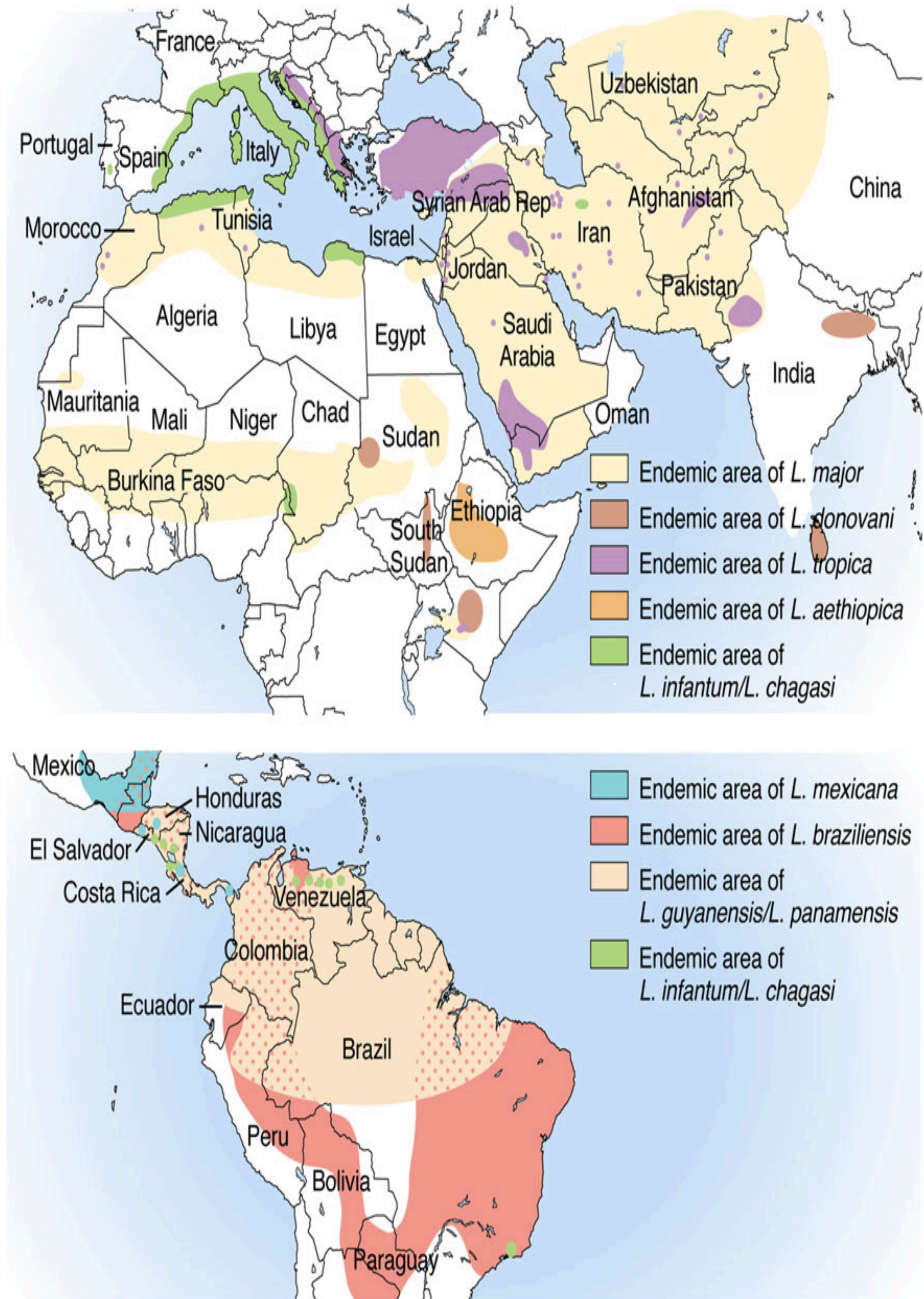


Figure 2.3: Maps of the geographic distribution of cutaneous leishmaniasis (CL).

(Source: Aronson *et al*., 2016; Clin Infect Dis. 63:e202-e264).

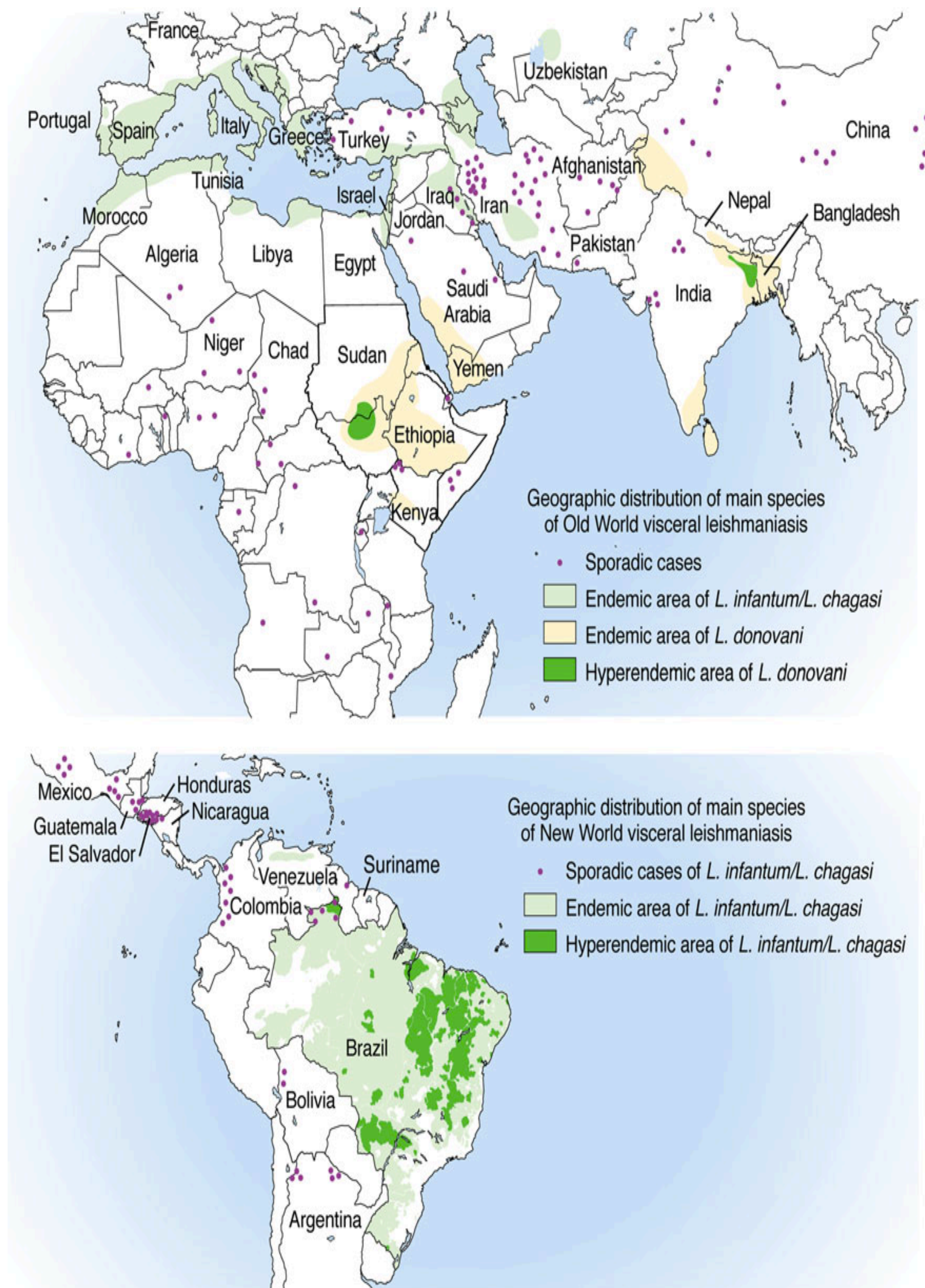


Figure 2.4: Maps of the geographic distribution of Visceral leishmaniasis (VL)

(Source: Aronson *et al*., 2016; Clin Infect Dis. 63:e202-e264).

2.5 **Types of Leishmaniasis**

Leishmaniasis term is used to represent spectrum of diseases ranging from the lethal visceral infection in VL or chronic ulcer of oriental sore in CL, or spreading to the mucous membranes to produce disfigurement in mucocutaneous leishmaniasis. The clinical outcome of infection depends upon infecting species of *Leishmania,* host genetic factors and immune status. A summary of clinical manifestations and geographic distribution of the *Leishmania* species is given in **Table 2.1.**

Table 2.1 Summary of clinical manifestations and geographical distribution of *Leishmania* species

Species	**Clinical manifestation**	**Geographical Distribution**
L. donovani	Visceral (kala-azar), PKDL	Old World: China, India, Bangladesh, Africa.
L. infantum	Visceral	Old World: North Central Asia, Northwest China, Uzbekistan, Middle East.
L. chagasi	Visceral	New World: South and Central America.
L. major	Cutaneous	Old World: Africa, Middle East, Northern Asia
L. mexicana	Cutaneous, Diffuse cutaneous, Mucocutaneous	New World: Southern Mexico, Belize, Northern Guatemala, Southern Texas.
L. amazonensis	Cutaneous, Mucocutaneous	New World: South and Central America.
L. braziliensis	Mucocutaneous, Cutaneous	New World: Throughout South America.
L. tropica	Cutaneous	Old World: India, South-West Asia, North and East Africa
L. panamensis	Mucocutaneous, Cutaneous	New World: Panama

L.venezuelensis	Cutaneous, Diffuse cutaneous	New World: Venezuela
L. guyanensis	Cutaneous, Mucocutaneous	New World: Brazil; Colombia; French Guiana

2.5.1 **Visceral Leishmaniasis (VL)**

VL (also known as kala-azar) is the most severe form of leishmaniasis and is often fatal if left untreated. VL is typically caused by *Leishmania donovani* (*L. donovani*) complex, which includes three species: *L donovani, L. infantum and L. chagasi*. Among them, *L. donovani* causes VL in the Indian subcontinent and East Africa, *L. infantum* in the Mediterranean basin and *L. chagasi* in South America (Berman, 1997). VL is ranked second in causing mortality and fourth in morbidity among all tropical diseases, with 20,000 to 40,000 deaths per year worldwide (Borghi *et al*., 2016, WHO 2016). Its symptoms include high fever, severe cachexia (weakness and wasting of the body), hepato-splenomegaly, pancytopenia and hypergammaglobulinaemia with hypoalbuminaemia **(Figure 2.5)**. Skin pigmentation can also be a feature (kala-azar: meaning black disease in hindi). After entering into the host, parasite invades and multiplies within macrophages (free mononuclear phagocytic cells) and affects the reticuloendothelial system including spleen, liver, bone marrow, and lymphoid tissues.

VL and HIV co-infection has now emerged as a major threat making clinical manifestation atypical leading to delayed diagnosis. Co-infected patients have usually higher parasite burden and they respond poorly to antileishmanial drugs (Berman, 2003; Desjeux & Alvar 2003). Further, relapses and involvement of uncommon sites of infection such as gastrointestinal or the upper respiratory tract are also frequent. Due to marked immune suppression in both VL and HIV infection, patients suffering

from VL are more susceptible towards development of HIV and vice versa. The rate of co-infected cases has been significantly increased, as in Northern Ethiopia, it has increased from 19% during 1998–1999 to 34% during 2006–2007 (Alvar *et al.* 2008).

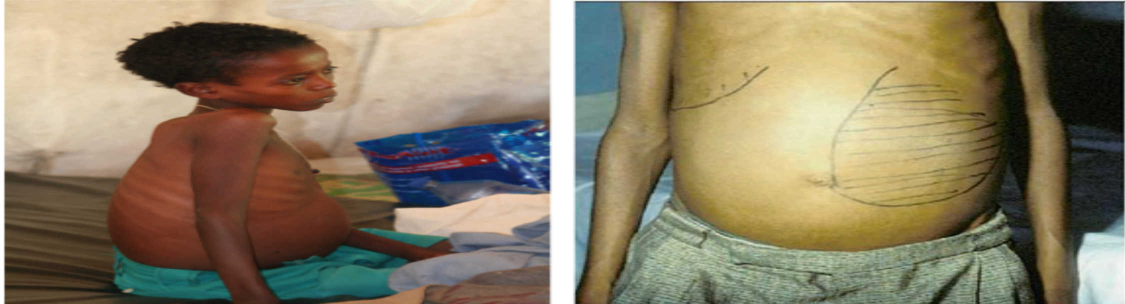


Figure 2.5: Patients with clinical manifestation of VL. Fever, hepato-splenomegaly and substantial weight loss are main clinical symptoms. Marked area shows spleen and liver enlargement. (Source: - Murray *et al*., 2005; Lancet 366: 1561–77 and WHO: http://www.who.int/leishmaniasis/visceral_leishmaniasis/en/)

Further, only a small proportion of *Leishmania* infection manifests as clinical disease and majority of them remain asymptomatic (~6–10 times more than reported cases of VL) (Singh *et al*., 2014); however, these cases can serve as a potential reservoir in transmission dynamics of VL (Sharma *et al*., 2000). Hence, elimination of VL highly depends on management of such cases.

2.5.2 **Post-Kala-Azar Dermal Leishmaniasis (PKDL)**

PKDL is a dermal sequel of VL or kala-azar that usually develops at uncertain times following VL treatment (months to several years in India and weeks to months in Sudan) (Salotra & Singh, 2006). It is characterized by appearance of hypopigmented macules, red papules or nodular rash, or a combination of these, mainly on the face followed by the trunk and limbs **(Figure 2.6).** PKDL is mainly reported in East Africa and the Indian sub-continent, where it develops in approximately 5–15% cured cases of VL in the Indian subcontinent and 50–60% of

cured VL cases in Sudan (WHO 2012; Ramesh *et al*., 2015). PKDL patients usually develop atypical presentations in case of HIV co-infection, such as large nodular lesions and the treatment is also difficult due to lack of proper regimen (Ramesh *et al*., 2014). In the Indian subcontinent, where transmission of VL is only anthroponotic, PKDL patients serve as important parasite reservoir, especially during an inter-epidemic period (WHO 2010; Ramesh *et al*., 2015). Hence, proper monitoring and treatment of PKDL is very important to eliminate VL.

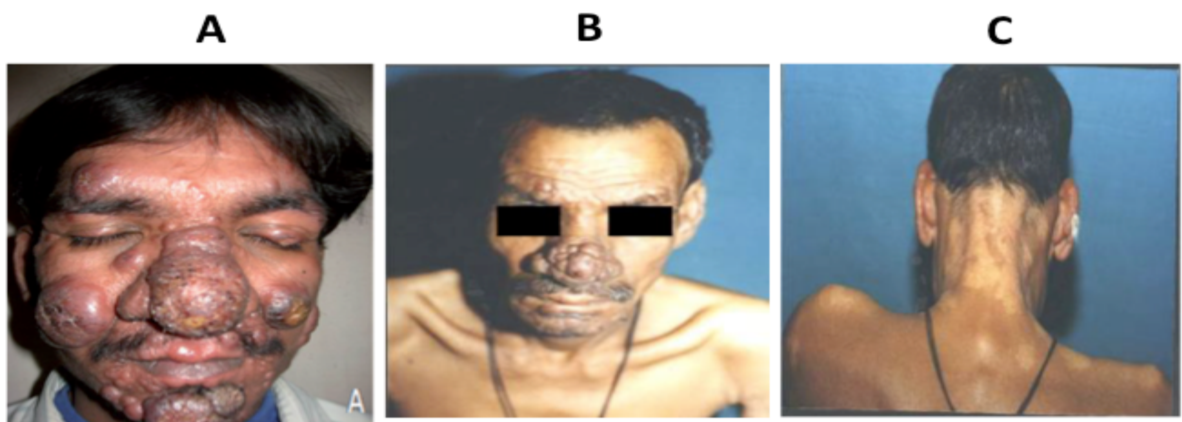


Figure 2.6: Clinical presentations in PKDL. (A) Tumor like nodular lesions on face. (B) Papular-nodular lesions present on face. (C) Hypopigmented macules on neck area (Source: - Ramesh *et al*., 2014; ActaDermVenereol 94:242-3 and Salotra & Singh., 2006; Indian J Med Res 123: 295).

2.5.3 **Cutaneous Leishmaniasis (CL)**

CL is the most common and widely prevalent form of leishmaniasis. It is characterized by development of relatively benign self-healing lesions at the site of sandfly bite, mainly on the exposed part of the body, such as the face, forearms and lower legs, which are easily accessible to sandflies **(Figure 2.7)**. CL is also known as 'oriental sore' and lesions are usually painless and non-pruritic. *L. major, L. tropica* and *L. aethiopica* mainly cause old world CL, whereas new world CL is caused by *L.*

braziliensis. CL infection can remain subclinical and appearance of clinical symptoms may occur after a variable incubation period that averages several weeks to months. These lesions take months to years for complete healing and generally leave depressed scars with altered pigmentation.

Figure 2.7: Clinical presentations in CL. Ulcerative skin lesions, with a raised outer border in CL patients. (Source:- Chappuis *et al*, 2007, Nature Rev Microbiol, 5:S7-15 and CDC: https://www.cdc.gov/parasites/leishmaniasis/health_professionals/)

2.5.3.1 **Variation in Cutaneous Leishmaniasis**

2.5.3.1.1 **Mucocutaneous Leishmaniasis (MCL)**

MCL is a dreaded sequela of new-world CL and it is occasionally reported from Sudan and other Old World foci. The members of *L. braziliensis* complex primarily cause MCL, also known as 'espundia'. In MCL mucosal involvement occurs affecting oral cavity, nose and pharynx, which causes progressive naso-oropharyngeal destruction with hideous disfiguring lesions, that may lead to permanent mutilation of the face **(Figure 2.8)** and also increasing the risk of secondary infection. Only a small proportion of patients (< 5%) with simple cutaneous leishmaniasis develop MCL. Initially it begins as simple skin lesions, which metastasize via the blood stream or lymphatics, particularly to the mucosa of the mouth and nose. The expression of MCL can occur while the primary lesions are still present or can also occur after several years of resolution of the original cutaneous lesions.

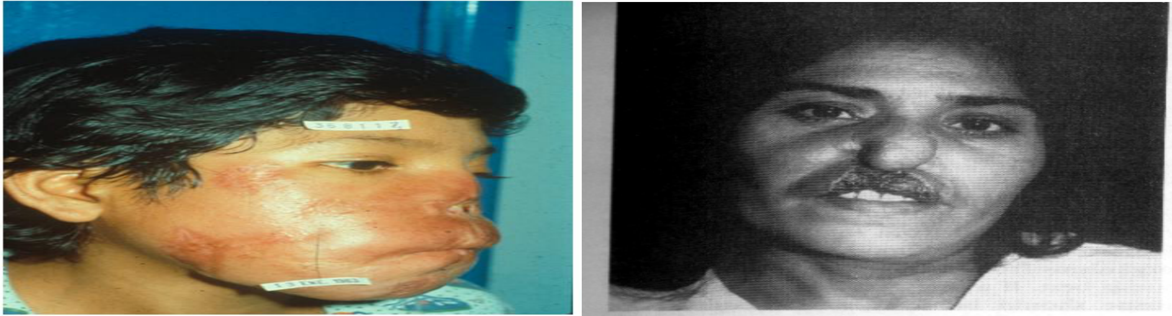


Figure 2.8: Patients with clinical manifestation of MCL.

(Source: - Neva and Brown, Basic Clinical Parasitology, 6th edition., published by Appleton and Lange, 1994).

2.5.3.1.2 **Diffuse Cutaneous Leishmaniais (DCL)**

DCL is chronic, progressive and a rare cutaneous infection with non-ulcerating nodules resembling lepromatous leprosy. These lesions can metastasize distally to cover large areas of the body **(Figure 2.9)**. Its main causative organisms are *L. aethiopica* (old world) and *L. mexicana* species complex (new world). It is reported to manifest only in the patients with impaired cell mediated immunity, otherwise the same parasite causes simple ulcerating lesions in general population.

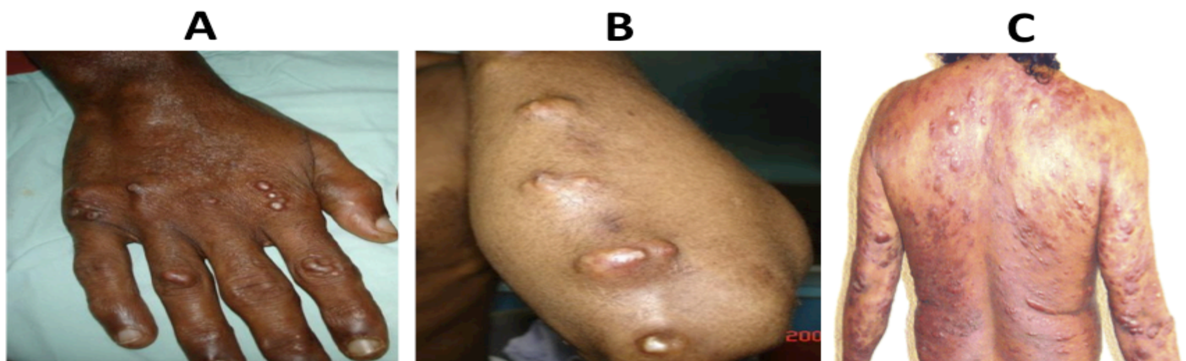


Figure 2.9: Clinical presentation in DCL: (A) Papules like lesions present on the dorsa of hands. (B) Multiple discrete nodules present on the elbows. (C) Multiple papules and nodules present all over the body. (Source: -Mehta *et al*., 2009; Dermatology Online Journal 15(4):9 and Calvopina *et al*., 2006; Am J Trop Med Hyg. 75:1074-77) .

2.5.3.1.3 **Leishmaniasis Recidivans**

It is a hypersensitive dermal response, characterized by the appearance of nodular lesions and satellite lesions at the margin of previously healed CL lesions. Its symptoms may arise decades after resolution of primary lesions and it is difficult to cure.

2.6 Diagnosis

The clinical signs and symptoms of VL are not specific enough to differentiate with other co-endemic diseases (Hercules *et al*., 2016). Similarly, diagnosis of PKDL and CL remains a major challenge. PKDL is often misdiagnosed and confused with other skin diseases, including vitiligo in hypopigmented form and as leprosy in nodular form (el Hassan *et al.*, 1992; Ramesh and Mukherjee, 1995, Verma *et al*., 2013a). CL may be confused with sporotrichosis, *Mycobacterium marinum* infection, sarcoidosis, leprosy, squamous cell carcinoma etc. (Vega-López, 2003). Hence, early and accurate diagnosis of VL, PKDL and CL is important for the effective treatment. Commonly used methods for their diagnosis include: - demonstration of parasite by microscopy, immunodiagnosis by detection of antileishmanial antibodies and molecular tests including detection of parasite DNA by PCR.

2.6.1 **Microscopic Examination**

The visualization of the amastigote form of the parasite by microscopic examination **(Figure 2.10)** of aspirates from lymph nodes, bone marrow or spleen in VL and tissue biopsy of lesions in PKDL and CL is the classical confirmatory test and considered as diagnostic gold standard. Although the specificity is high, the sensitivity of microscopy varies. In VL, it is higher for spleen (93–99%) than for bone marrow (53–86%) or lymph node (53–65%) aspirates (Babiker *et al*., 2007, Sundar, 2003). In PKDL, sensitivity of microscopy is 67–100% in nodular lesions, 36–69% in

papular lesions and 7–33% in macular lesions (Salotra & Singh 2006). In CL, sensitivity is 70-100% (Vega-López, 2003). The accuracy of microscopic examination is influenced by technical expertise and the quality of reagents, hence for accurate diagnosis, well-trained, competent microscope analyst and effective quality control (QC)/ quality assurance (QA) is needed.

Parasite culture from bone marrow or splenic aspirates of VL and tissue biopsy or slit aspirate of PKDL and CL is also a highly specific detection technique. However, the culture technique is tedious, time-consuming and expensive, therefore remains restricted to referral labs (WHO, 2010; Srividya *et al*., 2012; Vega-López, 2012).

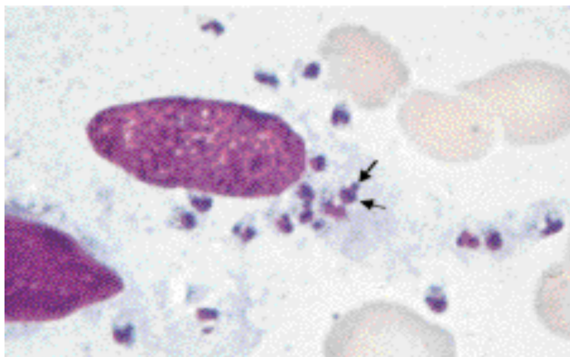


Figure 2.10: Demonstration of amastigotesby Microscopy in bone marrow sample of VL Patient. (Source:-Herwaldt BL, 1999; Lancet.354:1191-9)

2.6.2 **Immunodiagnostic Methods**

Serological tests are highly sensitive and most commonly used for the diagnosis of VL. Over the last years, several assays using different *Leishmania* antigens have been used for the detection of antileishmanial antibodies (Sato *et al*., 2016). To improve sensitivity and specificity, numerous recombinant antigens have also been used for the serological diagnosis. The major limitation of serology based diagnostic test is presence of antileishmania antibodies, which are detectable up to several years after cure; therefore, they cannot be used for diagnosis of relapse

infection of VL (Sundar & Rai 2002). Furthermore, as serological tests failed to distinguish between past and present infection they are not conclusive for diagnosis of PKDL (Salotra *et al*., 2002; Saha *et al*., 2005). However, they can be used as screening test for relapse cases of VL and PKDL positive cases can be further confirmed by reliable parasitological or molecular test.

2.6.2.1 **Direct Agglutination Test (DAT)**

It is routinely used for the detection of anti-leishmanial antibodies in VL patients. In DAT, formalin fixed whole promastigotes are trypsinized and stained with coomassie brilliant blue and when patient's serum is incubated with this whole parasite antigen, agglutination is observed the next day **(Figure 2.11A)**. It is easy to perform, widely applicable technique with high sensitivity (90-100%) and specificity (95-100%) (El Hassan *et al*. 1988; El Mustasim *et al.* 2006; Jacquet *et al.* 2006). It also showed high sensitivity (90.5%) and specificity (91.8%) in CL cases (Hailu, 2000). A fast agglutination-screening test (FAST) has been developed, which can rapidly detect (< 3 hrs.) anti-leishmanial antibodies in serum samples and on blood collected on filter paper. In this test, freeze-dried antigen and only one serum dilution is used, making the test less cumbersome and readily reproducible (Schoone *et al*., 2001).

2.6.2.2 **Immuno-Chromatographic Strip Test**

Introductions of rK39 immuno-chromatographic strip test (ICT) based rapid diagnostic test have facilitated the field applicability of serological methods. It is very easy to perform, quick, cheap, gives reproducible results and even field workers with minimal training can perform it reliably. After addition of serum/slit aspirate sample,

appearance of two visible bands in rK39 strip indicates the presence of anti-leishmanial antibodies **(Figure 2.11B)**. K39 antigen was obtained from *L. chagasi.* It is made up of 39 amino acid residues and encoded by a kinesin related gene. In India, rK39 is widely used for the diagnosis of VL and PKDL using serum and slit aspirate sample, with sensitivity ranging from 95 to 100 per cent (Singh *et al*., 1995; Salotra *et al*., 2002; Verma *et al*., 2013a). Apart from rK39, several other kinesin-related proteins such as K26, K9, KRP42, and KE16 have been tested for their serodiagnostic potential with variable success (Takagi *et al*., 2007; Mohapatra *et al*., 2010) but rK39 remains the most promising diagnostic antigen.

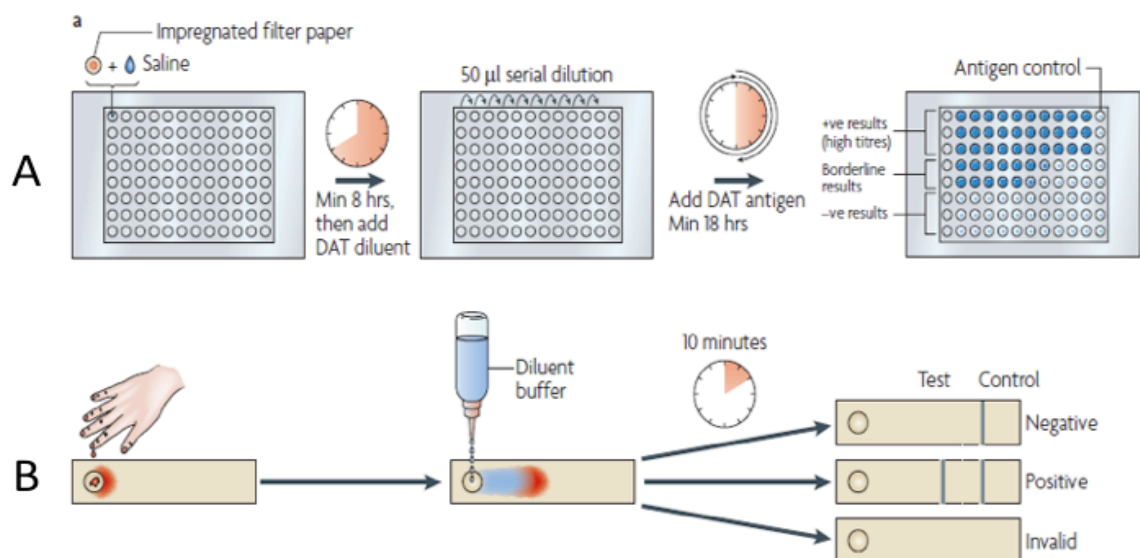


Figure 2.11: Serological tests for visceral leishmaniasis. (A) Direct agglutination test. (B) rK39 immuno-chromatographic strip test. (Source:-Chappuis *et al*., 2007, Nature Rev Microbiol, 5:S7-15).

2.6.2.3 **Enzyme-Linked Immunosorbent Assay (ELISA)**

ELISA is highly sensitive and widely used technique for serodiagnosis of almost all infectious diseases including leishmaniasis. Specificity of ELISA depends upon the antigen used, hence several antigens have been tried but crude soluble antigen

(CSA) of *Leishmania* is most commonly used. The sensitivity of ELISA using different concentrations of CSA (100 to 5,000 ng/ml) ranges from 80 to 100%, but cross-reactions with sera from patients with tuberculosis, trypanosomiasis, and toxoplasmosis have also been found (Cohen & Sadun 1976; Smrkovski *et al*., 1977; Choudhry *et al*., 1980; Singh *et al*., 1995; Kumar *et al*., 2001). ELISA is not commonly used in field conditions since it is quite expensive, laborious and/or require specific technical equipment (Srivastava *et al.* 2011).

2.6.2.4 **Antigen-Detection Tests**

Antigen detection tests are more specific in diagnosis of active *Leishmania* infection than antibody-detection tests as they avoid cross-reactivity and can distinguish active from past infections. However, detection of *Leishmania* antigen in serum sample of patient is complicated due to presence of high level of circulating immune complexes, antibodies, rheumatoid factor and autoantibodies, all of which may mask immunologically important antigenic determinants or competitively inhibit the binding of free antigen (Salotra & Singh 2006). A latex agglutination test (KATEX) detecting a heat-stable, low-molecular-weight carbohydrate antigen in the urine of VL patients has been developed (Sundar *et al.* 2005; Chappuis *et al.* 2006). It has shown sensitivity range between 68 and 100% and a specificity of 100% (Bagchi *et al*., 1998; Salotra & Singh 2006). However, its applications for the diagnosis of PKDL cases remains to be confirmed.

2.6.3 **Molecular Tests**

Molecular approaches have become increasingly relevant due to high sensitivity, specificity and limitations associated with parasitological and serological tests (Srividya *et al*., 2012; Paiva-Cavalcanti *et al*., 2015). There are several

advantages of PCR technique including requirement of small amount of target material, rapid detection, ability to differentiate between past and present infection, follow up of treatment as well as the assessment of the successful cure of VL and PKDL. The sensitivity of PCR has been found to be more than 90% in the diagnosis of VL, CL and PKDL (Salotra *et al*., 2003; Vega-Lopez, 2003; Salotra & Singh, 2006; Medley *et al*., 2015). Further, PCR is also capable of identifying relapses and reinfections in treated PKDL and VL patients that is not possible with serology based techniques. A variety of PCR methods for detection of parasites nucleic acids (DNA/RNA) have been developed for leishmaniasis (Schallig *et al*., 2002) and they can be performed on peripheral blood, splenic, lymph node smears, bone marrow, and serum samples (Motazedian *et al*., 2008). Different DNA sequences which are specific for *Leishmania* species (GP63, ITS1, HSP70, cysteine proteinases) or that have high copy number (such as kinetoplast DNA, ribosomal DNA or mini-exon genes) have been targeted for identification and/or detection of *Leishmania* (Srividya *et al*., 2012). One of the major drawbacks of conventional PCR and serology based detection techniques are their inability to differentiate between clinically active and asymptomatic infection, which can be overcome by using quantitative real time PCR (q-PCR) (Sudarshan *et al*., 2014). q-PCR is a highly sensitive and specific tool used in referral labs for detection/assessment of parasite load in VL, PKDL and CL patients. Remarkable sensitivity and specificity and ability to quantify parasite load at pre and post treatment stages indicated its robust potential in diagnosis of VL and PKDL and monitoring of the treatment outcome (Mary *et al*., 2004; Wortmann *et al*., 2005; Verma *et al*., 2010; Verma *et al*., 2013a). q-PCR has shown 100% sensitivity with blood samples of VL, tissue biopsy and slit aspirate samples of PKDL patients (Galai *et al*., 2011; Verma *et al*., 2013a; Sakkas *et al*., 2016). A quantitative PCR with

validated thresholds can be used to distinguish asymptomatics in endemic areas. However, the need for well-equipped laboratories and cost concerns still limits the use of q-PCR in the field in developing countries, and efforts are on going to overcome such issues.

Loop-mediated isothermal amplification (LAMP) has been developed as an alternative of PCR. It has the advantage of amplifying DNA with rapidity and high specificity under an isothermal condition, using basic equipment (Notomi *et al*., 2000; Nagamine *et al*., 2002; Verma *et al*., 2013b). It has great potential for field application as it is a faster, sensitive and less expensive technique (Verma *et al*., 2013b; Paiva Cavalcanti *et al*., 2015). Study carried out in India has shown that sensitivity of LAMP was 96.4% and 96.8% for VL blood and PKDL tissue sample respectively and its specificity was 98.5% (Verma *et al*., 2013b). Similarly, high sensitivity and specificity of LAMP assay were also observed for buffy coat DNA isolated from blood sample of VL patients in Bangladesh (Khan *et al*., 2012)

In different endemic regions, the sensitivity and specificity of diagnostic methods may vary, hence, during the selection of diagnostic test several parameters including specificity and sensitivity, cost effectiveness, availability of qualified personnel, equipment and field applicability should be considered.

2.7 **Drug Treatment**

At present there are no effective vaccines available for human leishmaniasis, making chemotherapy the only practical means of combating the disease. The present treatment of VL is not satisfactory as the available drugs are costly, toxic to the patients, require long duration of treatment, hospitalization and also associated with

increasing parasite drug resistance (Sundar 2001; Croft *et al*., 2006; Ramesh *et al*., 2015). All antileishmanials drugs have to be administered parenterally (except miltefosine). Moreover, response to antileishmanial drugs depends on host, parasite factors and regional variation. Some approaches/regimens are only effective against certain *Leishmania* strains/species and only in particular geographic regions, thus recommendations for VL treatment vary in different regions.

2.7.1 **Pentavalent Antimonials (Sb^V)**

The pentavalent antimonials [meglumineantimoniate (Glucantime) and sodium stibogluconate (Pentostam)] have remained the standard first line treatment for leishmaniasis for more than 60 years. They are still used as a first line treatment for VL in many parts of world, except in Indian subcontinent where in the state of Bihar this parasite has developed resistance and cure rate of VL has dropped to 36–69% (Sundar *et al*., 2000; 2002). Its common side effects are vomiting, nausea, anorexia, abdominal pain, malaise, myalgia, headache, arthralgia, and lethargy. Serious side effects such as nephrotoxicity, cardio toxicity and pancreatitis are rare but have also been reported (Thakur *et al*., 2004). The recommended dose of $Sb^V$is: 15-20mg/Kg of body weight per day for 21-28 days for VL and MCL, 10-20 days for CL and up to 2 months for PKDL (Ameen *et al*., 2010; WHO 2010).

The mode of action of antimonials is not yet clearly known. Sb^V are considered as prodrugs that require *in vivo* reduction to the leishmanicidal trivalent form [Sb(III)]. These SbIII inhibit macromolecular biosynthesis in amastigotes, possibly by the inhibition of fatty acid beta-oxidation and glycolysis (Berman *et al*., 1985, 1987). Antimony also induces efflux of the intracellular trypanothione, and consequently inhibits the enzyme trypanothionereductase (Wyllie *et al*., 2010), leading to the accumulation of reactive oxygen species (ROS). Sb^V binds to the ribose moiety and

can form stable complexes with adenine nucleosides, which further act as inhibitors of *Leishmania* purine transporters (Demicheli *et al*., 2002). However, due to several limitations including variable efficacy against VL and CL, toxic side effects and significant increase in drug resistance, use of antimonials has decreased worldwide (Croft *et al*., 2003b).

2.7.2 **Amphotericin B (AmB)**

Amphotericin B is a macrolide polyene antifungal antibiotic, discovered in 1956 from a bacterium, *Streptomyces nodosus*. It has been found to be highly effective in the treatment of VL cases that were caused by antimonial-resistant *L. donovani* (Thakur CP *et al*., 1999) and MCL cases that have not responded to antimonials. Efficacy of AmB is >95% in all regions and relapses or primary unresponsiveness of AmB treated patients are rare (Mondal *et al*., 2010). AmB complexes with 24-substituted sterols, such as ergosterol present in the cell membrane of the *Leishmania* parasite, leading to formation of pores, which alter ion balance and causes cell death (Robert *et al*., 2003). The recommended doses of AmB for VL is 0.75 to 1.0 mg/kg for 15 to 20 infusions either daily or on alternate days with a total dose of 1.5 to 2.0g (Sundar 2001). At present AmB is extensively used drug in Bihar for all sodium stibogluconate unresponsive cases and even as a first line drug (with cure rates > 95%) (Sundar 2001; Thakur *et al*., 2008). It has also shown good efficacy in the treatment of VL-HIV-coinfected cases (Sinha *et al*., 2011). Occasional relapses might occur but these patients can be successfully re-treated with the same drug (Giri 1994; Giri and singh 1994). However, there are several limitations for use of AmB as a first line drug including hospitalization for prolonged periods, need of infusions, high cost of the drug, requirement of close monitoring of patients due to incidence of adverse events. Due to these limitations, liposomal formulations of AmB are now preferred over the

conventional AmB (Sundar *et al*., 2014). Common side effect of AmB are infusion reactions, including high fever, chills, rigor and thrombophlebitis, occasionally serious toxicity also occurs e.g. thrombocytopenia, myocarditis, hypokalaemia, nephrotoxicity and even death (Sundar 2001).

Lipid formulations of amphotericin B (AmBisome is the best tested among these formulations) are as effective as the conventional AmB, and have negligible side effects. In the Bihar state of Indian subcontinent a single dose of 15 mg/kg of liposomal-AmB (Sundar *et al*., 2014) or 20 mg/kg administered in four doses of 5 mg/kg over 4–10 days (Burza *et al*., 2014) have shown 100% and 99.3% cure rate respectively. In East Africa, severe cases of PKDL are treated with 20-day course of 2.5 mg/kg per day dose of liposomal-AmB (Musa *et al*., 2005). These lipid formulations lead to targeted drug delivery to the macrophages of spleen, liver and bone marrow without compromising the efficacy of the drug. It is possible to administer high doses of liposomal-AmB over a short period of time and with high cure rate (Sunder *et al*., 2014). The only major drawback of AmBisome is high cost, which makes it unaffordable in VL endemic countries.

2.7.3 **Paromomycin (PMM) (Aminosidine)**

PMM is an aminoglycoside antibiotic with antileishmanial activity. It was isolated from bacterial pathogen *Streptomyces rimosus* var. Paromomycinus in 1950s. Paromomycin inhibits protein synthesis by targeting ribosomal subunits. It also appears to modify membrane polar lipids of *Leishmania* parasite thereby affecting membrane fluidity and permeability (Maarouf *et al*., 1995,1997). An in vitro study showed that it might also target mitochondria (Jhingran *et al*., 2009). PMM was first introduced in India in 2006 for the treatment of VL patients (Mondal *et al*., 2010). In the phase III trial of VL in India, a dose of 15mg/kg PMM sulfate (11 mg base) for 21

days, gave 94.6% cure rates, which was similar to the AmB. However, adverse effects with PMM treatment were more frequent as compared to the AmB (Sundar *et al*., 2007a; 2007b). Most common side effects are mild pain at the injection site (55%) and reversible ototoxicity (2%). Hepato and renal toxicities are rare. Since PMM is an aminoglycoside, emergence of resistance is also possible when used as monotherapy for VL. Paromomycin ointments can be used for the treatment of CL (WHO 2010).

2.7.4 **Miltefosine (MIL)**

Miltefosine is an alkyl phospholipid (hexadecylphosphocholine), which was originally developed as an oral anticancer drug. At present it is the only available orally effective antileishmanial drug, MIL was first introduced in India in 2002 for treatment of VL (Sundar *et al*., 2002) and was found to be highly effective at a dose of 50–100 mg/day for 28 days, giving cure rate of 94%, including antimony unresponsive VL cases (Sundar *et al*., 2002; Olliaro *et al*., 2005). Recommended dose of MIL for PKDL treatment is 2.5 mg/kg per day for children, 50mg twice daily for 90 days for adults and 2 mg/kg per day for 28 days for CL (WHO 2010). Although mechanism of action of MIL is not very clear, some studies suggest that this drug induces apoptotic-like process in *Leishmania* parasites, which causes cell death (Verma and Dey 2004; Paris *et al*., 2004). MIL can also possibly affect the phosphatidylcholine biosynthesis, calcium homeostasis and signal transduction in the parasite (Croft *et al*., 2003b). Its limitations are high cost, gastrointestinal side effects (anorexia, nausea, vomiting), occasional hepatic and nephrotoxicity. MIL is potentially teratogenic, hence women of child bearing potential should use contraception for the duration of treatment and for 3 months afterwards because of its half life of nearly one week (150 h). Due to long half-life, MIL is highly susceptible to development of resistance. Recent studies in

India have shown significant decline in the final cure rate and increase in the relapse rate of VL and PKDL cases after MIL treatment (Sundar *et al.*, 2012; Ramesh *et al.*, 2015).

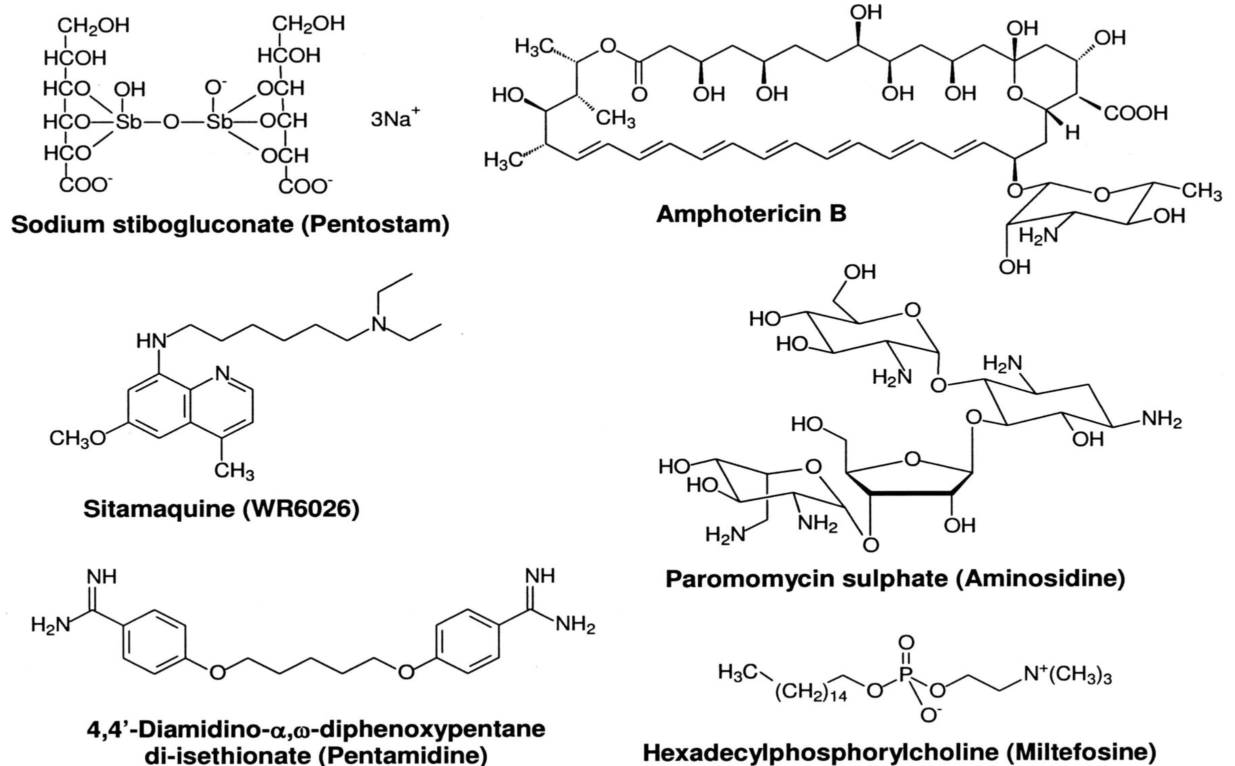


Figure 2.12: Chemical structures of currently used antilesihmanial drugs.
(Source: - Croft S *et al.*, 2006; Clin Microbiol Rev. 19:111-26.)

2.7.5 **Pentamidine**

Pentamidine was originally used for the treatment of African Trypanosomiasis and its antileishmanial activity was first demonstrated in 1939. It is administered intramuscularly or by intravenous infusion. Primary mode of action of pentamidine is uncertain but studies indicate that it may act on the genome of the parasite by inhibiting replication and transcription at mitochondrial level and by binding with kinetoplast DNA (Fries *et al.*, 2003; Mishra *et al.*, 2007). Safety is a major concern with pentamidine as it is associated with severe adverse effects including, diabetes

mellitus, severe hypoglycaemia, shock, renal toxicity and myocarditis. Although pentamidine can be used as a drug in all form of leishmaniasis, its high toxicity combined with decrease efficacy (Sundar 2001) has leads to complete abandonment of this drug to treat VL patients in India. However, it can still be used in combination therapy.

2.7.6 **Sitamaquine (WR6026)**

Sitamaquine (8-aminoquinoline analog) is another candidate for oral treatment. It was originally developed by the Walter Reed Army Institute of Research for malaria. Antileishmanial activity of sitamaquine was first identified in 1970s. Its mechanism of action is unknown but it might affect mitochondrial electron transport chain and may also stimulate nitric oxide production from macrophages (Buates *et al*., 1999). In a recent study, it has been shown that sitamaquine can also target succinate dehydrogenase causing oxidative stress in parasites (Carvalho *et al*., 2011). Sitamaquine has shown good efficacy in phase II clinical trials for VL (Jha *et al*., 2005). The common adverse events with this drug include cyanosis due to methaemoglobinaemia, vomiting and headache, nephrotoxicity and abdominal pain is rare and mostly observed in the patients receiving higher doses.

2.7.7 **Combination Therapy**

Combination therapy has the potential advantages of shortening the dose and duration of treatment, thereby increasing compliance and reducing the toxic effects and cost of drugs. It also reduces the probability of the emergence of drug-resistant parasites, thereby prolonging the therapeutic life span of the available medicines. Several trials of combinations have shown favorable results. Combination of antimonials and paromomycin resulted in a higher cure rate in VL cases of Bihar than

antimonials alone, to which lack of response is common (WHO 2010). In various phase 3 studies in India, different combinations have shown 98-99% cure rate (Sundar *et al*., 2008). The combination of paromomycin [15 mg/kg (11 mg base)] with sodium stibogluconate (20 mg/kg SbV) for 17 days has shown efficacy of 93% in East Africa and Yemen, Furthermore, similar efficacy was seen with this regimen in Sudan, Ethiopia and Kenya, suggesting that it could be used across the region (Seamen *et al*., 1993; Sundar & Chakravarty 2013). Combination is now also recommended therapy for the treatment of PKDL cases, particularly in those with extensive disease (Ramesh *et al*., 2014).

2.8 **Preventive Methods**

Efficient case management based on early diagnosis and treatment is the key to limit morbidity and prevent mortality caused by leishmaniasis (Matlashewski *et al*., 2011). Effective treatment of patients is required to control reservoir and transmission of disease in anthroponotic foci, particularly for PKDL cases, which serve as a reservoir of infection (Ramesh *et al*., 2015; WHO 2010). A study conducted in 1988 in India showed that the presence of even as few as 0.5 % PKDL patients during an epidemic might cause VL to become endemic (Dye & Wolpert 1988). Another challenge in the VL elimination is the presence of asymptomatic VL cases that can play a vital role in maintaining transmission dynamics of *Leishmania* infection (Sharma *et al*., 2000). Although, the actual estimate of such cases in the endemic area is difficult to assess, studies have shown the presence of asymptomatic cases in high endemic areas of Bihar in the range of 10 to 34% (Topno *et al*. 2010; Das *et al*. 2014; Sudarshan *et al*. 2014) and their conversion rate to symptomatic VL was 17.85 per 1000 persons (Topno *et al*. 2010). It is now essential to shift focus on these

"asymptomatic carriers" along with the PKDL cases or they might prove an important impediment towards VL elimination program. Suboptimal treatment of leishmaniasis must be avoided as it can lead to the development and spread of drug resistant parasites. In addition, vector control is also needed to be implemented wherever feasible. Sandflies are susceptible to the same insecticides that are used for Anopheles mosquitoes, the malaria vector. Some general control measures to avoid sandflies bite include: protective clothing, bed nets, insect repellants, insecticide in domiciliary and peridomiciliary transmission settings. Indian government has launched many programmes for the elimination of VL. Despite all efforts, VL still thrives in India. Now, there is consensus among the scientist that in long term, vaccine can become a major tool to control this disease; hence, development of an effective vaccine is urgently required.

2.9 *Leishmania* Macrophage Interaction

2.9.1 Entry into the Macrophages

The internalization of promastigotes by the macrophages is a receptor-mediated process and surface molecules of both parasite and macrophage trigger this process. Although, a definitive role of these receptors in natural infection is not fully understood (Da silva *et al*., 1989; Ueno *et al*., 2009), studies have shown that in the phagocytosis of promastigotes, first complement receptor (CR1), mannose-fucose receptor (MR), third complement receptor (CR3), Fc receptors (FcR) and the fibronectin receptors (FnRs) present on the surface of macrophages play important role ((Blackwell *et al*., 1985; Da Silva *et al*., 1989; Guy *et al*., 1993; Kane and Mosser., 2000). The third complement proteins (C3) of host bind to the surface metalloprotease-GP63 (also called as major surface protease, leishmanolysin) of the

parasite, which is expressed higher in metacyclic promastigotes as compared to the procyclic promastigotes (Kweider *et al*., 1989). GP63 converts C3 to the cleavage product C3b, which in turn mediates the opsonic recognition by the macrophage receptors CR1 and CR3 (Brittingham *et al*., 1996), finally leading to engulfment by the macrophages **(Figure. 2.13)**.

Successful entry of amastigotes into the macrophages differs from that by promastigotes. *Leishmania* amastigotes replicate inside the macrophages, eventually rupture the cells and continue to infect other healthy macrophages following host immune response. Studies have shown that amastigotes get coated with the IgG and they use these antibodies as opsonins for the adherence to the Fc receptors (FcγR) present on the surface of macrophages (Bosetto *et al*., 2007), which facilitate their entry into the cell **(Figure. 2.13).**

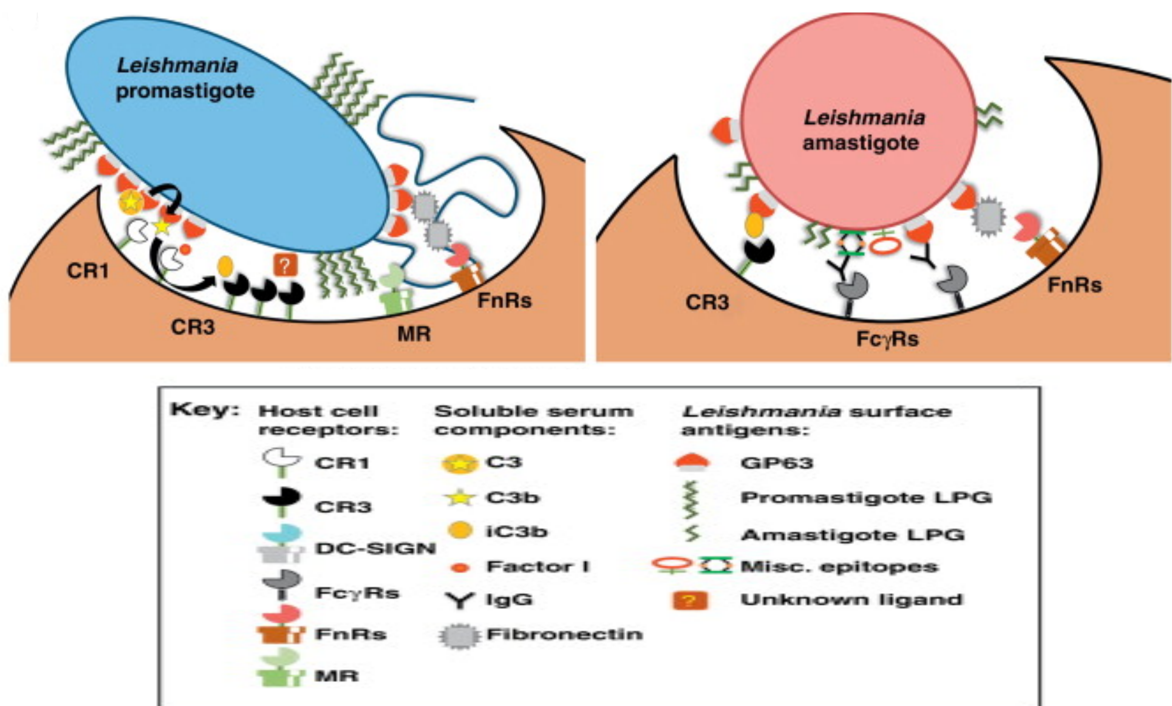


Figure 2.13: Receptor mediated phagocytosis of *Leishmania* parasite by phagocytic cells. (Source: - Ueno and Wilson., 2012; Trends Parasitol. 28:335-44).

Neutrophils are the first cells recruited to the sites of *Leishmania* infection following sand fly bite. They rapidly engulf the invading pathogen, however, studies have shown that neutrophils do not destroy the *Leishmania* parasites instead they get infected (Ribeiro-Gomes and Sacks, 2012). *Leishmania* infected apoptotic neutrophils can be taken up by macrophages that facilitate silent entry of parasites into the macrophages, which serve as the main host of parasite. By acting as a "Trojan horse" short-lived neutrophils transfer infection to the macrophages **(Figure 2.14).**

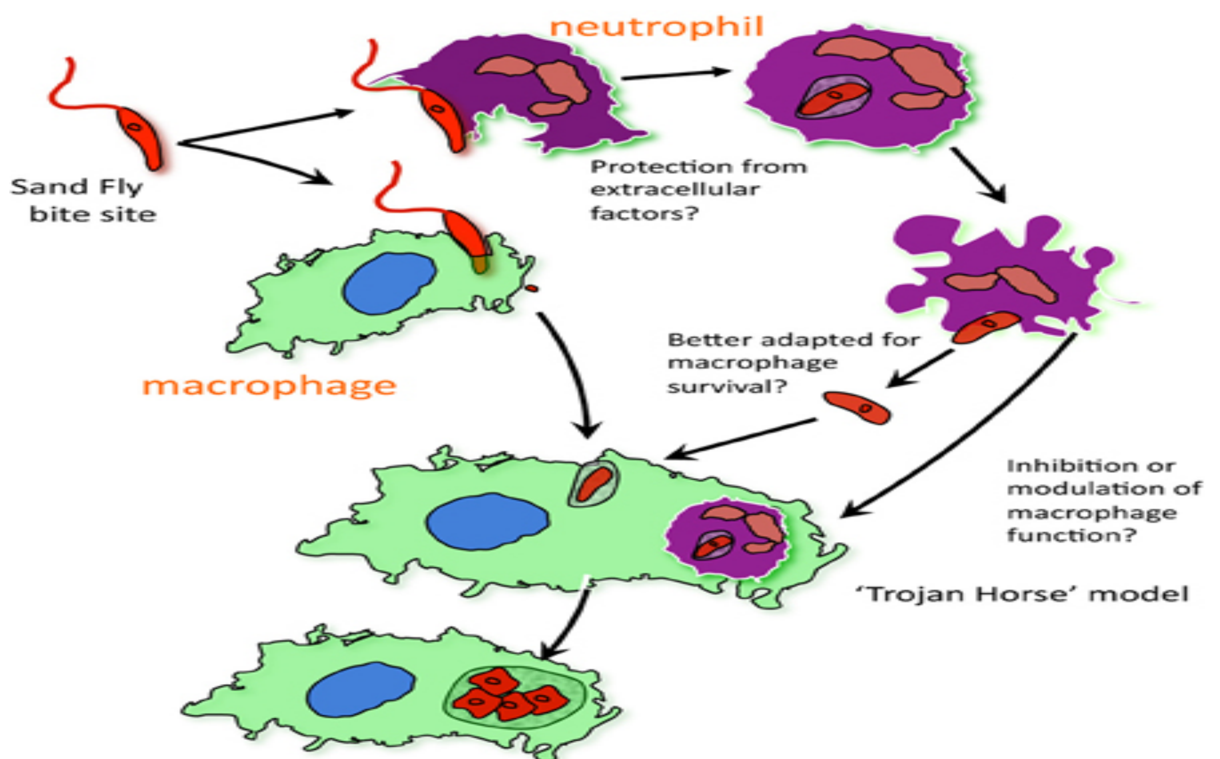


Figure 2.14: Transfer of *Leishmania* infection to the macrophages by neutrophils (Source: -Ribeiro-Gomes and Sacks 2012; Front Cell Infect Microbiol. 2:59).

2.9.2 **Intracellular Survival Strategies of *Leishmania* Parasite**

Leishmania parasite has developed several immune evasion strategies to survive in the hostile environment of phagolysosome, induced by host-parasite interaction. The infection of macrophages involves engulfment of *Leishmania* parasite

into a membrane bound structure phagosome, which fuses with secondary lysosome and results in the formation of phagolysosome. Maturation of phagosomes into phagolysosomes is essential to acquire the microbicidal properties required for the killing of parasite (Diana *et al*., 2013). Phagolysosome is an acidic compartment containing hydrolytic enzymes and micobicidal peptides. Studies have shown that lipophosphoglycan (LPG), the major promastigote surface glycolipid inhibits the process of phagolysosome biogenesis (Desjardins & Descoteaux, 1997; Lodge & Descoteaux, 2008). This inhibition represents an intramacrophage survival strategy used by parasite during their differentiation into the amastigotes, a stage which is adapted to survive under the acidic and hydrolase-rich conditions.

One of the major strategies used by *Leishmania* parasite to prevent its intracellular killing is inhibition of production of reactive oxygen and reactive nitrogen intermediates (Moore *et al*., 1993). Acid phosphatases produced by *Leishmania* parasite on its surface inhibit this burst. Macrophage protein kinase C (PKC) is associated with oxidative burst, resulting in the formation of reactive oxygen species (H_2O_2, OH^- and O^{2-}). It has been found that *Leishmania* LPG block PKC activity and also scavenge free oxygen radical (Descoteaux *et al*., 1992). These studies suggest that LPG molecule plays an important role against the anti-parasite effector mechanism of the host cell. Another important molecule of the parasite is cell surface metalloprotease Gp63 that inactivates proteolytic host enzymes and protects parasite from phagolysosomal degradation at acidic pH 4. Studies have shown that Gp63 can affect activated protein-1 and NF-kB, which are important molecules of the pathways that induce microbicidal response of the host cell (Contreras *et al*., 2010; Gomez *et al*., 2009). GP63 is also able to cleave a subset of macrophages soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins, which finally inhibit the cross-

presentation of *Leishmania* antigen and results in reduced T cell activation (Diana *et al*., 2013). Additionally, *Leishmania* parasite also inhibits the activation of mitogen-activated protein kinases (MAPKs) and JAK/STAT signaling pathways that are important for proper and optimal macrophage response (Liu & Uzonna., 2012). *Leishmania* infection also decreases the expression of MHC class-II molecules and co-stimulatory ligand B7.1 (Saha, *et al*., 1995; Reinger *et al*., 1990), which are essential for the effective antigen presentation and subsequent activation of type 1 T-helper lymphocytes.

2.10 **Immune Responses**

The pathology of *Leishmania* infection is determined by the parasite species and the host's genetic and immune factors. Cure in all forms of leishmaniasis is mainly affected through adaptive cellular immune response, which activates host macrophages to eliminate the parasite. Although infection of *Leishmania* in humans induces strong humoral responses, role of antibodies in providing protection against the disease is controversial and some studies have shown that they are associated with the non-healing forms of leishmaniasis (Tripathi *et al*., 2007). Analysis of serum sample of active VL patients revealed elevated levels of anti-leishmanial IgM, IgE, and IgG. A successful cure corresponded with a decline, most significantly, in the levels of IgE, IgG4, and IgG1 (Atta *et al*., 1998; da Matta *et al*., 2000). Binding of host IgG to the surface of amastigotes allows them to adhere with the Fc receptors of macrophages, engagement of Fc-receptors promotes parasite replication by driving IL-10 production (an inhibitory cytokine) in macrophages (Kane & Mosser, 2001). However, in CL, usually antibodies are present at low levels during the active phase of the disease (Romero *et al*., 2005). The control of leishmanial infection is mediated by a $CD4^+$ Th1 type immune response, and earlier studies in murine models of CL

have clearly shown dichotomy between Th1 response mediated protection and Th2 response mediated disease susceptibility (Sacks & Noben-Trauth, 2002). In human CL, disease progression is associated with Th1 type of cytokine profile and resistance with a Th1 profile; however in VL, immune response determining the disease vs. protection is not so well established and it shows a mixed Th1 and Th2 type of immune response (von Stebut & Udey, 2004; Reed & Scott, 1993).

Differentiation of naïve T-helper cells into Th1 or Th2-type effector cells chiefly depends on the priming during differentiation. IL-12 induces Th1 differentiation whereas IL-4 induces Th2 **(Figure. 2.15)**. Expression of Th1 cytokines is associated with cell-mediated immunity whereas Th2 cytokines are associated with humoral immunity. IL-12 and IFN-γ are signature cytokines of Th1 response. IL-12 plays a major immune-regulatory role in the development of protective cell-mediated immunity during leishmaniasis infection by activating macrophages to produce IFN- γ and TNF-α (Trinchieri, 1994; Watford *et al*., 2003) and by suppressing IL-4 and Th2 polarization.

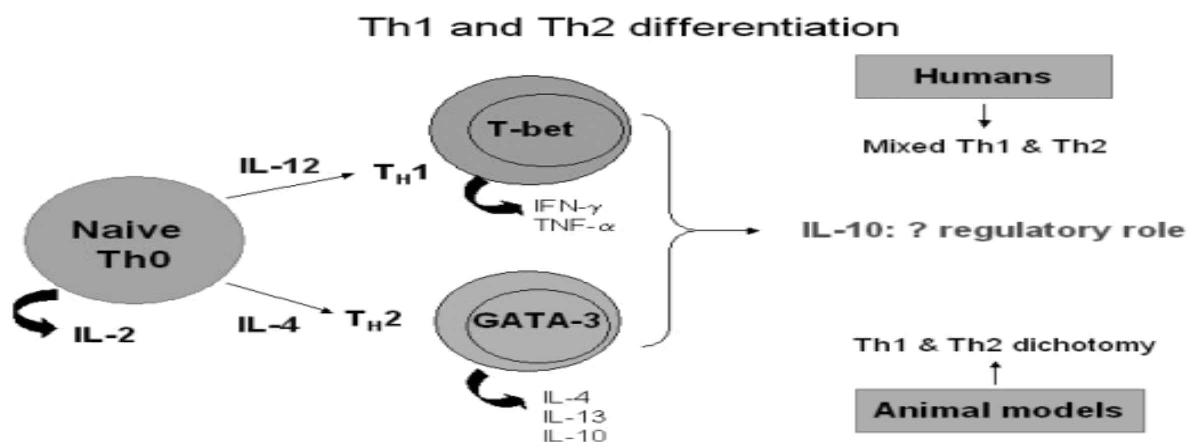


Figure 2.15: Th1 and Th2 dichotomy in leishmaniasis (Source:-Tripathi *et al*., 2007; FEMS Immunol Med Microbiol.51:229-42).

IFN-γ also plays very important role in providing protection against leishmaniasis infection. It activates macrophages for the generation of reactive oxygen and nitrogen species, which leads to the destruction of intracellular *Leishmania* parasite (Gannavaram *et al.*, 2014). Both $CD4^+$ and $CD8^+$ T-cell produce IFN-γ upon activation. Further, $CD8^+$ T-cell plays crucial role in the cure of VL by exerting its cytotoxic effect on infected cells (Kaushal *et al.*, 2014). Marked suppression in the cell-mediated immunity has been observed in the active cases of VL. It is reflected by decreased production of IL-12 and IFN-γ and failure of peripheral blood mononuclear cells (PBMCs) obtained from VL cases to proliferate and produce IFN-γ in response to *Leishmania* antigen (L-Ag). However, following cure, their proliferative capacity and cytokine responses to L-Ag is generally restored (Kumar *et al.*, 2012). In leishmaniasis, increased production of IL-4, IL-10 and IL-13 has been associated with disease promoting Th2 responses (Nylen *et al.*, 2007; Gannavaram *et al.*, 2014). IL-4 helps in leishmaniasis progression by inducing polarization of T-cells towards the Th2 subtype and by inhibiting macrophage function (Biedermann *et al.*, 2001). IL-13 shares many of the properties similar to the IL-4 cytokine and elevated level of both has been associated with VL (Nylen *et al.*, 2007; Sundar *et al.*, 1997). Further, IL-4 and IL-13 also result in up-regulation of arginase activity and the production of polyamines that favor intracellular parasite proliferation (Liu & Uzona, 2012) **(Figure 2.16)**. IL-10, produced by T regulatory (Treg) cells, macrophages, B cells and dendritic cells, plays a key regulatory role in dictating the final outcome of both CL and VL. Studies have shown that IL-10 suppresses the IFN-γ mediated microbicidal activity of macrophages thereby helping the disease progression (de Waal Malefyt *et al.*, 1991; Gannavaram *et al.*, 2014). In VL patients, strong correlation has been observed between parasite load and the level of IL-10, indicating IL-10 as a marker of

disease severity (Verma *et al*. 2010). Usually, VL is associated with increased production of multiple chemokines and cytokines and in active cases, elevated serum level of IL-1, IL-6, IL-8, IL-12, IL-15, IFN-γ and TNF-α has been found (Ansari *et al*., 2006 a; Nylen *et al*., 2007). Although the precise understanding of PKDL pathogenesis is still obscure, studies have shown enhanced expression of IL-10, TGF-β, IFN-γ, and TNF-α also at the lesion site of PKDL patients (Ansari *et al*., 2006 b).

T_{reg} and Th17 cells are now widely accepted subsets with important functions in induction and control of the inflammatory responses in leishmaniasis. $CD4^{+}CD25^{+}FoxP3^{+}$ T_{reg} cells are associated with pathogenesis of both VL and PKDL (Saha *et al*. 2007). They have been demonstrated to be the main source of IL-10 and TGF-β during active VL (Bhattacharya *et al*. 2016; Nylen *et al*., 2007), thereby promoting pathogen persistence. T_{reg} cells have also been found at the lesion site of CL and PKDL patients (Campanelli *et al*., 2006; Katara *et al*., 2011). Th17 cells are pro-inflammatory T helper cells, characterized by their ability to secrete IL-17 and IL-22. They play complementary role along with Th1 in providing protection against leishmaniasis (Pitta *et al*., 2009) by activating innate inflammatory mediators and acting synergistically with IFN-γ to strengthen Th1 response and preventing T_{reg} and IL-10+ cell expansion (Kolls *et al*., 2004; Nascimento *et al*., 2015).

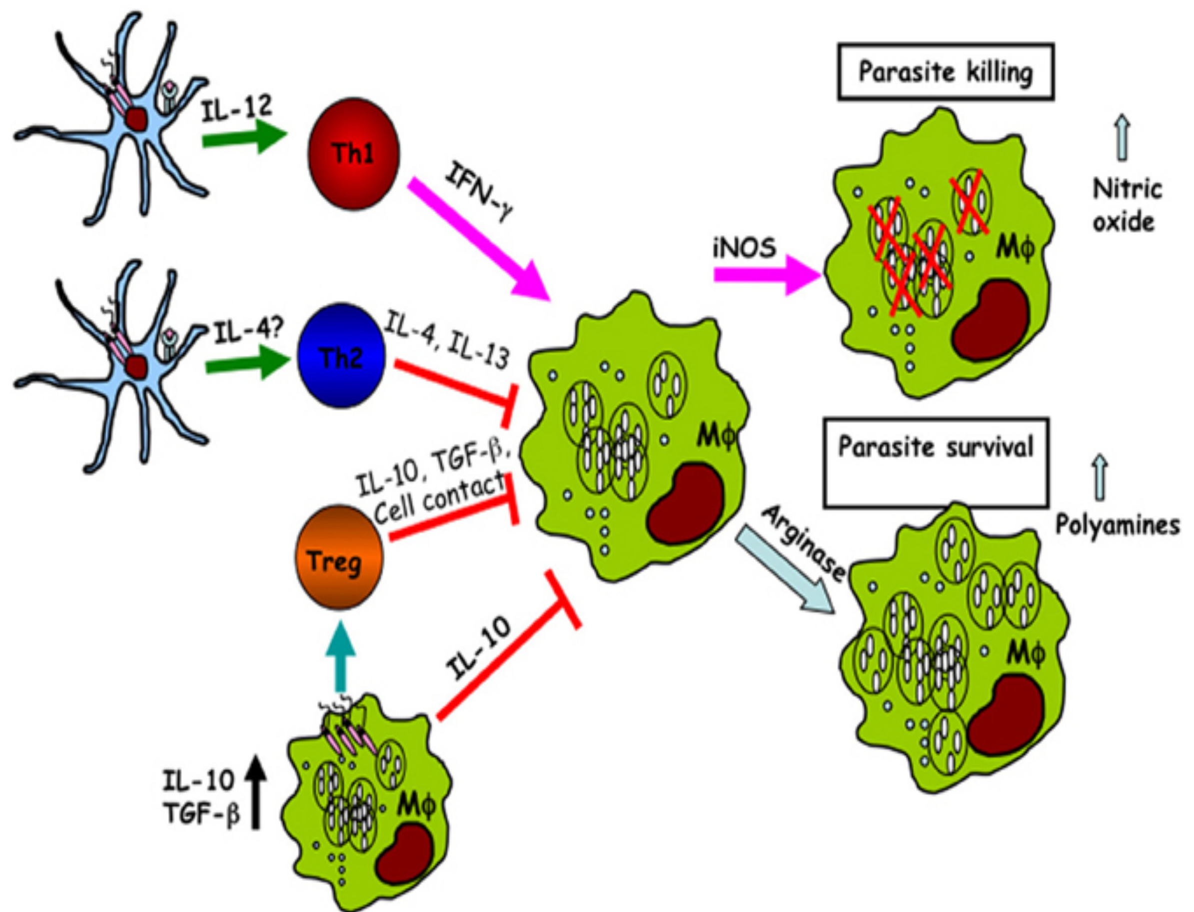


Figure 2.16: Different cytokines involved in activation/suppression of macrophage function during *Leishmania* infection (Source:- Liu and Uzona., 2012; Front Cell Infect Microbiol.12;2:83)

2.11 Vaccines for Leishmaniasis

Development of an anti-*Leishmania* vaccine has been a long-term goal but till now there is no licensed vaccine available against any form of human leishmaniasis. Advances in understanding of *Leishmania* infection pathogenesis and host protective immunity, along with the complete genome sequences of *Leishmania* parasite, has opened new avenues for vaccine research. However, still there are major challenges including translation of data obtained from animal models to clinical settings, and the transition of products from the laboratory to the field.

2.11.1 **Killed *Leishmania* Vaccine**

Killed *Leishmania* vaccine is an appealing candidate in terms of antigenicity, stable biochemical composition, low cost and safety (Giunchetti *et al*., 2008). Several studies have been carried out using killed *Leishmania* parasite as vaccine candidate in countries including Brazil, Colombia, Ecuador, Venezuela and the Islamic Republic of Iran against CL and in the Sudan against VL (Noazin *et al*., 2009; Kumar & Engwerda, 2014). Different formulations of killed parasites have been used, where they were tested either alone or in combination with adjuvant for clinical trials. Clinical trials in Brazil using autoclaved *Leishmania* parasite with BCG as an adjuvant showed reduction in the incidence of CL by 18–78% (Antunes *et al*., 1986; Sharifi *et al*., 1998). Similar trials were also conducted in Ecuador, Iran and Sudan with variable safety and efficacy (Bahar *et al*., 1996; Dowlati *et al*., 1996; Armijos *et al*., 1998; Khalil *et al*., 2000). Overall these trials pointed towards the practical shortcomings of using a non-standardized whole parasite vaccine approach and the relatively weak immunogenicity of the killed parasite preparations. As autoclaving can destroy many proteins, it might be the reason for reduced immunogenicity of these heat killed *Leishmania* parasites (Luca *et al*., 1999). Therefore, even after offering a safer and more stable alternative in comparison to the live vaccine, killed *Leishmania* vaccine does not mimic natural infection and is also less immunogenic.

2.11.2 **Subunit Vaccines**

In context of development of sub-unit vaccine, there has been significant progress in the identification and characterization of *Leishmania* antigens that induce protective immune responses. The immune responses induced by these recombinant

proteins can be potentiated and refined by formulation with the appropriate adjuvants (Reed *et al*., 2009). Several protein-based vaccine candidates of leishmaniasis including, GP63, A-2, p36/LACK, LCR1, gp46/M-2/Parasite Surface Antigen 2 (PSA-2), K26/HASPB1, KMP11 and ORFF, have been evaluated for their vaccine potential in animal models with varying degrees of success (Alvar *et al*., 2013) and only a few of them have progressed to clinical trials in dogs, non-human primates or in human preclinical studies (Singh *et al*., 2012). The fucose mannose ligand (FML), in formulation with saponin, has been found to be safe, immunogenic and protective in mice and hamster models (Palatnik-de-Sousa *et al.* 1994; Santos *et al.* 2003). After a series of canine VL field studies, now this formulation is a licensed vaccine for veterinary use as Leishmune® in Brazil (Parra *et al.* 2007). The excretory/secretory proteins of *L. infantum (*LiESAp) with muramyl dipeptide (MDP) were found to be protective in dogs model (Lemesre*et al.* 2005). Further, this *Li*ESAp-MDP vaccine formulation, elicited long-lasting protection against canine VL in a field trial with naturally infected dogs (Lemesre *et al.* 2007). The *Li*ESAp-MDP formulation is also licensed for commercial use under the name CaniLeish® for canine VL in Europe. PSA (promastigote surface antigen) protein is an active constituent of *Li*ESAp. It has been found that the sera of dogs vaccinated with *Li*ESAp and MDP, recognized immunodominant antigens belonging to PSA protein family, from *L. infantum* ESA (*Li*PSA-54S) and from *L. amazonensis* ESA (*La*PSA-38S). As a vaccine candidate *La*PSA-38S protein induced a mixed Th1 and Th2/T_{reg} cytokine response and granzyme B production in individuals pre-exposed with *Leishmania* parasite (Chamakh-Ayari *et al*., 2014).

Due to genetic polymorphism in the mammalian immune system, it was thought that a multicomponent vaccine would induce a better protective immune

response (Goto *et al*., 2011). Therefore, polyproteins or multicomponent preparations such as Leish-110f, KSAC, Q-proteins and Leish-111f came into existence. Leish111f, which is composed of *L. major* thiol-specific antioxidant (TSA), *L. major* stress-inducible protein-1 (LmSTI-1) and *L. braziliensis* elongation and initiation factor (LeIF), combined with adjuvant MPLA stable emulsion (MPL-SE), has been found to be protective in mouse models of VL and CL (Coler *et al*. 2007; Skeiky *et al*. 2002) but failed to protect canine VL caused by natural *L. infantum* (Gradoni *et al*., 2005). Leish-111F/MPL-SE formulation became the first defined vaccine candidate to reach phase-I and phase-II clinical trials in leishmaniasis patients and in healthy volunteers in different parts of world (Vélez *et al*. 2009; Llanos-Cuentas *et al*. 2010; Nascimento *et al*. 2010).

One of the major problems associated with subunit vaccine is that leishmaniasis is caused by different *Leishmania* species, having genetic variation and polymorphism; hence, it is necessary to select vaccine candidate antigens, which are conserved in all species. Further, the lack of stability and potency during the transportation of antigens is also a common problem

2.11.3 **Live Attenuated Vaccine**

Many successful vaccines for viral and bacterial infections including polio, MMR, small pox, influenza, BCG, cholera etc. are live pathogen vaccines. Till now in humans the best protection against leishmaniasis has been achieved by "leishmanization, which represents inoculation of a low dose of live *Leishmania* parasites in the hidden body part, to avoid development of deforming scars at visible site like face (Palatnik-de-Sousa, 2008). Although due to safety related issues leishmanization process was discontinued but it provided clear evidence that live

parasites can be used as vaccine candidate against leishmaniasis. Further, there is also an increasing consensus among vaccine researcher that parasite persistence may be important factor for development of effective protective immune response, which can only be achieved by immunization with live attenuated parasite. Several strategies have been used to develop live attenuated *Leishmania* parasite including, long-term in vitro cultures (Mitchell *et al*., 1984) chemical mutagenesis (Kimsey *et al*., 1993), γ-attenuation (Rivier *et al*., 1993) and parasite culture under drug pressure (Daneshvar *et al*., 2003). Although, these attenuated parasite strains showed substantial protection in murine model but due to lack of understanding regarding their nature of mutation, there are always chances of reversion back to virulent type, which makes them unsuitable for human use. As an alternative, genetically defined alteration in the *Leishmania* genome can be created using gene-targeted disruption strategy through homologous recombination. Several live attenuated *Leishmania* vaccine candidates have been generated using targeted gene deletion strategy **(Table 2.2).** They have been tested in the animal models with variable degree of success (Selvapandiyan *et al*., 2006, Gannavaram *et al*., 2014). Our laboratory has also developed centrin1 and p27 gene deleted live attenuated *Leishmania* parasites, which have been found to be safe, immunogenic and protective in animal models (Selvapandiyan *et al*., 2004, 2009; Dey *et al*., 2010, 2013; Fiuza *et al*., 2013, 2015). Details regarding their nature of attenuation and vaccine potential have been discussed in chapter 5. There are several advantages of live attenuated parasite vaccines. First, they mimic the natural course of infection without causing disease and are likely to induce immune responses required for protection. Second, in contrast to subunit vaccines, they deliver a complete spectrum of antigens, which increases the memory repertoire of the immune system. Third, by inducing a long-lasting subclinical infection, they assure persistence of

antigens for long time resulting in generation of antigen-specific memory immune response, which react immediately following infection.

Table 2.2 Genetically defined live attenuated *Leishmania* vaccine candidates

Organism	Gene/s knocked out	Mutant phenotype	Mutant survival in macrophage/animal
Leishmania major	dihydrofolate reductase - thymidylate synthase (*dhfr-ts*)	auxotrophic for thymidine	survival in macrophage is thymidine dependent, mutant incapable of causing disease in mice and rhesus monkeys, did not protect monkeys on challenge with virulent parasite.
L. major	leishmanolysin (*gp63 genes 1-7*)	deficient in leishmanolysin; no change in growth *in vitro*	showed normal development in sand fly, but delayed lesion formation in mice
L. major	galactofuranosyl transferase (*lpg1*)	deficient in LPG but contained normal levels of related glycoconjugates and GPI-anchored proteins	did not infect sand fly, mouse or macrophages
L. major	Golgi GDP-Man transporter (*lpg2*)	mutant lacked all phosphoglycans	unable to survive in sand fly, persisted indefinitely in mice with no disease, provided protection from challenge with virulent parasites in the absence of a strong Th1 response
L. mexicana	glucose transporter genes (*LmGT1, Lm GT2 & Lm GT3*)	Promastigotes showed reduced growth rate *in vitro*	reduced growth rate in sand fly mid gut, reduced infectivity in macrophages
L. mexicana	cysteine proteases (*cpa, cpb & cpc*)	deficient in cysteine protease; no change in growth *in vitro*	reduced infectivity in macrophages, attenuated virulence in mice and provided protection upon challenge with virulent parasite
L. donovani	partial knockout of *A2-A2rel* gene clusters	proliferation of mutants in culture compromised	attenuated virulence in mice
L. donovani	biopterin transporter (*BT1*)	biopterin transport abolished	reduced infectivity, parasite specific production of IFN-γ (cellular immunity - TH1 type response) and provided protection upon challenge with virulent parasite

2.11.4 **DNA Vaccine**

The concept of DNA vaccine is relatively new for leishmaniasis but it has several advantages in comparison to the recombinant protein vaccines such as stability, low costs of production, no need of cold chain for distribution, sustained

expression of relevant antigens and flexibility of combining multiple genes in a single construct (Kumar and Engwerda., 2014). Here, target genes encoding the vaccine candidate are cloned into a mammalian expression vector, and DNA is directly injected either intradermally or intramuscularly. Plasmid DNA is taken up by the host cells, where it is translated into the respective proteins. Bacteria-derived DNA plasmids are naturally immunogenic as their backbone contains non-methylated CpG motifs, which have been found to induce protective Th1 and CD8+ T cell responses (Palatnik-de-Sousa., 2008). DNA vaccines are in clinical trial for several infectious diseases (Alarcon *et al*., 1999; Hasan *et al*., 1999). In case of *Leishmania*, vaccination of animals with DNA encoding PSA-2, LACK and gp 63 antigens were found to be protective in mice models (Tabbara, 2006). However, till now no clinical trials have been conducted.

Gaps in the Existing Research

The current increase in the epidemic proportions of leishmaniasis and alarming emergence of resistance to currently available drugs exacerbates the need for development of vaccine. No effective vaccine is available against leishmaniasis, although, development of a vaccine has been a goal since a century. Attempts to develop a vaccine for humans using different strategies such as heat killed, subunit or DNA vaccines have not been successful. Studies have shown that the parasite persistence may be an important factor to develop long lasting protective immunity and it can be achieved by immunization with live attenuated parasites with known irreversible gene defect. In the digenetic life cycle of *Leishmania* parasite, several genes undergo the process of differential regulation to survive into two different host milieus. Amastigote specific genes are likely to be involved in the pathogenesis and intracellular survival of the *Leishmania* parasite. Further, deletion of amastigote specific gene can also lead to the generation of amastigote stage attenuated *Leishmania* parasites that can be used as potential vaccine candidate. However, till date, only a few amastigote specific genes have been characterized. In the present study, we have targeted an amastigote stage specific LdA1 gene, having up regulated expression at the amastigote stage for generation of live attenuated parasite. Furthermore, LdA1 gene was functionally characterized to understand its role in *Leishmania* life cycle.

Besides, centrin1 (growth regulating gene) and p27 gene (component of cytochrome c oxidase complex) deleted live attenuated *Leishmania* parasites (*Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$) have shown good potential as live attenuated vaccine candidates in animal models. However, as the animal models do not fully recapitulate the full

spectrum of human-parasite interactions, translation of results obtained from studies in the animal models remains a major challenge. Therefore, it became important to assess the vaccine potential of *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ for human use. In view of the above gaps in research, in the present study, we have evaluated their vaccine potential by determining the infectivity and the immune response generated by them in human PBMCs obtained from different clinical groups. To our knowledge, this is the first study where, two live attenuated vaccine candidates of VL have been evaluated for their capacity to elicit immune responses in cells from individuals with distinct clinical status, including naïve healthy, active VL, HVL and PKDL. The study helps to understand the correlates of protection induced by *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ in human PBMCs and their potential as a live attenuated vaccine candidate against human leishmaniasis.

Aims and Objectives

The aim of the present study is to generate live attenuated *Leishmania* parasites as vaccine candidates against leishmaniasis. Since amastigote specific genes are considered to be involved in the regulation of virulence of the parasite, in this study we will specifically target one such gene for generation of knockout mutants. Besides, the other goal of the study is to assess the vaccine potential of centrin1 and p27 gene deleted live attenuated *Leishmania* parasites (*Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$) for humans by evaluating the infectivity and immune responses elicited by them in the blood samples obtained from different clinical groups.

Objectives

1. Characterization of *Leishmania* amastigote specific gene (LdA1) by gene deletion/episomal expression study.

In order to investigate the potential applicability of LdA1 for generation of live attenuated vaccine candidate and its biological role in the life cycle of *Leishmania* parasites following studies will be carried out:

- Confirmation of upregulated expression of LdA1 in amastigote stage at protein level
- *In silico* analysis to predict structure and function of LdA1
- Immunofluorescence analysis to ascertain the subcellular localization
- Overexpression of LdA1 in *Leishmania* parasite with the help of expression plasmid and analysis of its effect on parasite growth/attenuation, phenotype and infectivity

- Deletion of LdA1 from *Leishmania* genome by homologous recombination method and its effect on parasite growth/attenuation, phenotype and macrophage infectivity

2. Evaluation of immune responses in human PBMCs elicited by live attenuated *Leishmania* parasites as vaccine candidates.

The present study will evaluate the vaccine potential of *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ for humans by assessing the infectivity and correlates of protection, induced by them in human blood samples obtained from different clinical groups. Towards this goal, following studies will be carried out:

- Determination of infectivity of *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$in human PBMCs differentiated macrophage
- Evaluation of immune responses induced by *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ in the human PBMCs obtained from healthy, healed VL and active cases of VL and PKDL subjects
- Analysis of phenotype of cytokine producing cells in response to *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ exposure

Characterization of *Leishmania* Amastigote Specific Gene (LdA1) by Gene Deletion/Episomal Expression Study for Vaccine Development.

4.1 **Introduction**

Visceral leishmaniasis (VL) is the second deadliest parasitic disease after malaria and afflicts the world's poorest people in developing countries. Control measures of VL are mainly dependent on chemotherapy, which is costly, toxic to the patients, requires long duration of treatment and hospitalization and is also associated with increasing drug resistance (Sundar *et al*., 2001; Croft *et al*., 2006; Ramesh *et al*., 2015). Further, usage of chemotherapeutics is only limited to the individuals with clinical symptoms; however, majority of the individuals living in the endemic areas remain with subclinical infection or as asymptomatic carriers (~6–10 times more than reported cases of VL) (Singh OP *et al*., 2014). Therefore, development of an effective vaccine is an urgent need.

In the digenetic life cycle of *Leishmania* parasite, distinct morphological and biochemical changes occur during the differentiation of promastigotes into amastigotes that include, loss of flagellum, rounding up, change in its glycoconjugate coat and expression of a set of metabolic enzymes optimally active at low pH (Charest and Matlashewski, 1994). These changes are accompanied by a change in temperature and pH from insect host to vertebrate host and a change from extracellular to intracellular life of the parasite (Glaser *et al*., 1991; Goyard *et al*., 2003; McConville *et al*., 2007). In order to survive in two different host milieus, several genes undergo the process of differential regulation (Flinn *et al*., 1992; Charest *et al*., 1994; Wu *et al*., 2000; Selvapandiyan *et al*., 2014). Studies have shown that as compared to the promastigotes, 3-9 % genes are highly or exclusively expressed at the amastigote stage and are essential for intracellular survival of the parasite (Charest *et al*., 1994; Bellatin *et al*., 2002; Nugent *et al*., 2004; McNicoll *et al*., 2006). These genes fall under different functional categories: genes involved in cytoskeleton and cell membrane;

stress response; cell cycle and proliferation; energy metabolism and phosphorylation; and amino acid metabolism (Selvapandiyan *et al*., 2014). Characterization of the genes, which are highly/specifically expressed at the amastigote stage may provide new insights into the mechanisms controlling the intracellular life stage and that can further lead to the identification of promising target required for the development of *Leishmania* cell lines attenuated at amastigote stage that may be potential vaccine candidates (Gannavaram *et al*., 2014; Selvapandiyan *et al*., 2014). Further, due to deletion of essential gene, such amastigote stage attenuated mutant parasites generally do not revert back to the virulent type in animal models, even under induced immune suppression conditions (Selvapandiyan *et al*., 2009).

Previously, amastigote specific genes like A2-A2 rel gene cluster (Zhang and Matlashewski, 2001), cathepsin L-like cysteine proteases (Mundodi *et al*., 2005), SIR2 (Silvestre *et al*., 2007) and p27 (Dey *et al*., 2010) have been characterized to elucidate their biological roles and evaluated for their potential to generate live attenuated parasites. These genes deleted mutant parasites displayed attenuation at the amastigote stage and as a vaccine candidate they were safe, immunogenic and protective in rodent models (Zhang and Matlashewski, 2001; Silvestre *et al*., 2007; Dey *et al*., 2010). Additionally, there are several other examples where amastigote stage attenuated parasites have been generated by gene deletion including, centrin 1 (Selvapandiyan *et al*., 2004), Adenine aminohydrolase (Boitz *et al*., 2012), carbamoyl phosphate synthase and Uracil phosphoribosyl transferase (Wilson *et al*., 2012), mitochondrial associated ubiquitin fold modifier protein Ufm1 (Gannavaram *et al*., 2012), Arabino-1,4-lactone oxidase enzyme (Manhas *et al*., 2014) etc. Vaccination strategies using live attenuated parasites is a promising approach as these mutant parasites can render protection against virulent infection by eliciting parasite specific immune response in

the host, just like after recovering from natural infection.

Studies using microarray and northern blotting technique have shown that expression of some genes is up regulated at the amastigote stage compared to the promastigote stage (Gannavaram *et al*., 2004; Srividaya *et al*., 2007). Among these genes, expression of a ~19 kDa protein encoding, single copy, A1gene (LdA1) was found to be highly up-regulated at the amastigote stage of the parasite (Gannavaram *et al*., 2004), which indicates that it might play functional roles at the infective stage of the parasite. Therefore, the main aim of the present study was to use LdA1 as a target gene for the generation of live attenuated *Leishmania* parasites using homologous recombination method. Further, we functionally characterized LdA1using bioinformatics and molecular biology approach to gain some insight into its potential biological role. We found that LdA1 is unique to *Leishmania* parasite, since no homolog is present in any other organism. Further, A1 single allele deleted parasites showed different phenotype and reduced growth as compared to the wild type; however, their ability to infect macrophages remains unaltered.

4.2 **Material and Methods**

4.2.1 ***In silico* analysis of putative LdA1 protein**

The putative LdA1 protein sequence was retrieved from UniProt. Physicochemical properties of the protein were predicted using ProtParam. A BLASTP search for LA1 protein was carried out to characterize and annotate the protein based on sequence homology (Altschul *et al*., 1990). The LdA1 protein was annotated using FFPred 3 (Yang and Zhang, 2015; Yang *et al*., 2015), in PSIPRED protein analysis workbench (Buchan *et al*., 2013; Cozzetto *et al*., 2016). Probable role of LdA1 in pathogenesis and survival of *Leshmania* was predicted based on assigned Gene Ontology terms by FFPred 3.

Structure based functional annotation enhances reliability of protein function prediction, as protein structures are evolutionarily more conserved than corresponding amino acid sequences. LdA1protein tertiary structure was predicted by implementing *ab initio* method through I-TASSER (Iterative Threading ASSEmbly Refinement) server (Zhang, 2014; Yang *et al*., 2015). The modeled structure was refined through molecular dynamics (MD) simulations in GROMACS v5.0 by applying Gromos96-53a6 force field (Van Der Spoel *et al*., 2005; Maharana *et al*., 2014). The simulation system was defined by solvating the model using SPC216 water in a cubic box with minimum distances 20 Å between the protein surfaces and box edges. The solvated system was added with appropriate counter ions at physiological ionic strength (0.15 M). The neutralized system was energy minimized using steepest descent integrator with restraints of 1,000 kJ mol^{-1} nm^{-1} force constant for a maximum of 3,000 steps to resolve weak van der Waals contacts and steric conflicts. All bond lengths and the geometry of water molecules were constrained with LINCs (Hess *et al*., 1997) and SETTLE algorithm (Miyamoto and Kollman, 1992), respectively. The energy-minimized model was equilibrated through position-restrained MD under NPT [constant number of particles (N), system pressure (P) and temperature (T)] conditions (300 K, 1 atm) for 1 nano second (ns). The equilibrated system was set for a production MD run of 50 ns under NPT conditions. The converged tertiary structure was evaluated using model validation tools such as PROCHECK (Laskowski *et al*., 1993), ERRAT (Colovos and Yeates, 1993), ProSA (Wiederstein and Sippl, 2007) and VERIFY3D (Lüthy *et al*., 1992). The probable function of the validated LdA1 protein model was predicted using COFACTOR (Yang and Zhang, 2015; Yang *et al*., 2015).

4.2.2 **Parasites cultures and growth kinetics**

The reference *Leishmania* strain *L. donovani*1S from Sudan (*Ld*1S) (WHO

designation, 103 MHOM/SD/00/1S-C12D) was used in the present study. Both promastigotes and amastigotes were maintained as described previously (Selvapandiyan *et al*., 2001, Gannavaram *et al*., 2004, Debrabant *et al*., 2004). Briefly, promastigotes were cultured in M199 medium (pH 7.4) containing 10% heat inactivated fetal bovine serum at 26 °C in a BOD incubator. Amastigotes were differentiated from promastigotes by gradually adapting them to grow at elevated temperature (37°C) and reduced pH (5.5) conditions. Once adapted to grow in these conditions as axenic amastigotes, the parasites were routinely shuttled between these two stages. Mutant parasites (described in 4.2.4 and 4.2.9) were grown in the same medium with added neomycin/hygromycin (40 µg/ml) antibiotics.

For determination of growth kinetics, stationary phase parasites were diluted to 2×10^{5} cells/ml in 5 ml of medium. Parasite concentration and viability were determined microscopically at every 24 hours intervals for 7 days.

4.2.3 **Cloning, sequencing, expression and purification of LdA1 protein**

Total genomic DNA was isolated from parasites by using GENOME DNA isolation kit (Promega Biosciences) according to the manufacturer's protocol. Full length LdA1 gene (Accession no: Linj.29.1020) was PCR amplified (PCR condition: 95°C- 5 min, 94°C- 45 sec, 68°C- 45 sec, 72°C- 45 sec and 72°C- 6 min for 36 cycle) using forward primer, 5'-ATGGACGCCGCCAGGAAACG-3' and reverse primer, 5'-CGAGAAAAAGACATGCAACAG-3'. PCR amplified complete ORF of LdA1 was cloned into pCR NT-TOPO T/A cloning vector (Invitrogen), sequence confirmed and expressed as 6X Histidine tagged protein in *E. coli*. LdA1 protein was purified using Ni-NTA affinity column (Qiagen) according to the manufacture's instruction. Protein products were analyzed by SDS-PAGE on a denaturing polyacrylamide gel and stained withR-250 Coomassie blue. Polyclonal rabbit antibodies to LdA1 were raised

using purified LdA1 protein with the help of Merck-Millipore.

4.2.4 **Plasmid construct for Over-expression/Episomal expression of LdA1 in *Leishmania* parasite**

The full-length open reading frame of LdA1 was PCR amplified with forward primer, 5'-GG**ACTAGT**ATGGACGCCGCCAGGAAACGTCACAGA-3', containing a *Spe*I restriction site (bold) and reverse primer, 5'-CC**ACTAGT**CTACGCGTAGTCCGGCACGTCGTACGGGTACGAGAAAAAGACATGCAA -3', containing a *Spe*I restriction site (bold) and hemagglutinin (HA) tag sequence (underlined). The resulting PCR product (~550 bp) was initially cloned into *pGEMT* Easy T/A cloning vector and sequence confirmed. The insert was excised from the sequence confirmed plasmid by digestion with *Spe*I and subsequently cloned into the *Spe*I site of *pKSNEO, a Leishmania* specific expression plasmid (Zhang *et al*., 1996). Presence of insert in *pKSNEO* was confirmed with restriction digestion. Mid-log-phase parasites were transfected with the LdA1 over-expressing plasmid construct (40 µg) by electroporation method

4.2.5 **Electroporation**

Mid log phase promastigotes (2X10^8 cells) were harvested by centrifugation, washed once with electroporation buffer and resuspended in ice-cold electroporation buffer (137mM NaCl, 21mM HEPES, 5mM KCl, 6mM glucose, 0.7mMNa2HPO4, pH7.4). 0.5 ml cells were taken in 2mm gap electroporation cuvette and 20µl of ice-cold plasmid DNA was added to it for transfection. The DNA was electroporated into *Leishmania* cells at 450 V, 500µF and 800π resistance. Following electroporation, cells were recovered in M199+10%FCS for 24hrs. After 24hrs, cells were washed

once with M199 and resuspended in M199 + 10% FCS + 20μg/ml G418 (selectable marker). The drug concentration was gradually increased to a final concentration of 200μg/ml for the selection of mutant cell lines. Parasites transfected with the empty vector pKSNeo were prepared as mock controls.

4.2.6 **Western blot analysis**

The protein lysate from promastigotes and axenic amastigotes was prepared using SDS lysis buffer (10mM Tris-HCl, 100mM NaCl, 5mM EDTA, 0.5% Triton X-100, 1 × protease inhibitor mixture and 5% (v/v) glycerol at pH 7.5) according to the procedure previously described (Vanila *et al*., 2015). In brief, parasites were washed thrice with ice cold PBS and lysed with buffer at 100 °C for 10 minutes. The lysate were snap chilled on ice and centrifugation was done at 13,000 × g at 4°C, the supernatant was collected and stored at -70 °C. Proteins from cell lysate were separated on 12% SDS-PAGE and blotted on nitrocellulose membrane. Blots were probed with anti-A1 (1:1000 dilution) or anti-HA (1:4000 dilution) followed by horseradish peroxidase-conjugated anti rabbit IgG (Sigma-Aldrich). Antibody binding was detected using enhanced chemiluminescence (ECL) detection Kit (GE Healthcare Life Sciences) and visualized on ChemiDoc system (Bio-Rad). Images scanning and quantitative assessment were carried out with “Image Lab 5.1 software”.

4.2.7 **Immunolocalization of LdA1**

For the immunofluorescence analysis, *L. donovani* promastigotes were washed thrice with PBS, fixed in 4% paraformaldehyde for 20 min, washed thrice again with PBS and allowed to attach on poly-L-lysine (Sigma Aldrich) coated glass cover slips. After adherence, cells were permeabilized with Triton-X 100 (0.25%) for 10 min, blocked for 1 h with 3% (w/v) bovine serum albumin (Himedia) and incubated for 1 h

with anti-LdA1 serum (1:200 dilution in 3% bovine serum albumin). After three washes with PBS, cells were incubated for 1h with FITC-conjugated anti-rabbit IgG (1:500 dilution). Cells were again washed with PBS and mounted in fluoroshield (Sigma Aldrich), containing 4'6-diamidino-2-phenylindole (DAPI) to stain both nucleus and kinetoplast and analyzed for fluorescence under the microscope (Nikon Eclipse TE2000-U). The negative control samples were processed in parallel by omitting primary antibodies that were used to negate background fluorescence, if any. Images were processed using Adobe Photoshop (version 5.5) for presentation purposes.

4.2.8 **Construction of DNA for LdA1 gene disruption by homologous recombination**

The construct was designed to replace 528 bp coding region of LdA1. The assembly of knockout construct was started with a gene targeting plasmid containing neomycin gene, as described previously for centrin1 gene (Selvapandiyan *et al*., 2004, Dey R *et al*., 2010). The 5' flanking fragment of LdA1 (5' FLK) was PCR amplified with forward primer, 5'-**AGATCT**ATTTAAATGCGCTGCTCTGCACGCATC-3', containing a restriction site for *BglII* (bold) and reverse primer, 5'-**GTCGAC**CTGCGGAGGGGCAACGAG-3' containing restriction site for *SalI* (bold). The 3' flanking fragment of LdA1 (3'FLK) was PCR amplified with forward primer, 5'-**ACTAGT**CTCGTGATACGTTCGCGTGC-3', containing a restriction site for *SpeI* (bold) and reverse primer, 5'-**GGTACC**CAAGAGGAGGGCGGGTGGAG-3', containing a restriction site for *KpnI* (bold). PCR amplified 5' and 3' flanking fragments were cloned into *pGEMT* Easy T/A cloning vector and sequence confirmed. The recombinant plasmid containing 3'FLK was digested with *SpeI* and *KpnI* and

5'FLK with *BglII* and *SalI* to obtain inserts. The targeting plasmid (Selvapandiyan *et al*., JBC 2004) was digested with *SpeI* and *KpnI* and the sequence confirmed 3'FLKof LdA1 from the above step was ligated into it followed by digestion of plasmid with *BamHI* and *SalI* (*BglII* and *BamHI* forms compatible end) and ligation of 5'FLK. The final homologous recombination construct was prepared by digestion of the plasmid with *SwaI* and *KpnI* and it contained a fragment from the 5'FLK of LdA1 gene, followed by the coding sequence of neomycin resistance gene, followed by a fragment of 3'FLK(5'FLK/NEO/3'FLK).

For the deletion of second allele of LdA1, homologous recombination construct 2 was prepared with hygromycin selectable marker. The above targeting plasmid (with 5'FLK/NEO/3'FLK) and pX63-HYG plasmid (Selvapandiyan *et al*., 2004) were digested with *SalI* and *SpeI*, resulting in release of neomycin and hygromycin gene respectively. The hygromycin gene was ligated into the plasmid in place of neomycin. The final homologous recombination construct 2 containing 5' and 3' flanking fragment of LdA1 and hygromycin gene was prepared by digestion of the plasmid with *SwaI* and *KpnI* (5'FLK/HYG/3'FLK), followed by gel purification of linear cassette.

All restriction enzymes used in the study were obtained from New England Biolabs (NEB) and antibiotics (G418 and hygromycin) from Sigma-Aldrich.

4.2.9 **DNA transfection and selection of LdA1 gene deleted mutants by PCR**

Transfections were carried out using *L. donovani* promastigote with 20μg of homologous recombination construct 1 (5'FLK/NEO/3'FLK) using electroporation method as described above. Selection of transfectants was performed by plating on semi solid plate of M199 medium containing noble agar with 100 μg/ml G418.

Individual clones were selected and transferred into tissue culture flasks containing M199 medium with added G418 (40µg/ml).To confirm the homologous recombination integration of cassette at the right place and replacement of LdA1 allele by neomycin^r gene, different diagnostic PCRs were performed. Total genomic DNA was isolated from mutants growing in the presence of G418 antibiotic and integration of neomycin^r gene was confirmed by PCR using forward primer, 5'-AGGATCTCCTGTCATCTCACCTTGCTCCTG-3' and reverse primer, 5'-AAGAACTCGTCAAGAAGGCGATAGAAGGCG-3' and presence of LdA1 gene in mutants was analyzed using forward primer, 5'-ATGGACGCCGCCAGGAAACG-3' and reverse primer, 5'-CGAGAAAAAGACATGCAACAG-3'. Integration of neomycin^r gene at the right place in the *Leishmaina* genome was confirmed using forward primer of gene present just before LdA1 (accession no. Linj.29.1010), 5'-CTTCCATGATGGGGCTTCTA-3'and reverse primer of neomycin gene. PCR amplified product was further cloned into *pGEMT* Easy vector and sequence confirmed.

Transfection with homologous construct 2 (5'FLK/HYG/3'FLK) was performed as described above and transfectants were selected in hygromycin antibiotics pressure (40µg/ml). To confirm the integration of hygromycin^r gene, PCR was performed using forward primer, 5'-ATGAAAAAGCCTGAACTCACCG-3' and reverse primer, 5'-CTATTCCTTTGCCCTCGGACG-3'.

4.2.10 ***In Vitro*** **macrophage infection**

To investigate infectivity of wild type and mutant parasites, primary peritoneal macrophages were harvested from 6 to 8 weeks old female BALB/c mice. Cells were washed with PBS and resuspended in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FCS (Gibco). 2×10^5 cells were plated in 8-well chamber

slides, and incubated at 37°C in 5% CO_2. Non-adherent cells were removed by washing after overnight incubation and adherent cells were infected with late-log-phase promastigotes at 1:10 macrophage to parasite ratio. After 6 h of infection, the remaining extracellular parasites were removed by washing and cells were further incubated in CO_2 incubator. The slides were fixed with methanol and stained with Diff- Quik solution after 24, 48, 72, 96 and 120 hours of infection. Percentages of infected macrophages were determined by counting a minimum of 300 macrophages under microscope (100X) and the numbers of amastigotes per cell were counted in 100 macrophages.

4.3 **Results**

4.3.1 **Putative LdA1 protein: sequence and structure based annotations**

The putative LdA1 protein is made of 175 amino acids and designated with UniProt identifier A4I4F1. The protein has 18961.2 Dalton molecular weight and theoretical isoelectric point (pI) of 6.59. The computed instability index of the protein is 49.96, which classify the protein as unstable. Sequence homology search revealed that LdA1 protein is unique to *Leishmania* genus and not present in any other organism. No putative conserved domains could be mapped to LdA1 protein through sequence homology search. The protein was observed to share orthology with *Leishmania* species sequences available at RefSeq database **(Figure 4.1).**

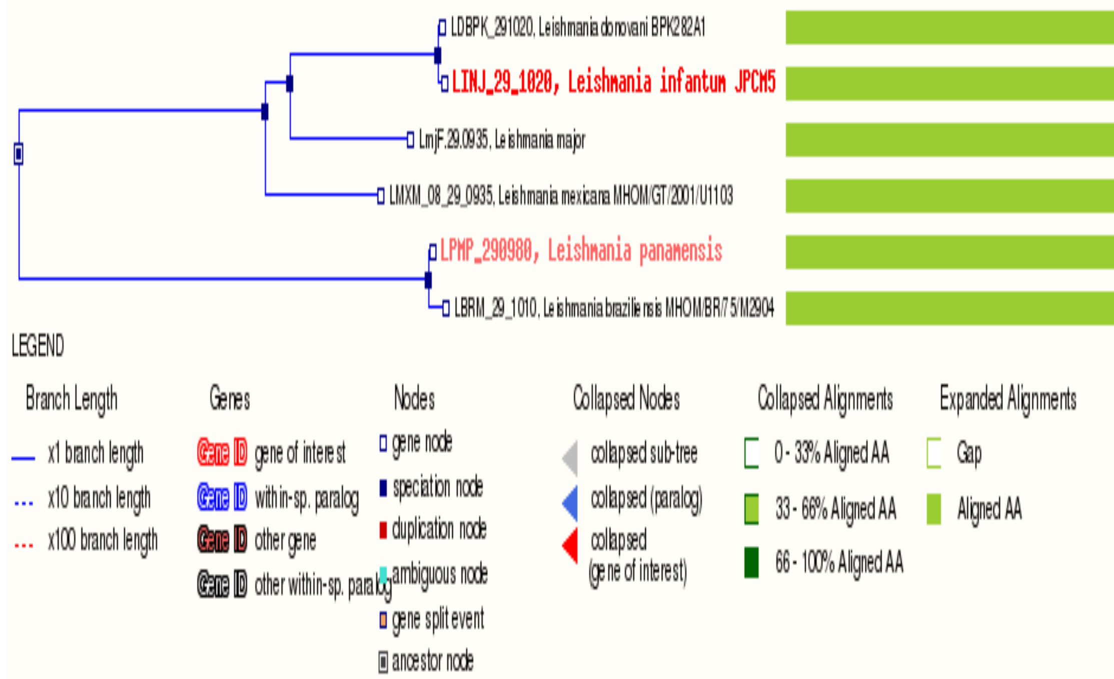


Figure 4.1: **Phylogenetic analysis showing evolutionary patterns of putative LdA1 protein in *Leishmania.*** Phylogenetic tree was constructed using Ensemblprotists gene tree database showing the different clusters of LdA1.

Sequence level functional annotations indicated that protein might be involved in the biological processes critical for survival of *Leishmania*. The list summarizing predicted involvement of LdA1 in various biological processes, molecular function and the cellular components is given as **table 4.1.**

Table 4.1: Sequence level functional annotations of putative LdA1 protein

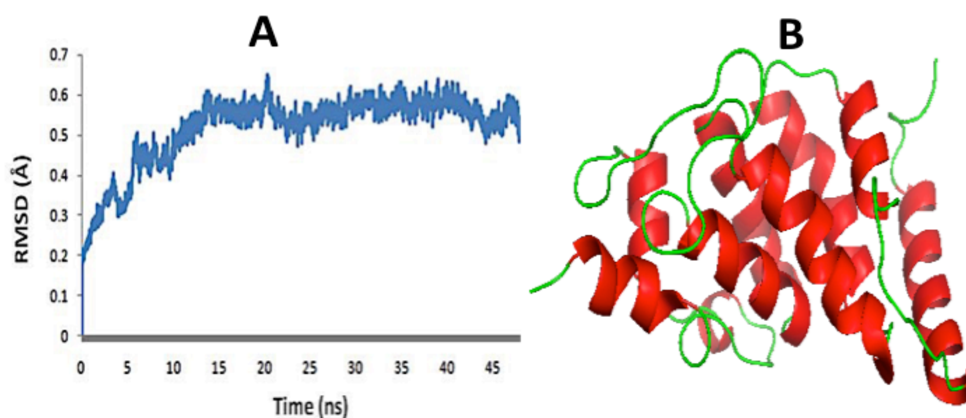
Score	GO term	RL	Domain	Description
0.932	GO:0019222	H	BP	Regulation of metabolic process
0.919	GO:0010468	H	BP	Regulation of gene expression
0.914	GO:0034645	H	BP	Cellular macromolecule biosynthetic process
0.894	GO:2001141	H	BP	Regulation of RNA biosynthetic process

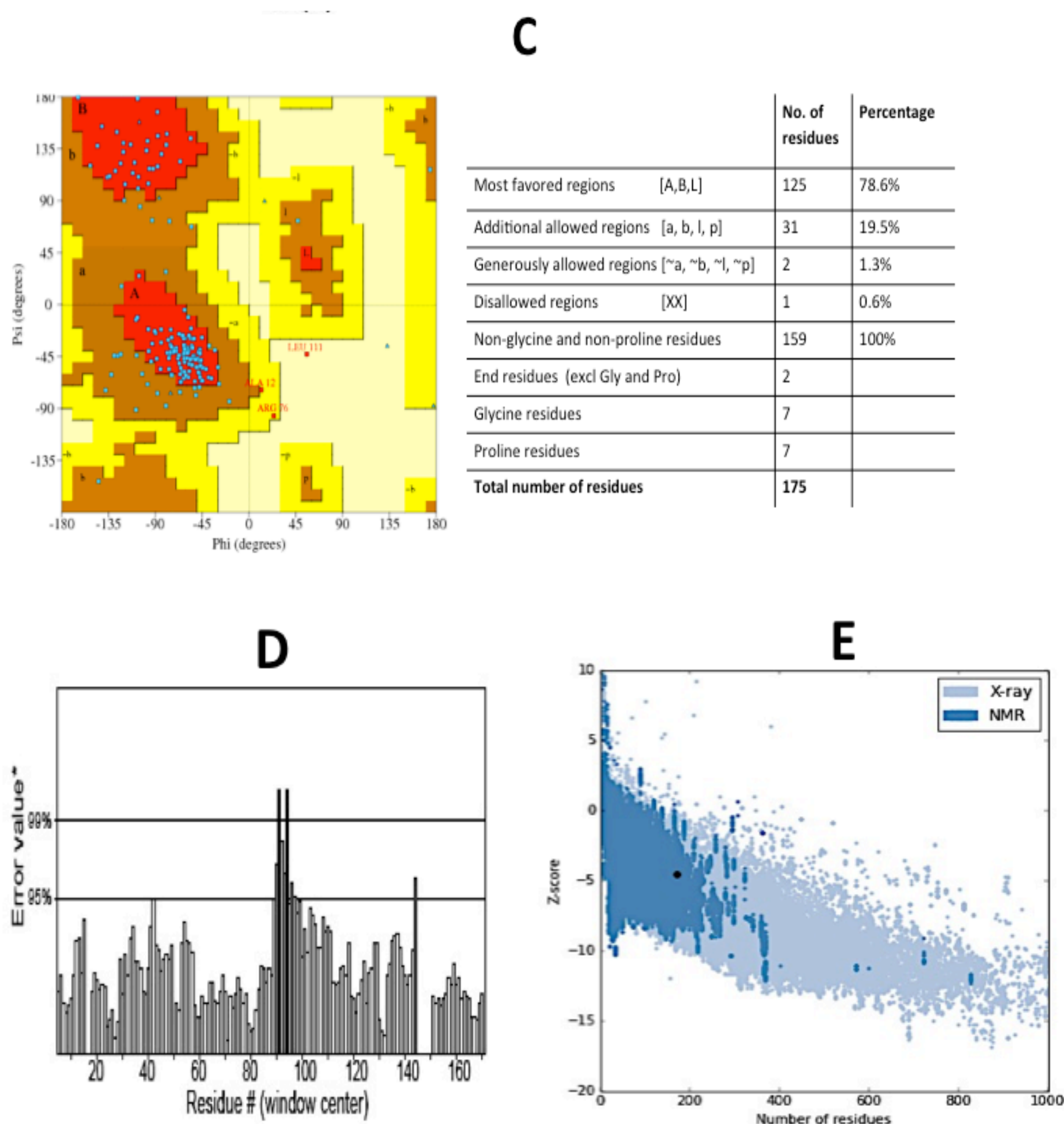
0.890	GO:0051171	H	BP	Regulation of nitrogen compound metabolic process
0.863	GO:0000981	H	MP	Sequence-specific DNA binding RNA polymerase II transcription factor activity
0.854	GO:0051252	H	BP	Regulation of RNA metabolic process
0.851	GO:0006355	H	BP	Regulation of transcription
0.754	GO:0003676	H	MF	Nucleic acid binding
0.690	GO:0009059	H	BP	Macromolecule biosynthetic process
0.688	GO:0005730	H	CC	Nucleolus
0.686	GO:0008092	H	MP	Cytoskeletal protein binding
0.661	GO:0016604	H	CC	Nuclear body
0.595	GO:0003677	H	MF	DNA binding
0.567	GO:0003723	H	MF	RNA binding
0.563	GO:0016020	H	CC	Membrane
0.519	GO:0031328	H	BP	Positive regulation of cellular biosynthetic process
0.514	GO:0010628	H	BP	Positive regulation of gene expression
0.701	GO:0005634	H	CC	Nucleus
0.610	GO:0044444	H	CC	Cytoplasmic part
0.569	GO:0044260	H	MP	Cellular macromolecule metabolic process
0.672	GO:0005515	H	MF	Protein binding
0.542	GO:0051234	H	MF	Establishment of localization

RL- Reliability level for that GO term (H-high), CC- cellular component, MF- molecular function, MP- metabolic processes, BP-biological processes

Five models of putative LdA1were computationally generated using the I-TASSER server. The C-scores for the predicted models range from -5 to -2.94. The model 1 was selected and was observed to be of moderate quality during model validation (Overall 96.9% residues were in allowed region, 3.1 % residues in disallowed region, ERRAT Overall quality factor**: 83.832). Therefore, the 3D model was refined through 50 ns molecular dynamics simulations. A stable root mean square

deviation (RMSD) after 10 ns of production run (**Figure 4.2A**) ensured the model refinement process has adequately converged to report a reliable 3D model of LdA1protein. Validation of refined putative LdA1structure (**Figure 4.2B**) showed quality of LdA1 protein model has significantly improved after molecular dynamics simulations. After refinement 99.4% amino acids of the protein structure were seen in favorable regions of the Ramachandran plot (**Figure 4.2C**). The residues in the disallowed region decreased from 3.1 % to 0.6%. The ERRAT overall quality factor improved from 83.8% to 94.9%. It is established that good high-resolution structures generally produce values around 95% or higher while for lower resolutions (2.5 to 3A) the average overall quality factor is around 91%. The ERRAT overall quality factor of refined LdA1 protein (94.9%) indicates the predicted structure is of good quality (**Figure 4.2D**). Subsequent model validation through ProSA-web analysis (Z-score: -4.55) (**Figure 4.2E**) and VERIFY3D (84.57% residues ≥ 2.0 3D/1D profile) also established the structure is of good quality for structure based functional annotations. Both sequence and structure level function predictions shared some important biological functions together including, macromolecule and nucleic acid metabolic processes and protein binding.





	No. of residues	Percentage
Most favored regions [A,B,L]	125	78.6%
Additional allowed regions [a, b, l, p]	31	19.5%
Generously allowed regions [~a, ~b, ~l, ~p]	2	1.3%
Disallowed regions [XX]	1	0.6%
Non-glycine and non-proline residues	159	100%
End residues (excl Gly and Pro)	2	
Glycine residues	7	
Proline residues	7	
Total number of residues	**175**	

Figure 4.2: *In silico* characterization of putative LdA1 protein.(A) Root mean square deviation (RMSD) of backbone atoms of putative A-1 proteins during 50 ns production run. (B) 3D structure of LdA1 protein after 50 ns production run. (C) Ramachandran plot of LdA1 showing most favored regions (red), additional allowed region (yellow), generously allowed region (white) and rest as disallowed region. Stastical parameters are given in the table below. (D) ERRAT validation of LdA1 simulated model . (E) ProSA results showing the Z- score of LdA1 protein simulated model is comparable with the experimental structures of similar length.

4.3.2 **Recombinant LdA1 protein preparation, antibody generation**

In an effort to prepare LdA1 protein, full-length open reading frame of LdA1 (528bp) was PCR amplified **(Figure 4.3A)** using gene specific primers. PCR amplified product was cloned, sequence confirmed and expressed as 6X-His tagged LdA1 protein of~20kDa molecular size. LdA1 protein was purified using Ni-NTA affinity resins and presence of single band on SDS page confirmed its purity **(Figure 4.3B)**. Polyclonal antibodies against LdA1 were raised in rabbit for further studies.

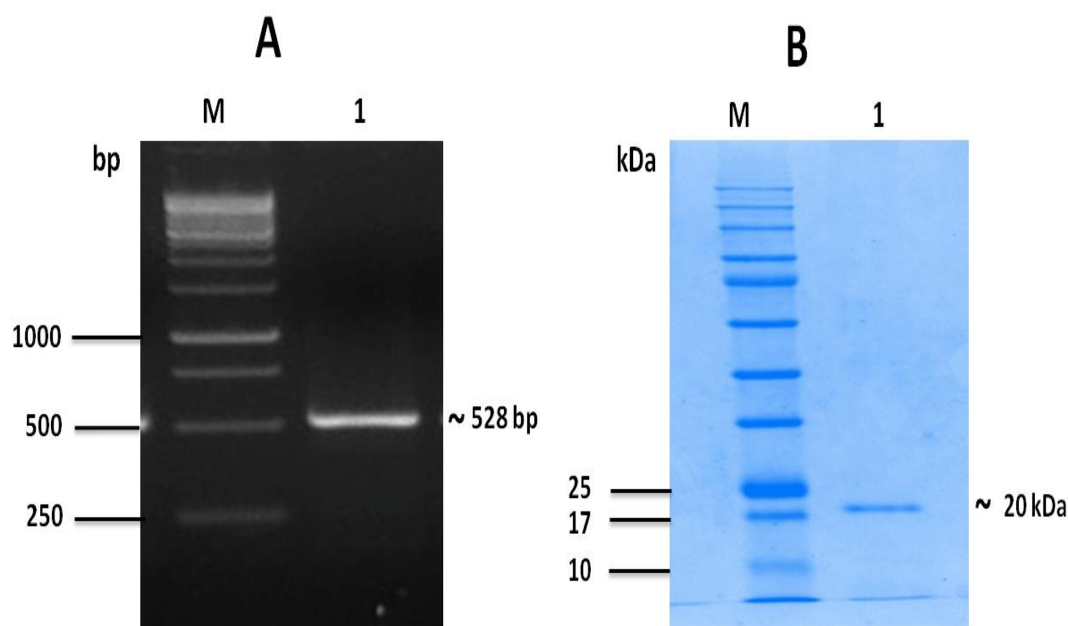


Figure 4.3: Preparation of recombinant LdA1 protein. (A) PCR amplification of LdA1 from *Leishmania* genomic DNA. M: 1kb molecular weight marker; Lane 1: PCR amplicon of LdA1. (B) SDS gel page showing the purified recombinant LdA1 protein. M: Protein molecular weight markers (in kDa), Lane 1: purified LdA1 protein.

4.3.3 **Confirmation of up regulated expression of LdA1 at amastigote stage**

Studies have shown up regulated expression of LdA1 in amastigotes at RNA level by northern hybridization (Srividya *et al* 2007, Gannavaram *et al*., 2004). In the

present study, we analyzed expression profile of LdA1 in promastigote and amastigote at protein level by western blotting. Western blot analysis was performed with the cell lysate of promastigotes and amastigotes using polyclonal anti-rLdA1 antibody, which detected LdA1 protein of ~19 kDa molecular size in the whole cell lysate. The expression of LdA1 at protein level was found to be up regulated by approximately 3 fold at amastigote stage compared to the promastigote **(Figure 4.4)**.

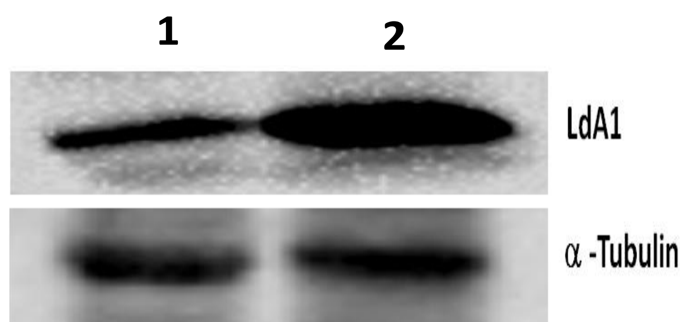


Figure 4.4: Comparison of expression of LdA1 between promastigote and amastigote stage using western blot. Lane 1: Lysate of parasite at promastigote stage probed with anti-LdA1 antibody, Lane 2: Lysate of parasite at amastigote stage probed with anti-LdA1 antibody. α - tubulin was used as an endogenous control.

4.3.4 **Immunolocalization of LdA1 in *Leishmania* parasite**

To ascertain the subcellular localization of LdA1 in *L. donovani,* immunofluorescence analysis was carried out at both promastigote and amastigote level. A punctate staining pattern of FITC labeled secondary antibody against anti-rLdA1 was observed near the DAPI stained kinetoplast **(Figure 4.5)**, which indicates that LdA1 is predominantly localized close to the kinetoplast of both promastigotes (**Figure 4.5A**) and axenic amastigotes (**Figure 4.5B**). No fluorescence was obtained with pre-immune rabbit serum.

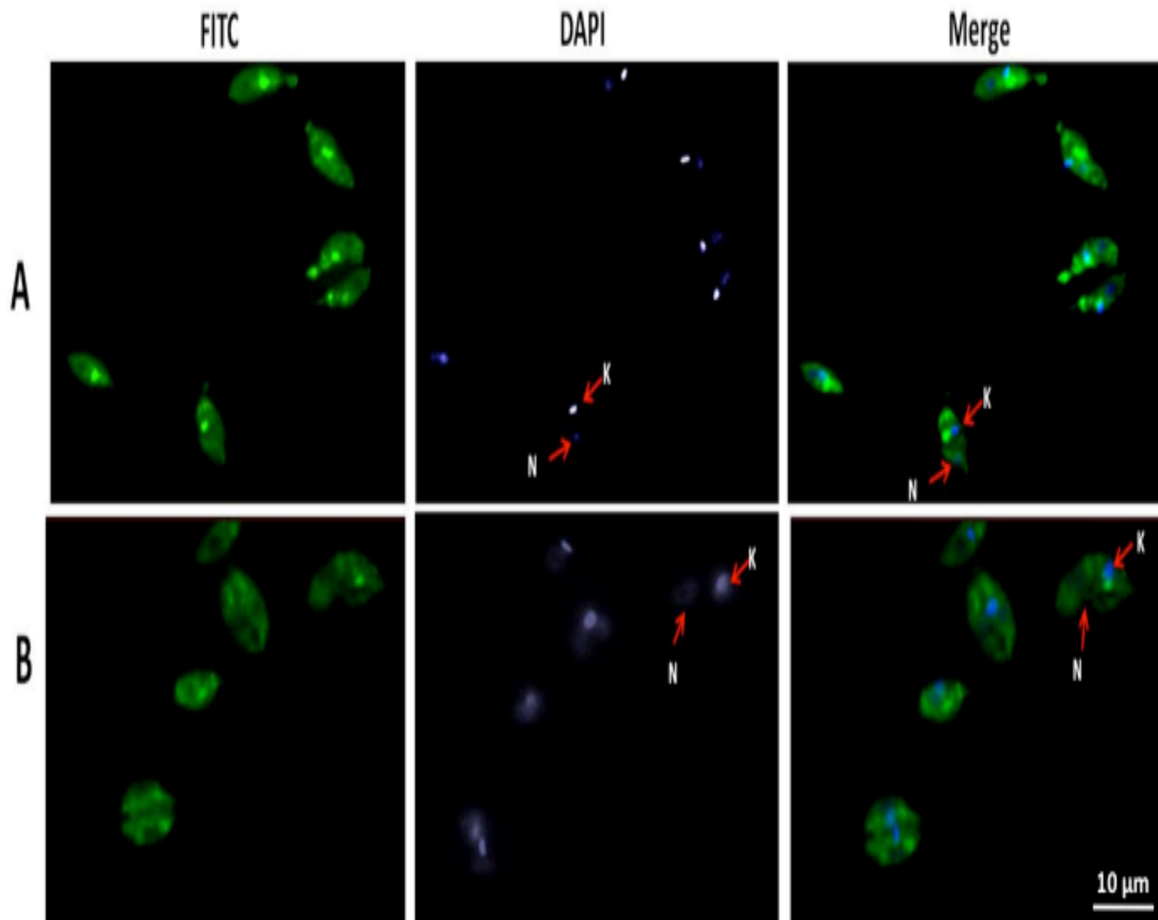


Figure 4.5: Immunolocalization of LdA1 in promastigotes and axenic amastigotes. To determine the subcellular localization, immunofluorescence analysis was performed with anti-LdA1 (green signal). DAPI (blue) was used to stain the nucleus (N) and kinetoplast (K). (A) Localization of LdA1 in promastigotes. (B) Localization of LdA1 in axenic amastigotes.

4.3.5 **Episomal expression of LdA1 and its effect on parasite growth and phenotype**

LdA1 gene was PCR amplified with HA tag gene sequence (~550 bp) **(Figure 4.6A)** and sub-cloned into *pGEMT* Easy T/A cloning vector. Sequence confirmed *pGEMT* clone was digested with SpeI to release LdA1 insert **(Figure 4.6B),** which

was further cloned into *Leishmania* specific expression plasmid *pKSNEO* for over-expression **(Figure 4.6C).** Further, right orientation of LdA1 in *pKSNEO* was confirmed by sequencing. *L. donovani* promastigotes were transfected with this over-expression construct and the transfectant were selected under G418 pressure. Over-expression of LdA1 was confirmed by western blotting with anti-HA and anti-LdA1 antibody **(Figure 4.6D).** Parasites transfected with over-expression construct (LdA1-OE) showed 2 to 3 fold higher expression of A1 in comparison to the mock-transfected parasite **(Figure 4.6D)**.

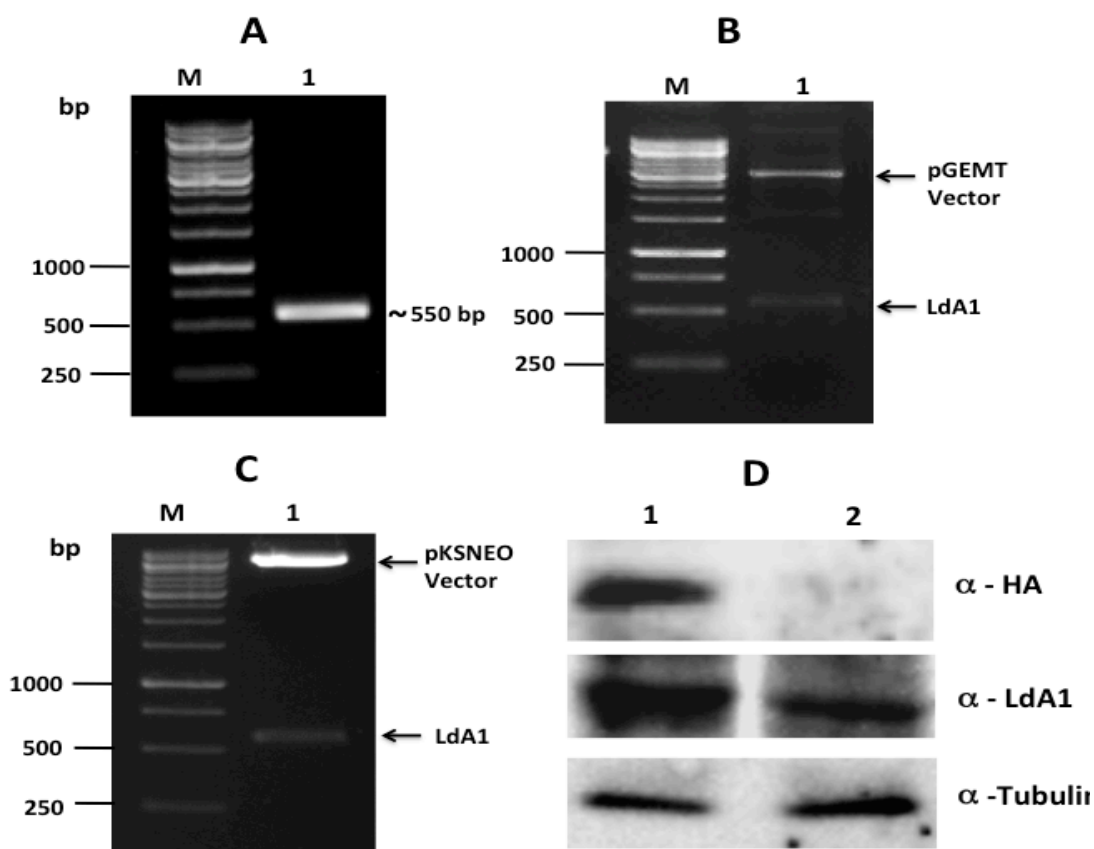


Figure 4.6: Over-expression of LdA1 in *Leishmania* parasite by using *Leishmania* specific expression vector *pKSNEO*. (A) PCR amplification of LdA1. M: 1kb molecular weight marker; Lane 1: PCR amplicon of LdA1 with HA tag sequence. (B) Release of LdA1 insert from *pGEMT* plasmid after restriction digestion. M: 1kb molecular weight marker; Lane 1: *SpeI* digestion of *pKSNEO* plasmid containing

LdA1 insert. (C) Confirmation of presence of LdA1 insert in *pKSNEO* plasmid by restriction digestion. M: 1kb molecular weight marker; Lane 1: *Spe*I digestion of *pKSNEO* plasmid containing LdA1 insert. (D) Western blots using anti-HA and anti-LdA1 antibody to confirm the over-expression in *Leishmania* parasite. Lane1: Lysate of parasites transfected with *pKSNEO* plasmid alone; Lane2: Lysate of parasites transfected with *pKSNEO* plasmid containing LdA1 insert. α- tubulin was used as an endogenous control.

Next we examined effect of over-expression on phenotype and growth of the parasites at both the life stages. No difference was found in the growth rate and phenotype of LdA1 overexpressors and the mock-transfected controls at both promastigote **(Figure 4.7A)** as well as amastigote stage **(Figure 4.7B).**

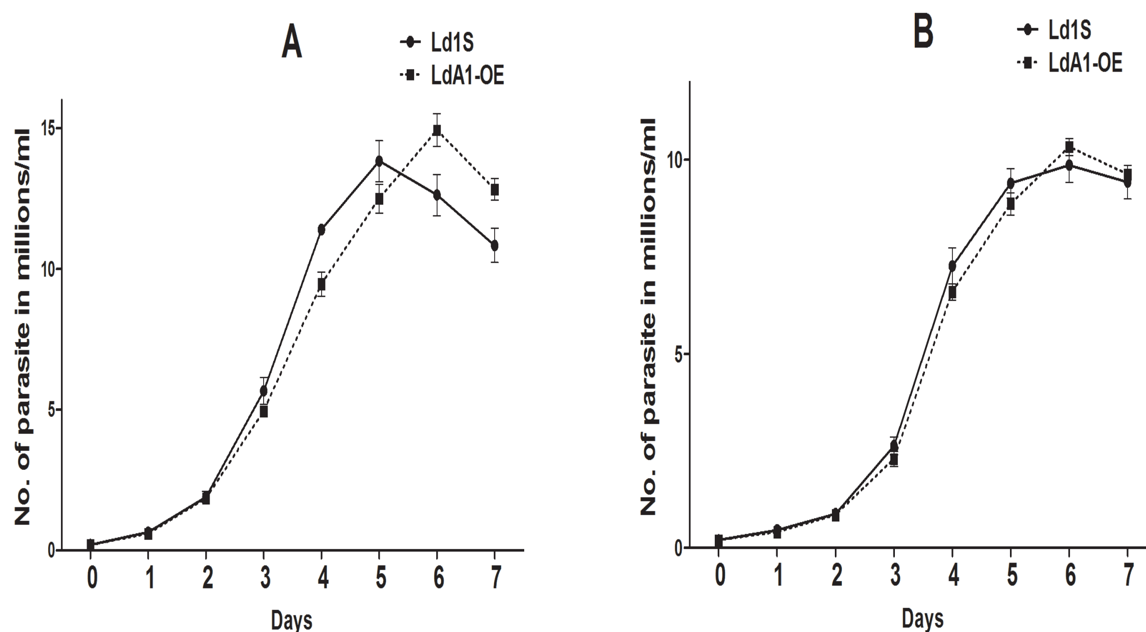


Figure 4.7: Growth curve of mock transfected (Ld1S) and LdA1 overexpressing (LdA1-OE) mutants. Experiment was repeated thrice independently and data are given in mean ± SEM.

4.3.6 **Formation of constructs for deletion of LdA1 by homologous recombination**

A targeted gene replacement strategy was used for the deletion of LdA1 in *L. donovani.* The 5' and 3' flanking fragment of LdA1 (5'FLK and 3'FLK) was PCR amplified **(Figure 4.8A,B)** and cloned into *pGEMT* Easy T/A cloning vector **(Figure 4.8C,D)** to confirm the fidelity of nucleotide sequence.

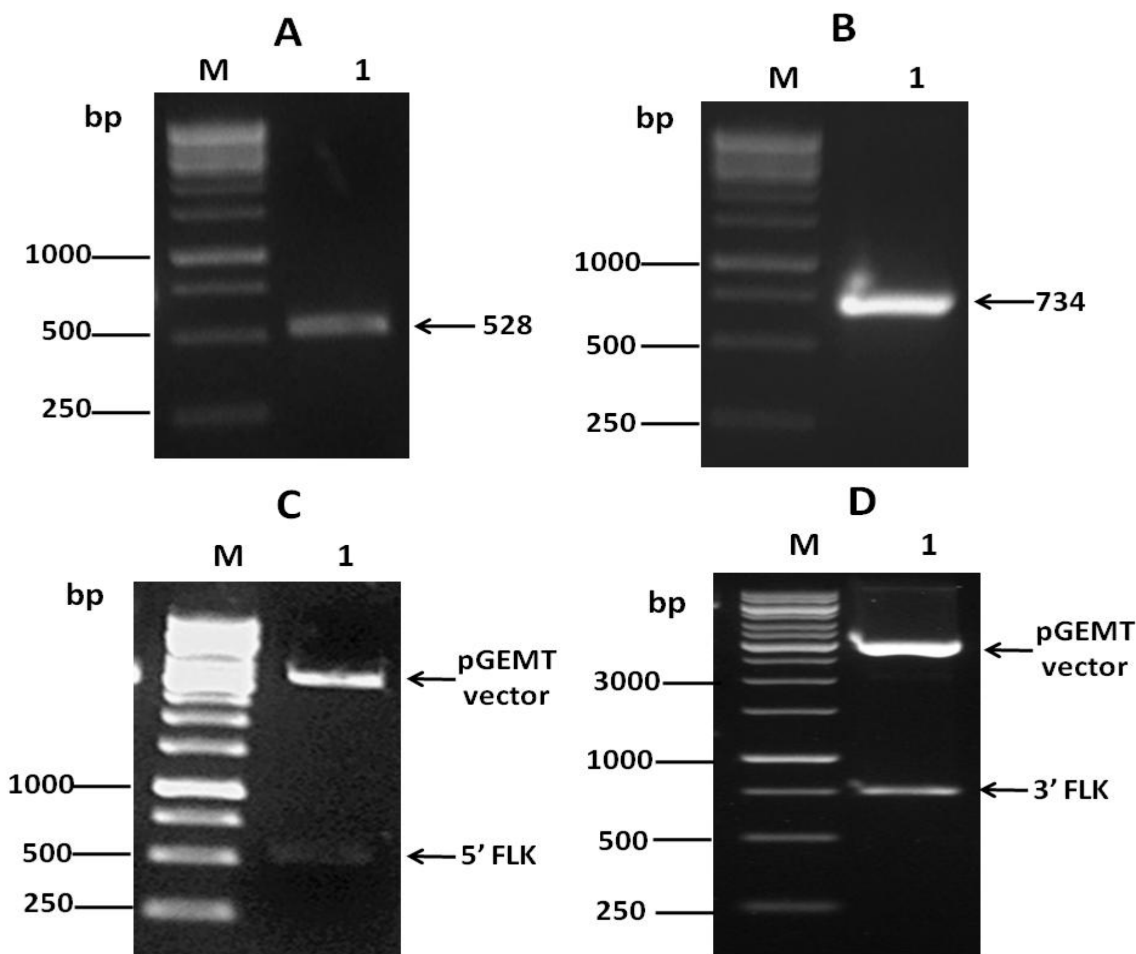


Figure 4.8: PCR amplification and cloning of 5' and 3' flanking regions of LdA1 gene. (A) & (B) PCR amplification of 5' and 3' flanking regions respectively. M: 1kb molecular weight marker; Lane 1: PCR amplicon. (C) & (D) Confirmation of presence of 5' and 3' FLK insert respectively in *pGEMT* plasmid by restriction digestion. M: 1kb molecular weight marker; Lane 1: SpeI digestion of *pGEMT* plasmid containing 5'FLK and 3' FLK inserts.

In order to generate homologous recombination constructs, these sequence confirmed 5'FLK and 3'FLKwere further cloned into the knockout targeting plasmid **(Figure 4.9A,B)** as described in the methodology.

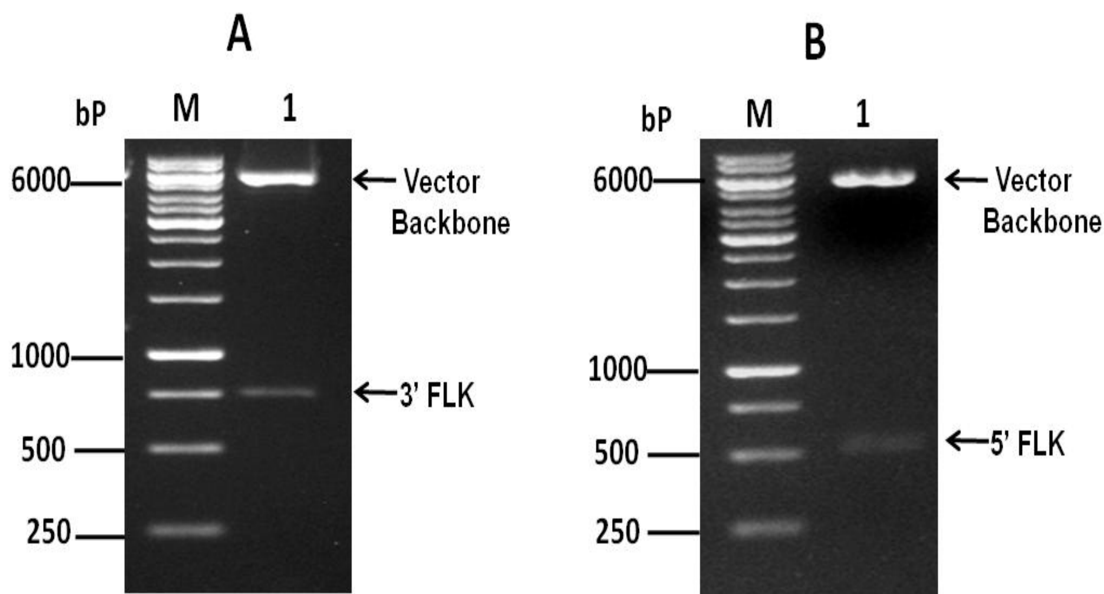


Figure 4.9: Cloning of 3' and 5' flanking regions in knockout targeting plasmid. (A) Confirmation of presence of 3' and 5' FLK insert in knockout targeting plasmid by restriction digestion. M: 1kb molecular weight marker; Lane 1: SpeI-KpnI of digestion knockout targeting plasmid containing 3' FLK inserts. (B) Confirmation of presence of 5'FLK insert in knockout targeting plasmid by restriction digestion. M: 1kb molecular weight marker; Lane 1: SwaI-SalI digestion of knockout targeting plasmid containing 5'FLK inserts.

Two homologous recombination constructs with two different antibiotic resistance genes were prepared for transfection **(Figure 4.10)**, construct1 (2.1 kb) with neomycin resistance gene **(Figure 4.10 and 4.11A)** and construct2 (2.3 kb) with hygromycin resistance gene **(Figure 4.10 and 4.11B)** along with 5' and 3' flanking region of LdA1.

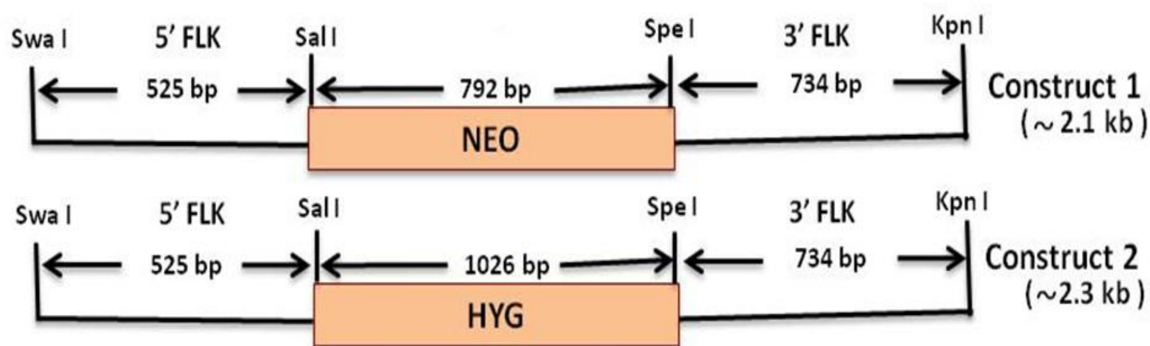


Figure 4.10: Schematic diagram showing design of constructs for LdA1 gene disruption from *Leishmania* genome. Construct 1 and 2 containing neomycin and hygromycin genes, respectively, flanked on the 5′ and 3′ sides with 5' and 3'- flanking regions (FLK) of LdA1 respectively. The position of restriction sites and the lengths of the antibiotic-resistant genes and FLK have been indicated.

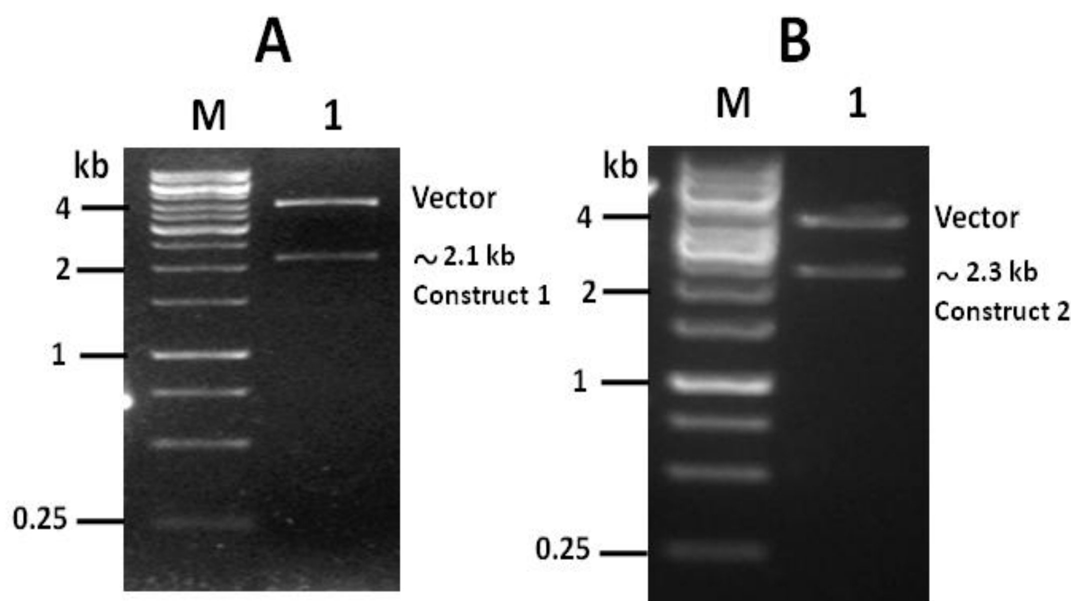


Figure 4.11: Generation of knockout constructs for homologous recombination. (A) & (B) Restriction digestion of knockout vector to generate homologous recombination constructs1 (5'FLK/NEO/3'FLK) and constructs2 (5'FLK/HYG/3'FLK) respectively. M: 1kb molecular weight marker; Lane 1: *Swa*I and *Kpn*I digestion of knockout vector containing construct 1 and construct 2.

4.3.7 **Generation of LdA1 single deleted mutant parasites by transfection with knockout constructs**

To delete LdA1 gene, *Leishmania* parasite was first transfected with knockout construct1 using electroporation method, which resulted in the deletion of single allele of LdA1 (LdA1$^{+/-}$) and generation of G418 resistant parasites. Single allele deletion of LdA1 was confirmed by using PCR, which was found positive for both, neomycin gene **(Figure 4.12A)** as well as LdA1. Further, the successful integration of neomycin gene at the right place was verified with diagnostic PCR and an amplified product of expected size (~1.4kb) confirmed the integration **(Figure 4.12B)**.

In an attempt to delete the 2nd allele of LdA1, heterozygous clones (LdA1$^{+/-}$) were transfected with construct2. However, even after several attempts no null mutants could be generated.

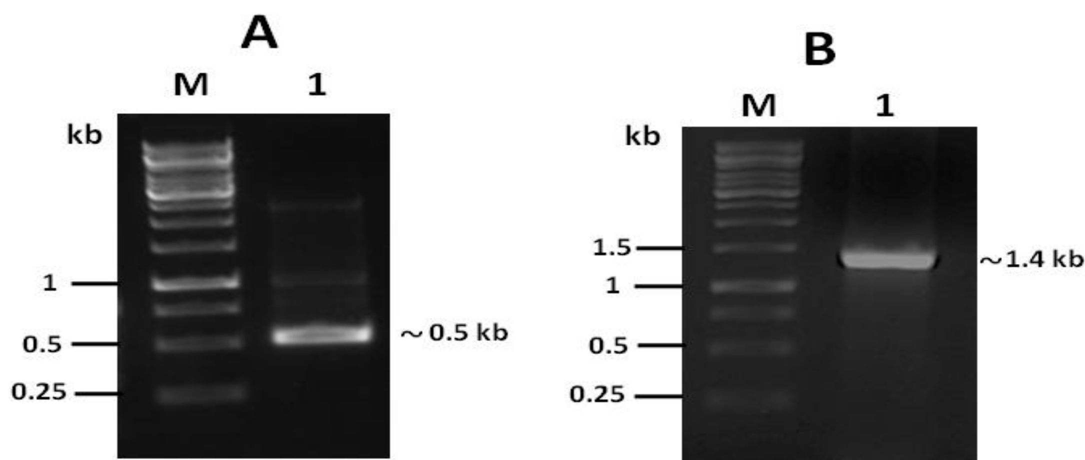


Figure 4.12: Confirmation of single allele deletion of LdA1. (A) Confirmation of integration of neomycin gene into *Leishmania* genome by PCR. M: 1kb molecular weight marker; Lane 1: PCR amplicon of neomycin gene. (B) Confirmation of integration of neomycin gene on the place of LdA1 by diagnostic PCR, as described in the methodology section. M: 1kb molecular weight marker; Lane 1: PCR amplicon of desired size.

4.3.8 **Effect of LdA1 single allele deletion on parasites phenotype and growth**

We examined the effect of $LdA1^{+/-}$ on phenotype and growth of parasite at promastigote as well as amastigote stage. $LdA1^{+/-}$ mutants showed different morphology (more rounded instead of regular elongated) and appeared to be smaller in size **(Figure 4.13A)** with reduced motility as compared to the wild type parasite. Phenotypic changes were also seen at the amastigote stage **(Figure 4.13B)**.

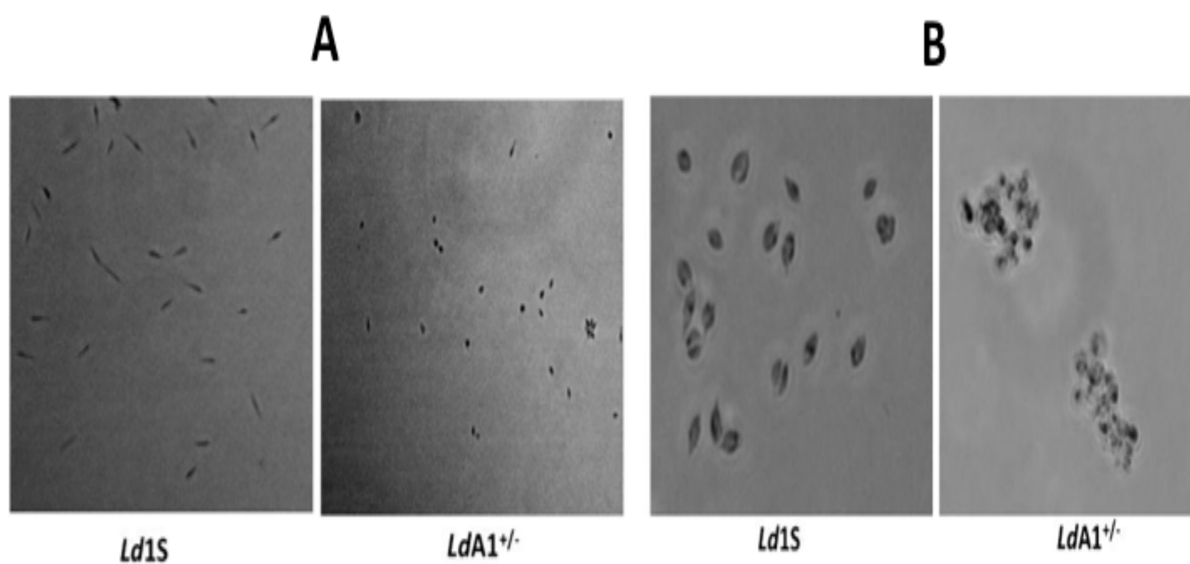


Figure 4.13: Effect of LdA1 single allele deletion on phenotype of parasites. Differences in phenotype of wild type parasites (Ld1S) and LdA1 single allele deleted ($LdA1^{+/-}$) mutants in log phage culture: (A) promastigotestage and (B) amastigote stage.

Further, we observed that the growth of *Ld*A1$^{+/-}$ parasite was also reduced as compared to the wild type at both the life stages **(Figure 4.14)** and this reduction of growth was more evident at the amastigote stage as they had tendency to form clumps together **(Fig. 4.13B)**.

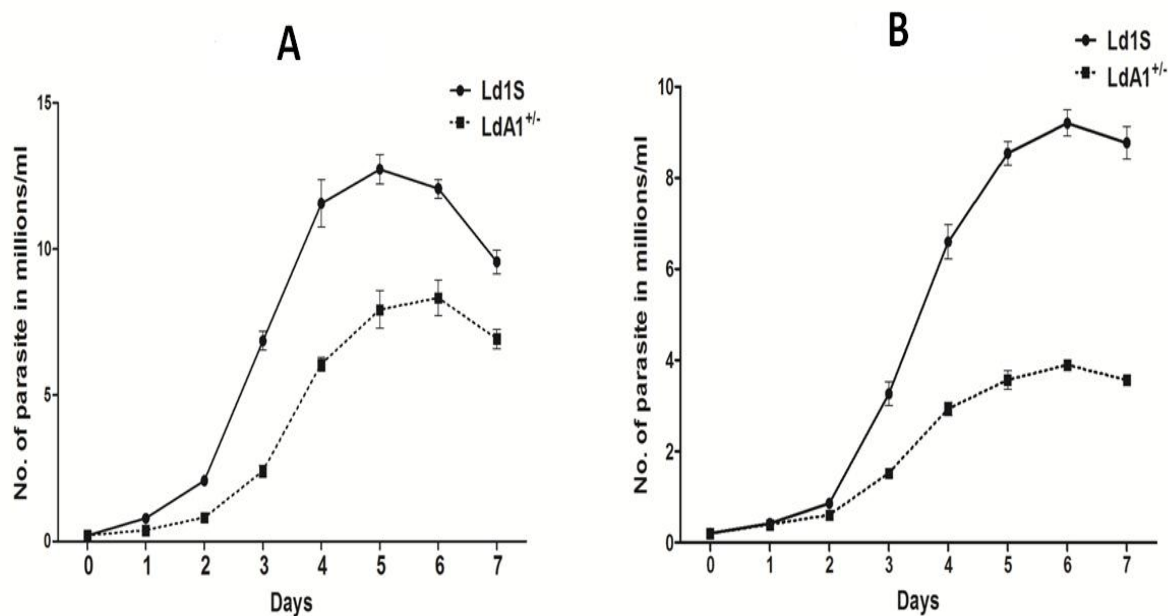


Figure 4.14: Effect of LdA1 single allele deletion on growth of parasites. Growth curve of wild-type (Ld1S) and LdA1 single allele deleted ($LdA1^{+/-}$) mutants at (A) promastigote and (B) amastigote stage. Experiment was repeated thrice independently and data are given in mean ± SEM.

4.3.9 **Evaluation of macrophage infectivity of mutant parasites**

In order to study the effect of single allele deletion and over-expression of LdA1 on parasite infectivity, mouse peritoneal macrophages were infected with mutant parasites and compared with the infection achieved with the wild type **(Figure 4.15A)**. After 6 h of infection more than 60% of macrophages were infected with the parasites (contained atleast one amastigote) and there was no significant difference in the percentage infectivity of wild type (Ld1S), $LdA1^{+/-}$and LdA1-OE **(Figure 4.15A,B**) at this time point. However, there was significant decrease in the percentage of infected macrophages with $LdA1^{+/-}$ as compared to the wild type and LdA1 over-

expressing parasite after 72 h of infection and this trend remained the same after the each time intervals tested (96 and 120h of infection) **(Figure 4.15B)**.

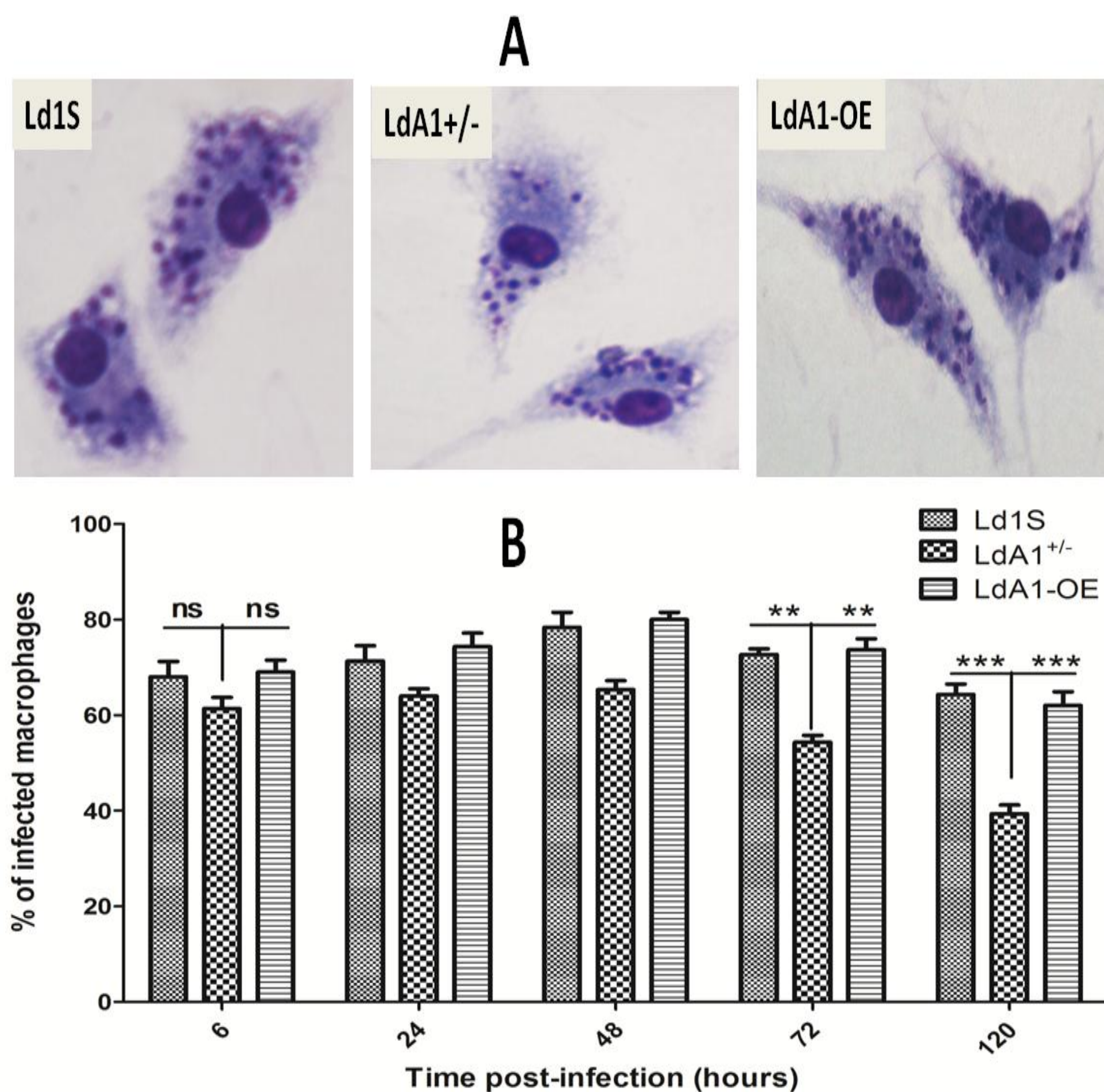


Figure 4.15: *In vitro* infectivity of mutant parasites. Mice peritoneal macrophages were infected with wild type (Ld1S), LdA1 single allele deleted ($LdA1^{+/-}$) or LdA1 overexpressing parasites (LdA1-OE) for 6 hours at a ratio of 10:1 (parasites: macrophage). (A) Macrophages infected with wild type, $LdA1^{+/-}$ or LdA1-OE, respectively after 6 hrs of infection and staining with Diff-Quik, (B) Bar graph showing the percentage of macrophages infected with Ld1S, $LdA1^{+/-}$ and LdA1-OE at the given time interval. Values represent mean ± SEM from three independent

experiments. Significance between different groups was tested using two-way ANOVA followed by post hoc ‘t’ test with Bonferroni correction. $P < 0.05$ was considered statistically significant and ** = P<0.01, ***= P< 0.001, ns= non significant.

4.3.10 **Evaluation of survival of mutant parasites inside macrophages**

Next we examined the effect of LdA1 deletion on the survival of *L. donovani* amastigotes by counting number of amastigotes in the infected macrophages. We observed that LdA1$^{+/-}$ parasites show significant decline in growth as the days progressed **(Figure 4.16)**, whereas wild type control and LdA1-OE parasites continue to grow normally inside macrophages **(Figure 4.16)**.

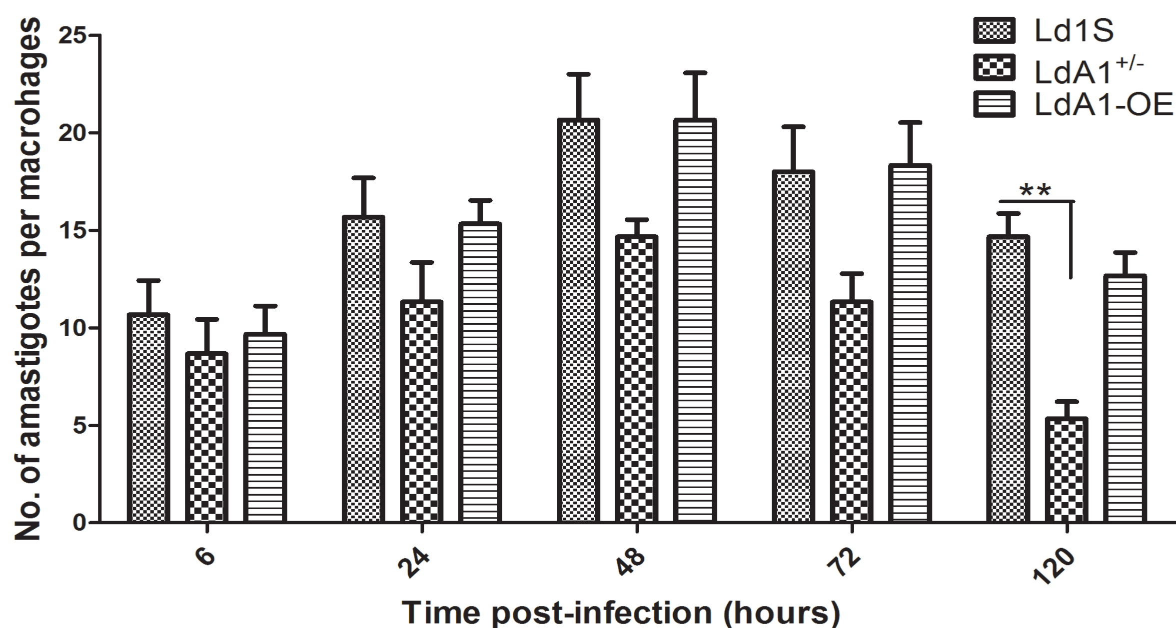


Figure 4.16: Effects of LdA1 deletion on the replication of *L. donovani* amastigotes inside macrophages. Mice peritoneal macrophages were infected with wild type (Ld1S), LdA1 single allele deleted (LdA1$^{+/-}$) or LdA1 overexpressing parasites (LdA1-OE) for 6 hours at a ratio of 10:1 (parasites: macrophage). Bar graph showing the number of intracellular amastigotes present in infected macrophages at the given time interval.

Values represent mean ± SEM from three independent experiments. Significance between different groups was tested using two-way ANOVA followed by post hoc 't' test with Bonferroni correction. $P < 0.05$ was considered statistically significant and ** = $P<0.01$.

4.4 **Discussion**

Amastigote specific/over-expressing genes are considered as potential virulent genes and they are likely to be involved in the pathogenesis and intracellular survival of the *Leishmania* parasite (Nugent PG *et al*., 2004;McNicoll F *et al*., 2006;Selvapandiyan*et al*., 2014). However, till date, only few amastigote specific genes have been characterized. In the present study we describe characterization of a single copy, amastigote stage over-expressing LdA1 gene to evaluate its potential for the generation of live attenuated parasites and its role in *Leishmania* life cycle. The LdA1gene was originally identified by arbitrary primed PCR and its expression was found to be up-regulated at the amastigote stage of parasite at RNA level (Gannavaram *et al*., 2004; Srividaya *et al*, 2007). Further, assessment of *in vivo* expression of LdA1 mRNA in bone marrow aspirate of VL and tissue lesions of post kala-azar dermal leishmaniasis (PKDL) and CL patients revealed that the gene transcript was present in all the three forms of leishmaniasis and its expression was substantially higher in VL, a deadly form of leishmaniasis in comparison to the dermal manifestations including CL and PKDL (Paresh *et al*., 2010), which indicated that it might be involved in the disease pathogenesis. In the present study, the potential role of LdA1 in *Leishmania* life cycle was elucidated via *in silico* analysis and generation of mutant parasites that either overexpressed or were deficient for LdA1 proteins.

To gain some insight into the probable molecular function of LdA1 and its

role in pathogenesis and survival of parasites, *in silico* analysis was performed. Sequence homology analysis revealed that LdA1 protein is highly conserved in the genus *Leishmania,* as no significant similarities were found with proteins of any other organism, not even with the closely related trypanosomatids. It is known that many of the amastigote specific genes have no homologs in other system and their functional analysis a major challenge (Uliana*et al*., 1999). Prediction of structure is an important step to determine the function of an unknown protein and homology modeling is a method of choice to decipher the structure of such proteins. Since LdA1 did not show homology with any other protein of the existing database, in the present study, we used *ab intio* modelling and molecular dynamics simulations to propose the first 3D structure of the LdA1. Ramachandran plot was developed to assess the quality of the structures for LdA1. The plot statistics showed the distribution of amino acids in the allowed and disallowed regions signifying the stability of the developed model and accounted its reliability. The predicted 3D structure enhanced our understanding in predicting potential function of LdA1 and it can be considered as a critical step towards the characterization of the novel LdA1 gene. Both sequence and structure level function prediction suggested the essential role of LdA1 in *Leishmania* parasite life cycle and it might be vital for the parasite survival. Deriving useful information from bioinformatics analysis critically depends on the ability to assign accurate biological roles to genes and their products. However, the only conclusive way for the characterization of protein function is by experimental determination.

Previously, up-regulated expression of LdA1 was shown at RNA level in amastigote stage (Srividaya *et al*., 2007; Gannavaram *et al*., 2004); however, studies have shown that there is a no linear relationship between expression levels of a given gene at mRNA and the proteins level due to post transcriptional regulation in

Leishmania parasites (McNicoll F *et al*., 2006; Clayton C *et al*., 2007; Haile S *et al*., 2007). Therefore, we investigated the expression profile of LdA1 between the two life stages of the parasites at protein level. Up regulated expression of LdA1 at amastigote stage was also observed at protein level, which was in agreement with the data at RNA level. Further, in an effort to examine the localization of LdA1 in *Leishmania* parasite, Immunofluorescence analysis was performed. It was found that LdA1 is primarily localized near kinetoplast, similar localization was seen in the case of centrin 1 gene, which was found to be associated with the basal body (Selvapandiayn *et al*., 2001).

Genes with up-regulated expression at amastigote stage have been previously characterized for their potential role in growth and/or virulence of the parasite (Mundodi *et al*., 2005; Dey R *et al*., 2010; Selvapandiyan *et al*., 2014). One of the common ways to explore the biological function of a protein is to genetically alter the cell either for the overexpression of protein of interest by episomal expression or under expression by gene deletion. To determine the effect of overexpression on phenotype changes associated with growth of the parasite, LdA1 was episomally expressed to generate stage independent LdA1 overexpressing *Leishmania* parasites. There was no significant difference on the growth and morphology of the LdA1 overexpressing parasites in comparison to the wild type, indicating an unaffected metabolic rate. Further, we examined the effect of LdA1 gene deletion on growth and attenuation of the parasites. Targeted gene disruption methods have been used earlier to delineate the function of many *Leishmania* genes and for generation of live attenuated parasites (Papadopoulou *et al*., 1996; Uliana *et al*., 1999; Selvapandiyan *et al*., 2004; Dey R *et al*., 2010; Taheri *et al*., 2010; Manhas *et al*., 2014). In the present study, to replace both the alleles of LdA1, two gene knockout constructs were prepared with two different antibiotic resistance markers. We successfully deleted

single allele of LdA1 using knockout construct1 containing neomycin antibiotic resistance marker; however, we could not generate null mutants (LdA1$^{-/-}$) using second replacement construct even after several attempts. Similar lack of viability has been previously seen after deletion of one allele of single copy number genes (Mundodi *et al*., 2005; Tatheri *et al*., 2010). Repeated inability to create null mutant of LdA1 was suggestive of gene essentiality. Therefore, we used the single allele deleted LdA1$^{+/-}$ mutants for further functional studies. LdA1$^{+/-}$parasites showed reduced size and sluggish movement compared with the wild type parasites. In addition to the altered morphology, they also exhibited slower growth kinetics compared to the wild type, indicating the effect of deletion on metabolic processes of the parasite. Since deletion of single allele of LdA1 affected both growth and phenotype of the parasite, next we examined its effect on the macrophage infectivity. We observed that infectivity of LdA1$^{+/-}$mutants was not significantly reduced, as initially, percentage of infected macrophages was comparable to the wild type. However, the number of LdA1$^{+/-}$ amastigotes inside macrophages significantly decreased in comparison to the wild type, which indicated that their capacity to survive inside the macrophages is reduced and they were not able to multiply as efficiently as the wild type parasite.

4.6 **Conclusion**

This study demonstrates that LdA1 is a amastigote stage over-expressing gene and it is unique to *Leishmania* genus. It might play some essential role in life cycle of *Leishmania* parasite, as was evident from the change in phenotype and growth kinetics of LdA1 single allele deleted mutant parasites at both the life stages and their inability to multiply efficiently as amastogotes inside the macrophages. Essentiality of LdA1 was further supported by *in silico* predictions as well as inability to delete both the alleles.

Evaluation of immune responses in human PBMCs elicited by live attenuated Leishmania parasites as vaccine candidate

5.1 **Introduction**

Currently no effective vaccine is available for visceral leishmaniasis (VL), which is potentially fatal. However, the epidemiological observations that individuals recovered from a *Leishmania* infection develop lifelong immunity against reinfection, suggest the possibility of developing a prophylactic vaccine. In past decades, several vaccination strategies have been tried, mainly against the cutaneous leishmaniasis (CL), these include killed or live attenuated *Leishmania* parasites, recombinant proteins or DNA encoding *Leishmania* proteins (Srineevas *et al*., 2014; Okwar*et al*., 2009); however, none of them have shown efficacy in humans. In VL, a complete *Leishmania* cDNA expression library injected into mice was found to be more protective than any sub pools of the library plasmids or a subunit, indicating that the whole parasite makes the best vaccine (Melby *et al*., 2000). Studies have also shown that persistence of parasite is an important factor in developing long lasting immunity (Working Group on Research Priorities *et al*., 2011; Okwor, 2008; Bogdan, 2008) also a durable protective immunity can be induced by live attenuated parasites. The major advantage of such parasites is that they are taken by the host cells similar to the virulent parasites, can deliver several protective antigens as compared to the subunit or recombinant vaccine and survive in the host without causing pathology (Okwor *et al*., 2009). Limited persistence of the parasites and the immunomodulatory functions exerted by the attenuated parasites on the antigen presenting cells (Bhattacharya *et al*., 2015) can help the host to develop strong memory immune response with a strong memory. Live attenuated parasites developed by genetically defined irreversible deletions are safer compared to the parasite lines that have been developed through other means.

It has been reported that centrin1 and p27 gene deleted live attenuated parasites (*Ld*Cen1$^{-/-}$and *Ld*p27$^{-/-}$) have good potential as vaccine candidate against leishmaniasis in animal models (Selvapandiyan *et al*., 2004, 2009; Dey *et al*., 2010, 2013; Fiuza *et al*., 2013,2015). Centrin1 is a cytoskeletal, calcium-binding protein and plays essential role in centrosome duplication or segregation (Selvapandiyan *et al*., 2001). *Ld*Cen1$^{-/-}$ amastigote showed failure of cytokinesis, resulting in large multinucleated cells and undergoes process of programmed cell death; however, growth of *Ld*Cen1$^{-/-}$ promastigotes remained unaffected (Selvapandiyan *et al*., 2004). *Ldp*27 is a 27kDa mitochondrial membrane protein, required for ATP synthesis by oxidative phosphorylation and is expressed in the metacyclic and amastigote stages of the *L. donovani*. Deletion of *Ld*p27 gene (*Ld*p27$^{-/-}$) resulted in significantly lower cytochrome c oxidase activity and reduced ATP synthesis in the intracellular amastigotes compared with the wild type (Dey *et al*., 2010). Attenuation of growth and virulence in both live attenuated vaccine candidates occurs specifically at the intracellular amastigote stage of the parasites. The major advantage of these two mutant parasites is that both can be easily propagated as promastigotes and upon infection of host cells undergo limited replication as amastigotes without causing pathology. The complete clearance of *Ld*Cen1$^{-/-}$ parasites after 10 weeks of infection in spleen and liver of human VL model hamsters and susceptible BALB/c mice and immune compromised SCID mice showed its safety. Mice immunized with *Ld*Cen1$^{-/-}$ were protected against virulent parasite challenge and upon challenge they displayed induction of protective Th1 response. Further, *Ld*Cen1$^{-/-}$ immunized mice were also cross protected against challenge with *Leishmania braziliensis* that causes mucocutaneous leishmniasis (Selvapandiyan *et al*., 2009). In dogs, *Ld*Cen1$^{-/-}$ showed better immunogenicity than Leishmune (a commercially available vaccine in Brazil)

and comparable reduction in parasite load after virulent challenge (Fiuza *et al*., 2013,2015). Similarly, *Ld*p27$^{-/-}$parasites were found to be safe, immunogenic and protective against homologous and heterologous *Leishmania* species in mice models (Dey *et al*., 2010, 2013). Both *Ld*Cen1$^{-/-}$and *Ld*p27$^{-/-}$ induce production of pro-inflammatory cytokines in mice macrophages by classical activation and also skew antigen presentation abilities of the macrophages more towards effective Th1 response that favor development of protective immunity (Bhattacharya *et al*., 2015). Lack of knowledge regarding clear biomarkers associated with immunological protection in humans remains a barrier for development of an effective vaccine. However, studies have shown that *Leishmania* antigens that elicit higher IFN-γ and TNF-α response in healed VL (HVL) as compared to active VL cases have better potential as vaccine candidates (Singh *et al*., 2012) and the vaccine candidates that provide strong protective efficacy in experimental models, generally induce Th1 recall response in HVL PBMCs (Kumar R *et al*., 2010).

As the animal models do not fully recapitulate the full spectrum of human-parasite interactions, translation of results obtained from studies in the animal models remains a major challenge (Kedzierski *et al*., 2006; Garg*et al*., 2006). This study was undertaken to evaluate the vaccine potential of *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ live attenuated parasites using human blood samples obtained from different clinical groups including, active VL, healed VL (HVL), PKDL and healthy individuals.

5.2 **Material and Methods:**

5.2.1 **Study subjects and ethical consideration**

Study was carried out in 4 groups; Naïve healthy (n=15), active VL (n=7), Healed VL (n=15) and PKDL (n=15) patients. Patients reported at the Safdarjung

hospital, New Delhi, showing clinical symptoms of disease were included in PKDL and VL Active VL cases were diagnosed with the clinical features such as fever, anemia, weight loss, hepato-spleenomegaly and pancytopenia with positive rK39 immunochromatographic strip test **(Figure 5.1A).** VL was confirmed by microscopic analysis of *Leishmania* amastigotes in bone marrow aspirates sample, and/or with a qPCR test for *Leishmania* DNA as reported earlier (Salotra et al. 2001; Verma et al. 2010). Active PKDL cases were identified by the presence of characteristic lesions such as macular, papular and nodular or mixed/polymorphic forms. They were initially subjected to rapid rK39 test **(Figure 5.1A)** and positive cases were further confirmed for PKDL by demonstration of *Leishmania* amastigotes by direct microscopy in slit aspirates/skin tissue sample **(Figure 5.1B)**, and/or with a qPCR test for *Leishmania DNA* (Verma et al. 2010). Individuals unexposed to *Leishmania* parasite i.e. living in VL non-endemic areas and negative for rk39 strip test **(Figure 5.1A)** were included in the naïve healthy group while those having a previous history of VL that was cured at least 1 year before the recruitment in the study and positive for rk39 test were included in the HVL group **(Figure 5.1A)**. Naïve healthy individuals were included because they are never exposed to *Leishmania donovani* and serve as negative controls while HVL and PKDL cases are already exposed and are presumably immune to recurrence of VL. All patients were seronegative for HIV.The study was approved by and carried out under the guidelines of the Ethical Committee of the Safdarjung Hospital, India. Written informed consent for participation was obtained from all participants including the healthy subjects.

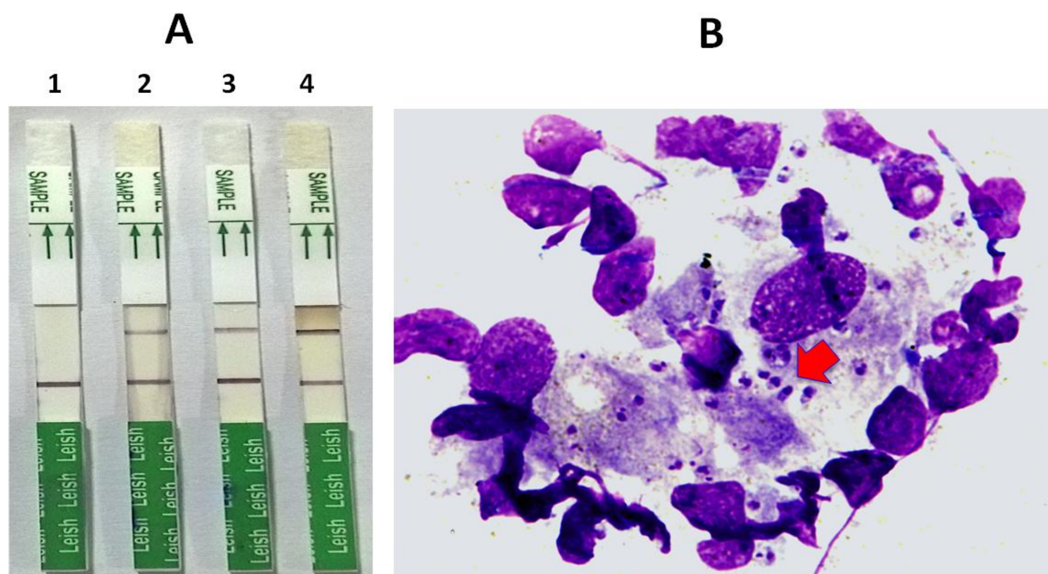


Figure 5.1: Diagnostic confirmation of cases for the recruitment in the study. (A) Initial screening of cases was done on the basis of rk39 test by using serum sample. Appearance of two bands in rk39 test indicates the presence of antibodies against *Leishmania* parasite. Lane showing: 1.Healthy; 2.VL; 3.HVL and 4. PKDL. (B) Microscopic demonstration of amastigotes in slit aspirate sample of PKDL patients by using Giemsa stain.

5.2.2 **Parasite culture and molecular analysis to confirm the absence of *Leptomonas* in all cultures**

Leishmania donovani promastigotes were cultured according to the procedure previously described (Bhandari *et al*., 2001; Selvapandiyan *et al*., 2001). In brief, wild type *Ld*1S was cultured in M199 medium containing 10% heat inactivated fetal bovine serum while *Ld*Cen1$^{-/-}$and *Ld*p27$^{-/-}$ were grown in the same medium with added antibiotics; hygromycin (40µg/ml) and G418 (40µg/ml) for *Ld*Cen1$^{-/-}$and G418 (40µg/ml) for *Ld*p27$^{-/-}$. All parasite cultures were incubated at 26°C in a BOD incubator and early stationary phase promastigotes were used in the study.

For the confirmation of pure cultures of *Leishmania* parasites, genomic DNA

was isolated from *Ld*1S, *Ld*Cen1$^{-/-}$and *Ld*p27$^{-/-}$cultures by using standard phenol-chloroform method and PCR was performed to amplify heat shock protein-70 (HSP70) gene as described previously (Srivastava *et al.*, 2010). PCR amplified product was digested with enzyme HaeIII and restriction fragment length polymorphism (RFLP) pattern analysis was done to confirm the absence of leptomonas in all cultures (Selvapandiyan *et al*., 2015, Srivastava *et al.*, 2010).

5.2.3 **PBMCs isolation and stimulation with parasites**

Heparinized blood was collected from all the study subjects and PBMCs were isolated from blood by density gradient centrifugation with Ficoll-Hypaque (Sigma-Aldrich) method. The cells were cultured in RPMI 1640 supplemented with 10% FCS, glutamine, HEPES and penicillin (100 U/ml), and streptomycin (100 µg/ml). $2x10^5$ PBMCs were plated in each well of 96 well flat bottom tissue culture plates and incubated in triplicate with (i) only media as unstimulated control, (ii) PHA (1 µg⁄mL) as a positive control, (iii) $1x10^4$ live wild type *L. donovani* (*Ld*1S), (iv) $1x10^4$live *Ld*Cen1$^{-/-}$ or (v) $1x10^4$live *Ld*p27$^{-/-}$parasites. All parasite cultures were washed three times with PBS before incubation with PBMCs. After 120 hours of incubation in a 5% CO_2 humidified atmosphere at 37°C, supernatants were collected and stored at -70°C until further analysis.

5.2.4 **Multiplex ELISA for cytokine estimation**

Cytokine levels in PBMC culture supernatants were determined using Human Cytokines, Bio-PlexPro™ (Bio-Rad) kit according to the manufacturer's protocol. Briefly, a 50µl cell supernatant sample was incubated with antibody coupled beads. Immune complexes were washed, incubated with biotinylated detection antibody and finally, with streptavidin-phycoerythrin prior to assessing cytokine concentration titers. Manufacturer provided standards were used to prepare the standard curve for

each cytokine. A total of 8 human cytokines representing either a pro-inflammatory (IL12, IL2, TNF-α, IFN-γ, IL6 and IL17) or an anti-inflammatory (IL4 and IL10) immune response were analyzed. Cytokine levels were determined using a multiplex array reader from Luminex™ Instrumentation System (Bio-Plex Workstation from Bio-Rad Laboratories). The cytokines concentration was calculated using software provided by the manufacturer (Bio-Plex Manager Software).

5.2.5 **Preparation of human peripheral blood macrophages and *in vitro* macrophage infections**

Infectivity of WT, *Ld*Cen1$^{-/-}$and *Ld*p27$^{-/-}$ parasites was investigated using human PBMCs derived macrophages. PBMCs were isolated from the healthy blood sample as described above and plated in 24 well (1.5X10^6/well) plate containing lysine coated cover slips and incubated for adherence at 37^0 C with 5% CO_2 for 4 hours in RPMI 1640 with 10% FCS. After removing the non-adherent cells by washing with PBS, the plate was further incubated for 8 days in complete RPMI medium supplemented with human MCSF (50ng/ml) for differentiation of macrophages. After every 48 hours cells were washed and fresh medium was added. On day-8 macrophages were infected with early stationary phase parasites at 1:10 macrophage to parasite ratio. After 6 hours of infection, cells were washed to remove non-internalized parasites. Cells adhered to cover slips were fixed in 100% methanol and stained with Diff Quik staining. Percentages of infected macrophages were determined by counting a minimum of 300 macrophages per sample under microscope (100X). Macrophage infectivity assay was performed in three technical and biological replicates for each parasite.

5.2.6 **Intracellular staining and flow cytometry**

To determine the cellular source/s and levels of cytokines, flow cytometry was performed with the blood PBMCs obtained from naïve (n=6) and HVL (n=6) individuals. PBMCs were stimulated with parasites as described above. Phorbolmyristate acetate (PMA) (50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) were added for 6hours in PBMCs culture and used as positive controls. To block cytokine secretion, cultures were treated with Golgi Stop (1ug/ml, BD Biosciences) and further incubated for an additional 4 hours. After incubation, cells were washed and surface stained with the following antibodies: CD3-PerCPcy5.5, CD4-PE-CF594 and CD8-APC-H7 for 20 minutes at $4^{o}C$. Surface stained cells were fixed and permeabilized using BD Cytoperm/cytofix kit (BD Biosciences) according to manufacturer's instructions, washed in permeabilization buffer (BD) and stained for the presence of intracellular IFN-γ, IL-17 and IL-10 using PE-Cy7, Alexa Fluor® 647 and PE conjugated antibodies (BD) respectively. Isotype matched control antibodies were used to detect non-specific binding to the cells. Following intra cellular staining, samples were acquired on FACS Aria and analyzed using FACS Diva software (BD Biosciences). 7AAD staining (BD Biosciences) of a limited number of samples confirmed that the gated lymphocytes were >95% viable for both healthy and HVL group stimulated with parasite. Lymphocytes were gated on the basis of forward and side scatter. From these lymphocyte population $CD3^{+}$ T-cells were gated to determine frequencies of $CD4^{+}$ and $CD8^{+}$ T-cells and for further analysis of IFN-γ, IL-10 and IL-17 expression.

5.2.7 **Statistical analysis**

Statistical analysis was performed using Graph Pad Prism 5 software (San Diego, USA). Statistical significance was determined by Mann-Whitney U test. Correlation was evaluated using Spearman's rank correlation test. P values of less

than 0.05 were considered significant.

5.3 **Results**

5.3.1 **Confirmation of pure cultures of *Leismania* parasites**

There are reports, which suggest the presence of *Leptomonas* in *Leishmania* parasite cultures (Srivastava *et al*., 2010; Selvapandiyan *et al*., 2015). Hence, before evaluation of infectivity and immunogenicity of live attenuated and wild type parasites, their cultures were checked for the potential *Leptomonas* contamination. Genomic DNA was isolated from all cultures (*Ld*1S, *Ld*Cen1$^{-/-}$and*Ld*p27$^{-/-}$) and PCR was carried out to amplify HSP 70 gene (Srivastava *et al.*, 2010). PCR amplified 1.4kb product of HSP-70 gene (**Figure 5.2A**) was further digested by using Hae III enzyme. RFLP analysis confirmed the banding pattern corresponding to *Leishmania* parasite (Selvapandiyan *et al*., 2015) for wild type as well as both live attenuated parasites (**Figure 5.2B**); indicated, that all *Leishmania* parasite cultures were pure and completely free of *Leptomonas* contamination.

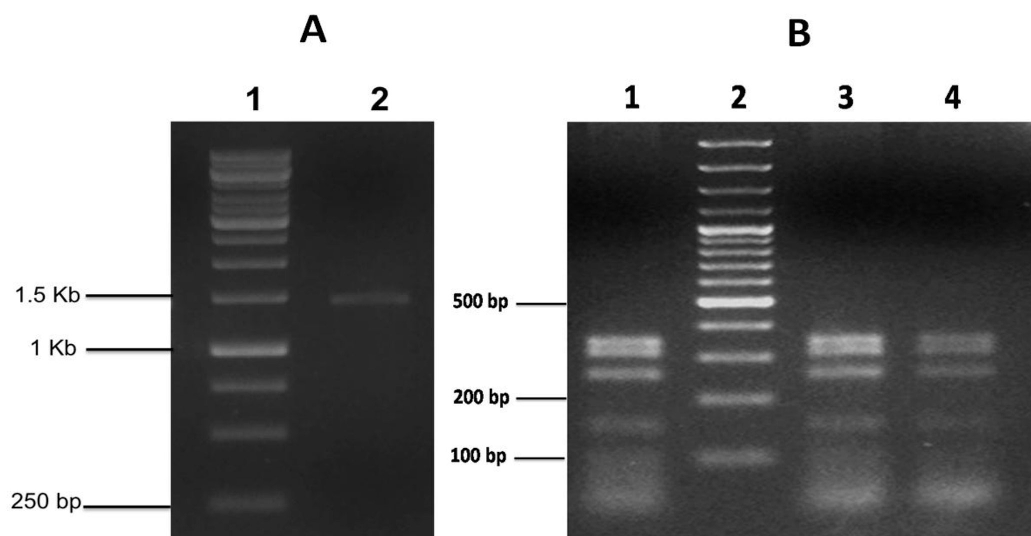


Figure 5.2: PCR-RFLP analysis of HSP-70 gene from different parasite cultures.

(A) Genomic DNA was isolated from *Ld*1S, *Ld*Cen1$^{-/-}$and *Ld*p27$^{-/-}$parasite cultures and PCR was done to amplify the HSP-70 gene. Lane showing: 1. 1kb ladder, 2. PCR amplified fragment of 1.4 kb size.(B) The PCR amplified products were digested with the restriction enzyme HaeIII and analyzed on 2% gel. Lane showing: 1. *Ld*1S, 2. 1kb marker, 3. *Ld*Cen1$^{-/-}$ and 4. *Ld*p27$^{-/-}$.

5.3.2 Determination of infectivity of *Ld*Cen1$^{-/-}$and *Ld*p27$^{-/-}$parasites in human PBMCs differentiated macrophages

Macrophages serve as host cell for survival and replication of *Leishmania* parasite and after infection they are recruited to the site of infection and their interaction with the parasite significantly influence disease outcome. Previously it has been shown that both *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ infect mice macrophages effectively (Bhattacharya *et al*., 2015; Selvapandiyan *et al*., 2009,Dey*et al*., 2010). In the present study, we have determined infectivity of the parasites and compared to the wild type infection, in human PBMCs differentiated macrophages. Macrophages were infected with early stationary phase promastigotes at 1:10 macrophage to parasite ratio. Following this protocol, > 70% of the population was infected after 6 hours of infection with either *Ld*Cen1$^{-/-}$ or *Ld*p27$^{-/-}$ and an average of 5 to 6 parasites were phagocytosed per cell (**Figure 5.3**). Further, the percentage of macrophages infected with the live attenuated parasites was similar to that of the wild type (**Figure 5.3)**.

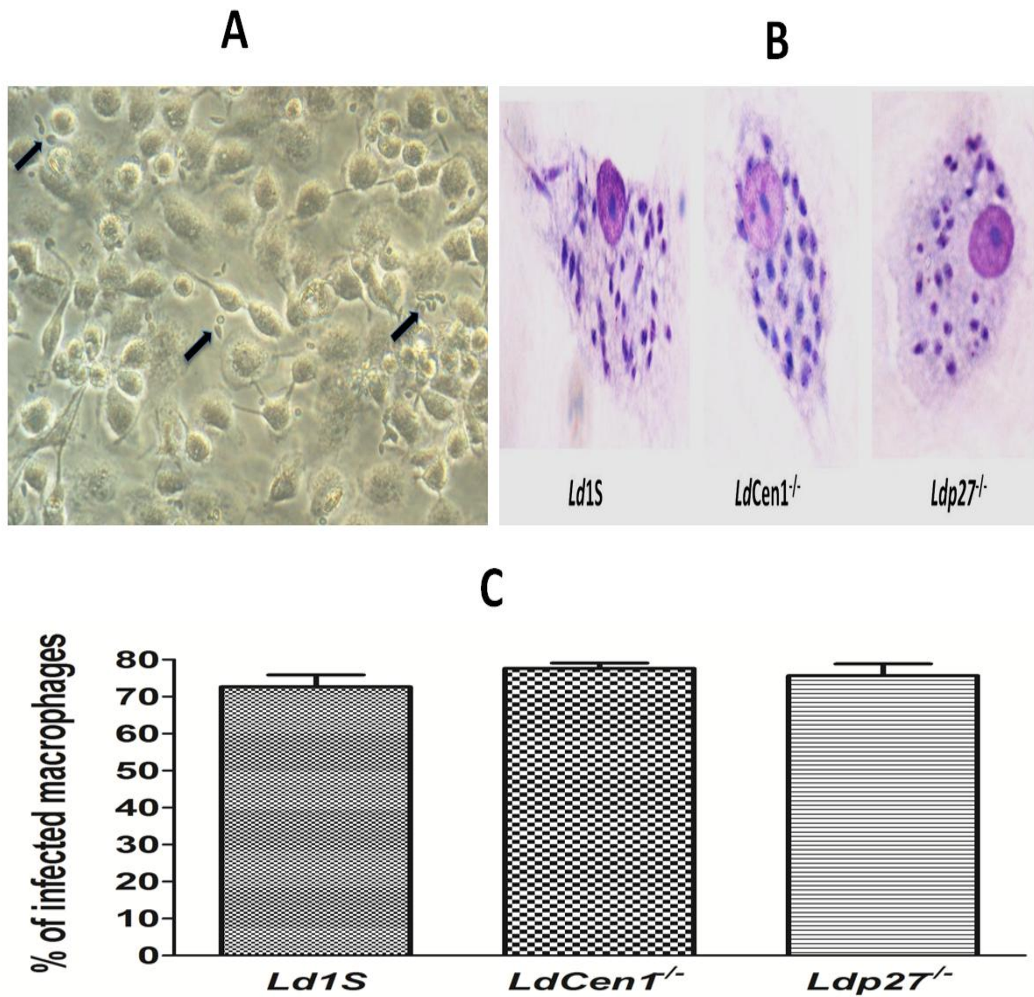
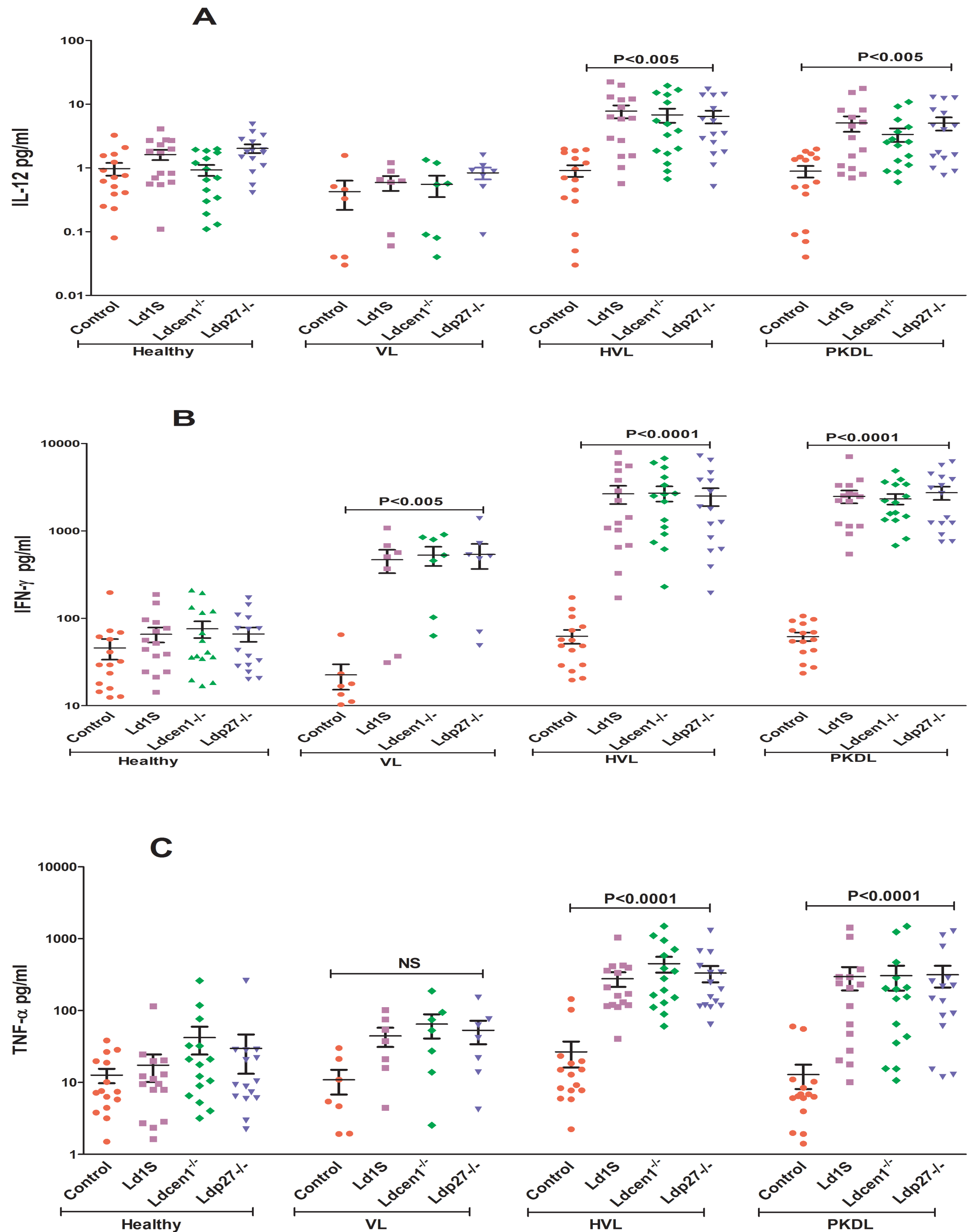


Figure 5.3: *In vitro* macrophage infectivity of live attenuated *Leishmania* parasites. Human PBMCs differentiated macrophages were infected with wild type (Ld1S), *Ld*Cen1$^{-/-}$or *Ld*p27$^{-/-}$parasites for 6 hours in a ratio of 10:1 (parasites: macrophage). (A) Human PBMCs differentiated macrophages and presence of unengulfed parasites (arrow sign) outside the macrophages. (B) Macrophages infected with *Ld*1S, *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$respectively after staining with Diff-Quik, (C) Percentages of infected macrophages determined by counting a minimum of 300 macrophages per sample under microscope (100X). Results are shown as mean ± SEM for three cover slips for each treatment and are pooled from three different experiments.

5.3.3 **Evaluation of pro-inflammatory cytokines response induced by *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ in PBMCs**

Cytokine response to *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$was evaluated in the culture supernatant of blood PBMCs of Naïve healthy (n=15), HVL (n=15), PKDL (n=15) and active VL (n=15) patients. Th1 response was evaluated by measuring level of cytokines IL-12, IFN-γ, TNF-α and IL-2, (**Figure 5.4).** It was observed that both *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ induced significantly higher levels of IL-12 in the PBMCs from the HVL and PKDL subjects when compared to the control uninfected cells ($P< 0.005$) **(Figure 5.4A).** No significant stimulation of IL-12 was observed in the VL group or healthy naïve subjects **(Figure 5.4A)**. Further, both *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$stimulated very high levels of IFN-γ in HVL and PKDL as compared to the control uninfected cells ($P<0.0001$) (**Figure 5.4B**). In this study significantly higher stimulation of IFN-γ was found inresponse to *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$in the VL group also ($P<0.005$) although level of stimulation was lower in comparison to HVL and PKDL (**Figure 5.4B**). Significantly higher stimulation of TNF-α was found only in HVL and PKDL ($P<0.0001$) group in comparison to the control uninfected cells (**Figure 5.4C**). Additionally, significantly higher stimulation of IL-2 in response to parasite exposure was observed in HVL, PKDL and healthy subjects; however, level of significance was higher in HVL and PKDL ($P<0.0001$) as compared to the healthy subjects ($P<0.005$) **(Figure 5.4D)**. IL-6 showed significantly higher level of stimulation ($P<0.001$) in HVL, PKDL and healthy groups with similar level of stimulation in all the three groups (**Figure 5.4E**). Significantly higher level of stimulation of IL-17 was seen only in HVL and PKDL ($P<0.005$) while no stimulation was seen in healthy and VL groups (**Figure 5.4F**). Moreover, stimulation of all cytokines measured in the study showed by both *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites was similar to that by the wild type

parasites with no significant difference among the attenuated parasite and virulent infections (**Figure 5.4**). As expected, PHA used as a positive control showed very high stimulation as compared to the control unstimulated cells for IFN- γ, TNF-α, IL-6 and IL-17 in PBMCs of all groups.



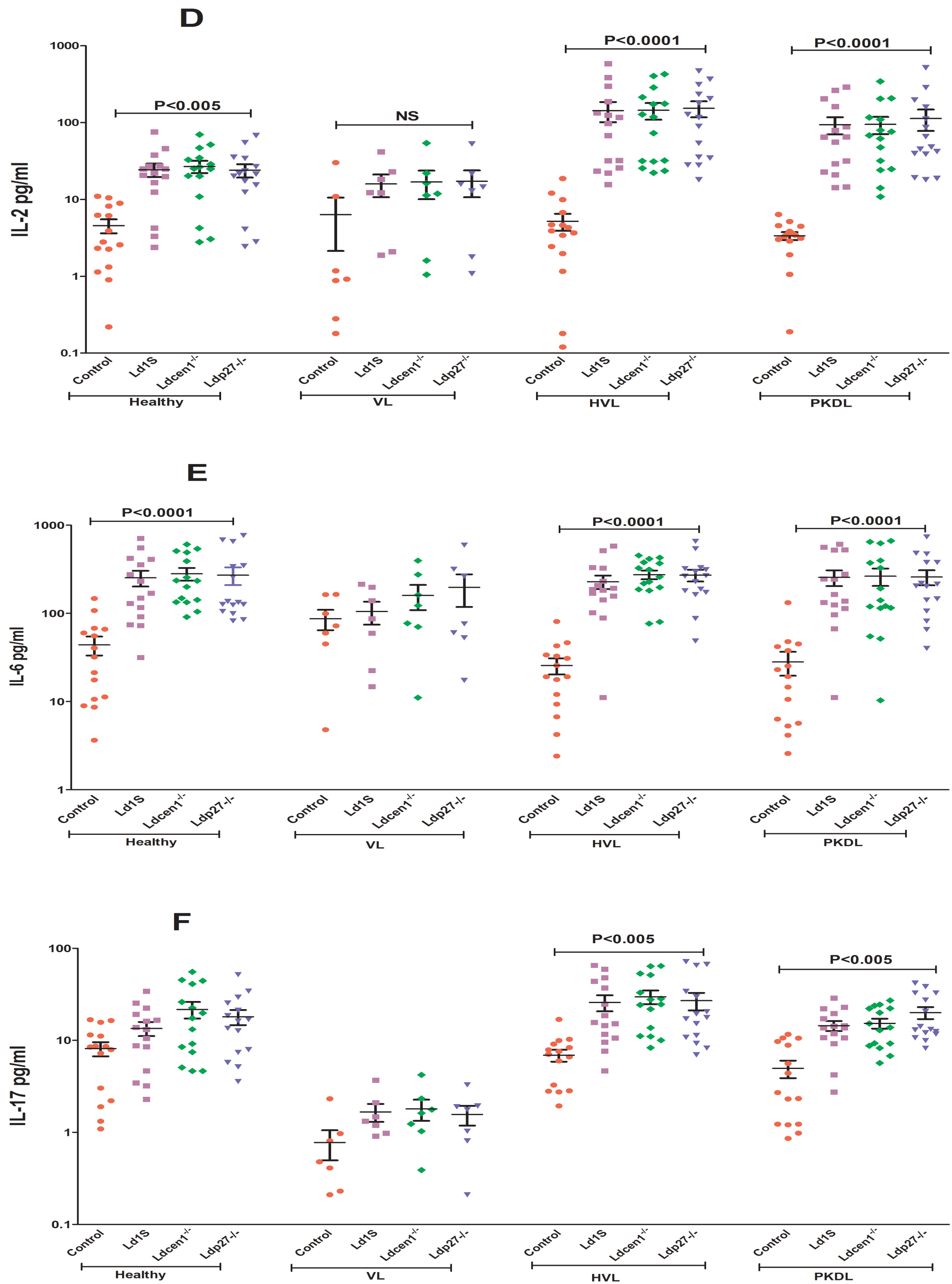
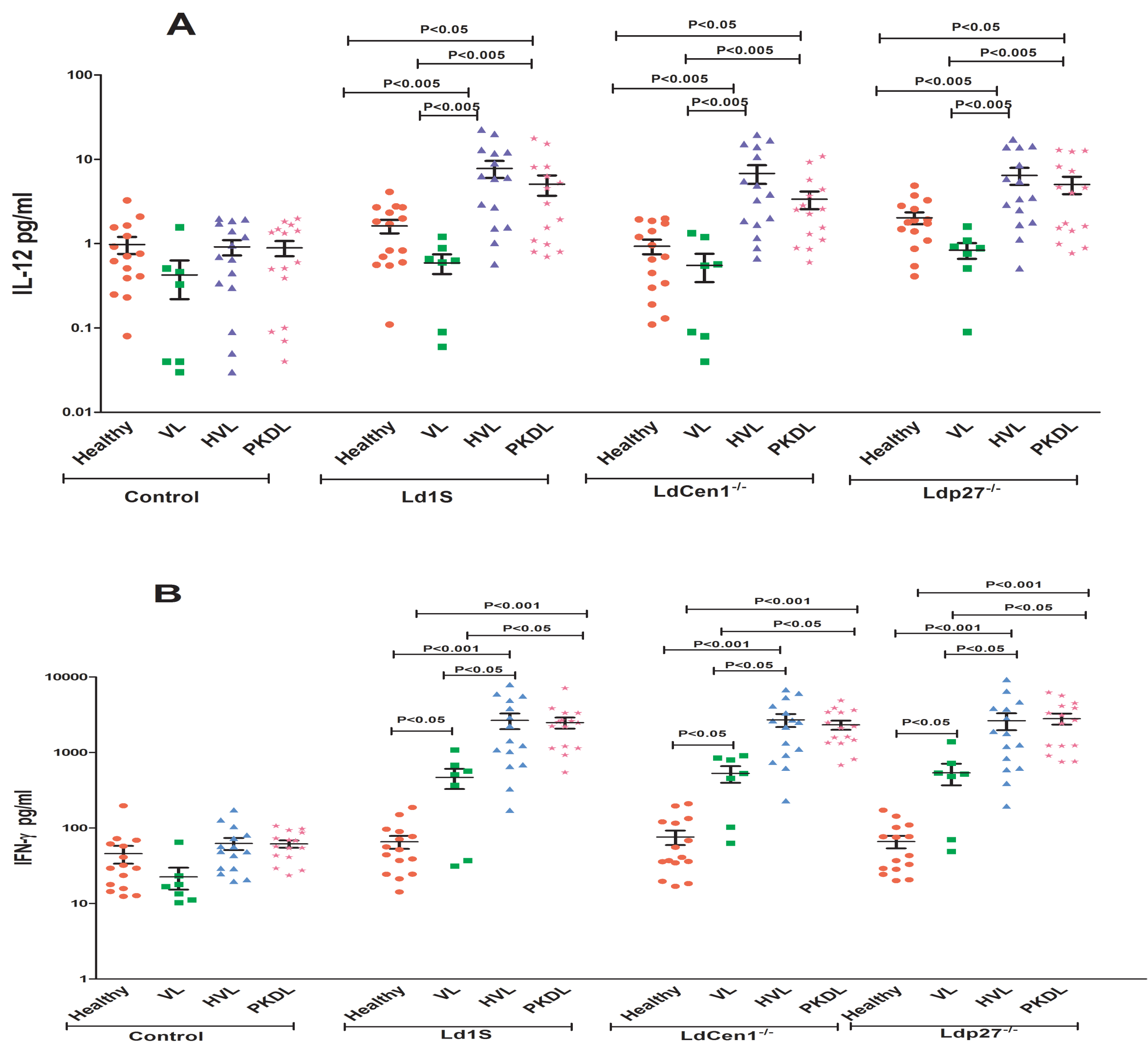
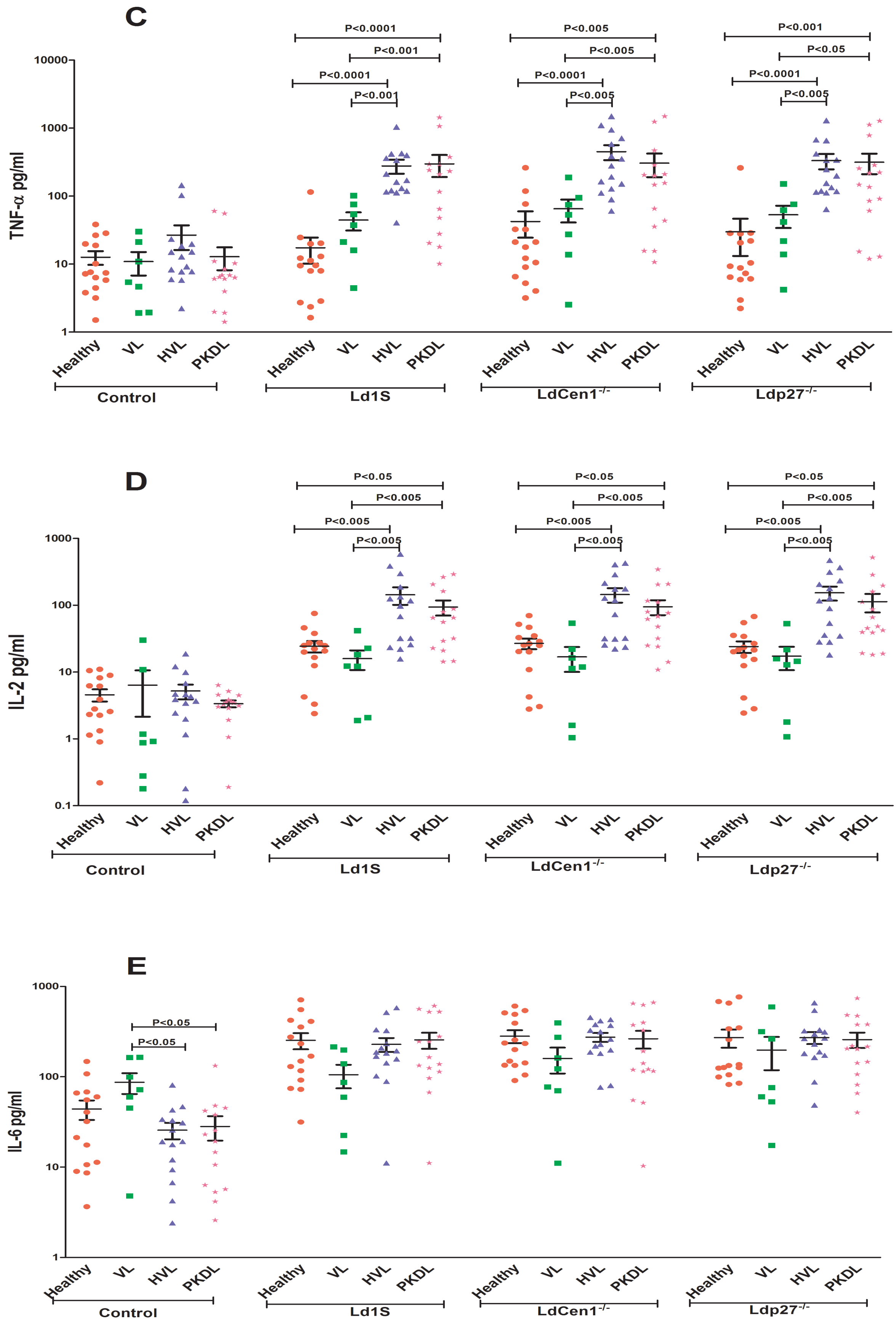


Figure 5.4: Level of pro-inflammatory cytokines stimulated by wild type (*Ld*1S), *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$parasites in culture supernatant of PBMCs obtained from

healthy, VL, HVL and PKDL patients. The results are expressed as scattering of individual values and data are given in Mean±SEM (pg/ml) of (A) IL-12, (B) IFN-γ, (C) TNF-α, (D) IL-2, (E) IL-6 and (F) IL-17. Significance was determined by Mann-Whitney U test. $P < 0.05$ is considered statistically significant.

Furthermore, the data was compared between different clinical groups and we observed that stimulation in response to *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ and wild type parasites in HVL and PKDL groups was significantly higher for IL-12, IL-17, IFN- γ and TNF-α as compared to the healthy group; however, no difference was found in the level of significance for IL-2 and IL-6 (**Figure 5.5**).




C
TNF-α pg/ml
P<0.0001
P<0.001
P<0.0001
P<0.001
P<0.005
P<0.005
P<0.0001
P<0.005
P<0.001
P<0.05
P<0.0001
P<0.005
Healthy
VL
HVL
PKDL
Control
Ld1S
LdCen1-/-
Ldp27-/-
D
IL-2 pg/ml
P<0.05
P<0.005
P<0.005
P<0.005
E
IL-6 pg/ml
P<0.05
P<0.05

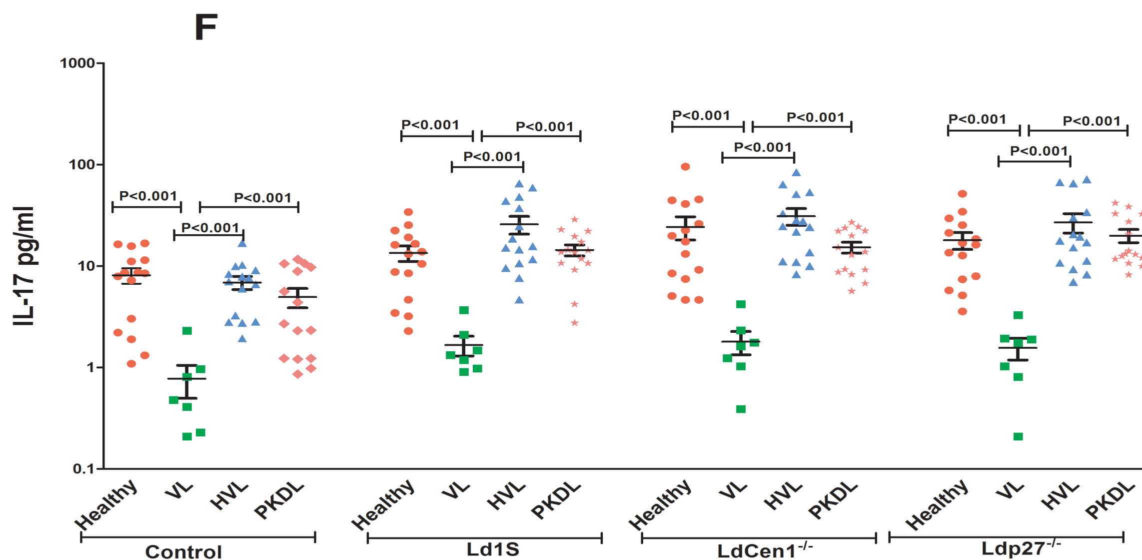


Figure 5.5: Level of pro-inflammatory cytokines stimulated in culture supernatant of PBMCs obtained from healthy, HVL, VL and PKDL groups by wild type (*Ld*1S), *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$parasites. The results are expressed as scattering of individual values and data are given in Mean±SEM (pg/ml) of (A) IL-12, (B) IFN-γ, (C) TNF- α, (D) IL-2, (E) IL-6 and (F) IL-17. Significance was determined by Mann-Whitney U test. $P < 0.05$ is considered statistically significant.

As compared to the VL group stimulation of all cytokines was significantly higher for HVL and PKDL groups. No significant difference in stimulation of cytokines was found between healthy and VL group except for IFN- γ (**Figure 5.5**).

5.3.4 **Correlation between Th1 cytokines**

We determined the correlation between different Th1 cytokines upon stimulation with *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ and wild type parasites. PBMCs of HVL group showed positive pair wise correlation between the two critical Th1 cytokines i.e. IL-12 and IFN-γ after exposure to *Ld*1S (r=0.73, P<0.005), *Ld*Cen1$^{-/-}$ (r= 0.69, P<0.005) and

*Ld*p27$^{-/-}$ (r=0.67, P<0.005) (**Figure 5.6**). However, no significant correlation was found among other cytokines.

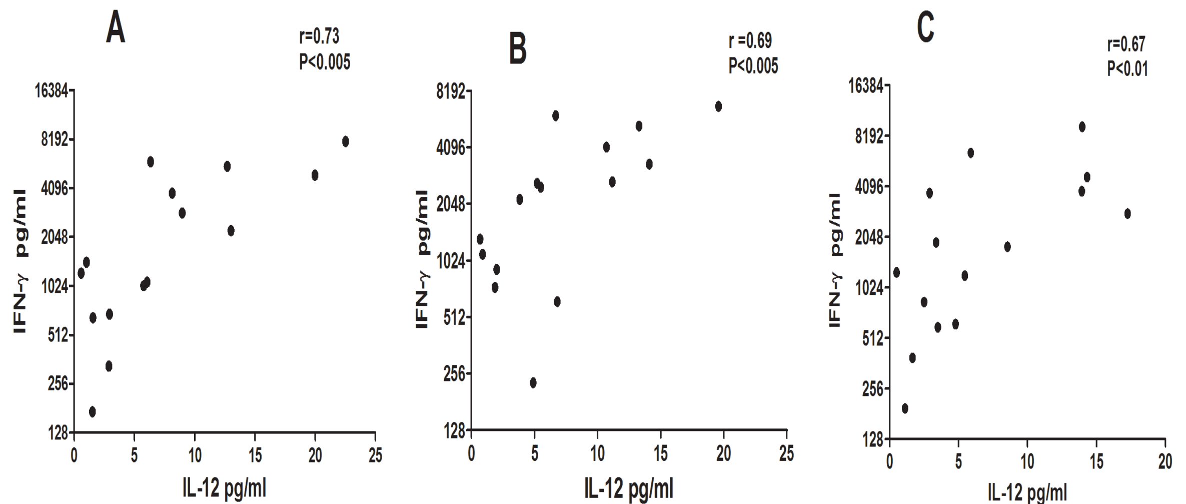


Figure 5.6: Pairwise correlation of IL-12 and IFN-γ cytokines stimulated in response to parasites in culture supernatant of PBMCs obtained from HVL. (A) wild type (*Ld*1S), (B) *Ld*Cen1$^{-/-}$ and (C) *Ld*p27$^{-/-}$. Significance was determined by Spearman's rank correlation test. P < 0.05 is considered statistically significant.

5.3.5 Evaluation of anti- inflammatory cytokines response induced by *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ in PBMCs

Anti-inflammatory response was evaluated by analyzing stimulation of IL-4 and IL-10, both being immunosuppressive cytokines that promote VL pathogenesis. There was no induction in levels of IL-4 or IL-10 in response to *Ld*Cen1$^{-/-}$ or *Ld*p27$^{-/-}$ in any of the groups examined in comparison with the control uninfected cells (P>0.05), similar to the response to wild type parasite **(Figure 5.7).**

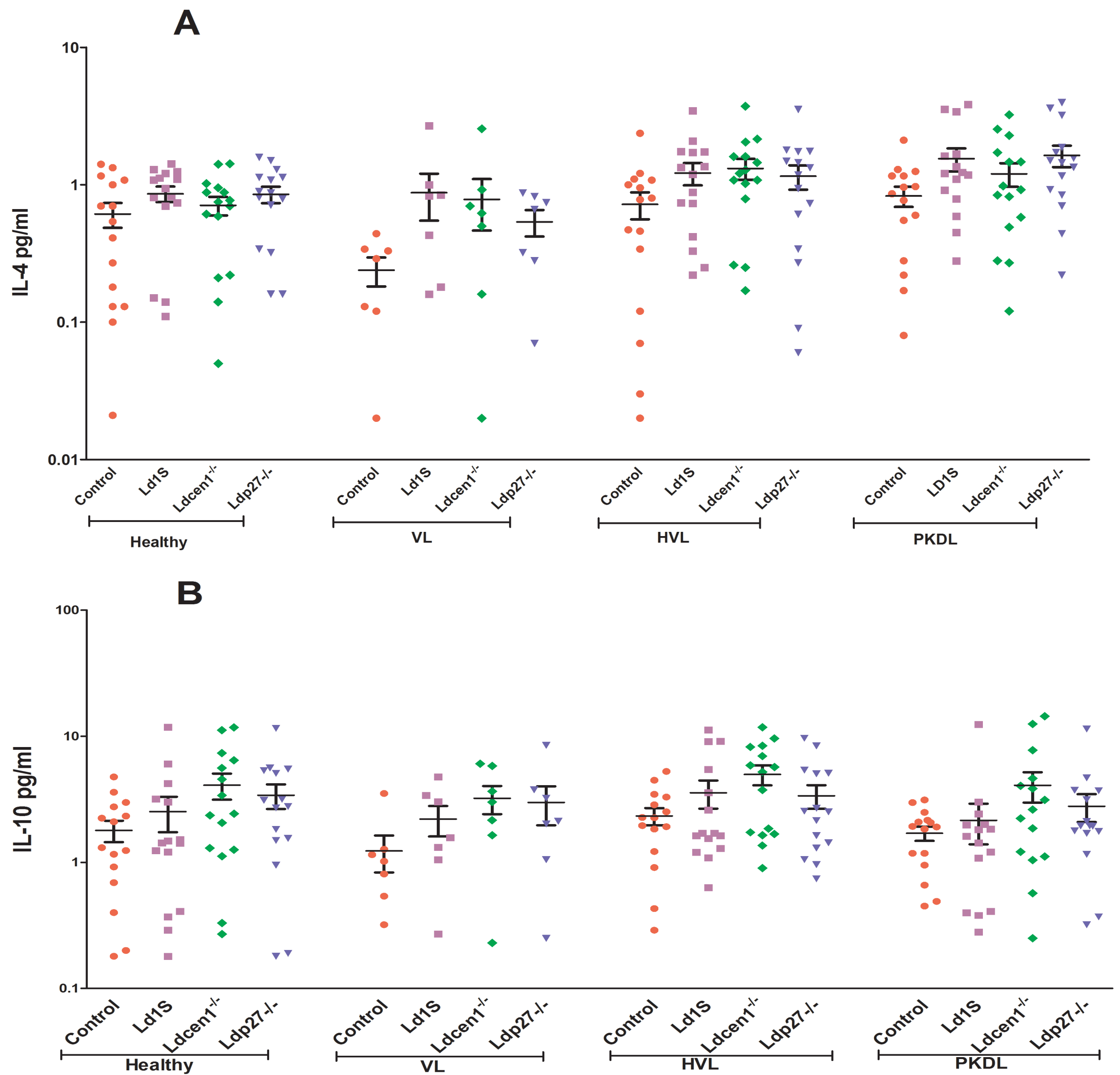


Figure 5.7: Level of anti-inflammatory cytokines stimulated by wild type (*Ld*1S), *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites in culture supernatant of PBMCs obtained from Healthy, HVL, VL and PKDL patients. The results are expressed as scattering of individual values and data are given in Mean±SEM (pg/ml) of(A) IL-4 and (B) IL-10. Significance was determined by Mann-Whitney U test. P < 0.05 is considered statistically significant.

Positive control with PHA showed significantly higher stimulation only for IL-

10 and this stimulation was found in all the groups including healthy and VL. Further, no difference was found in stimulation of IL4 and IL-10 among healthy, VL, HVL and PKDL groups (**Figure 5.8).**

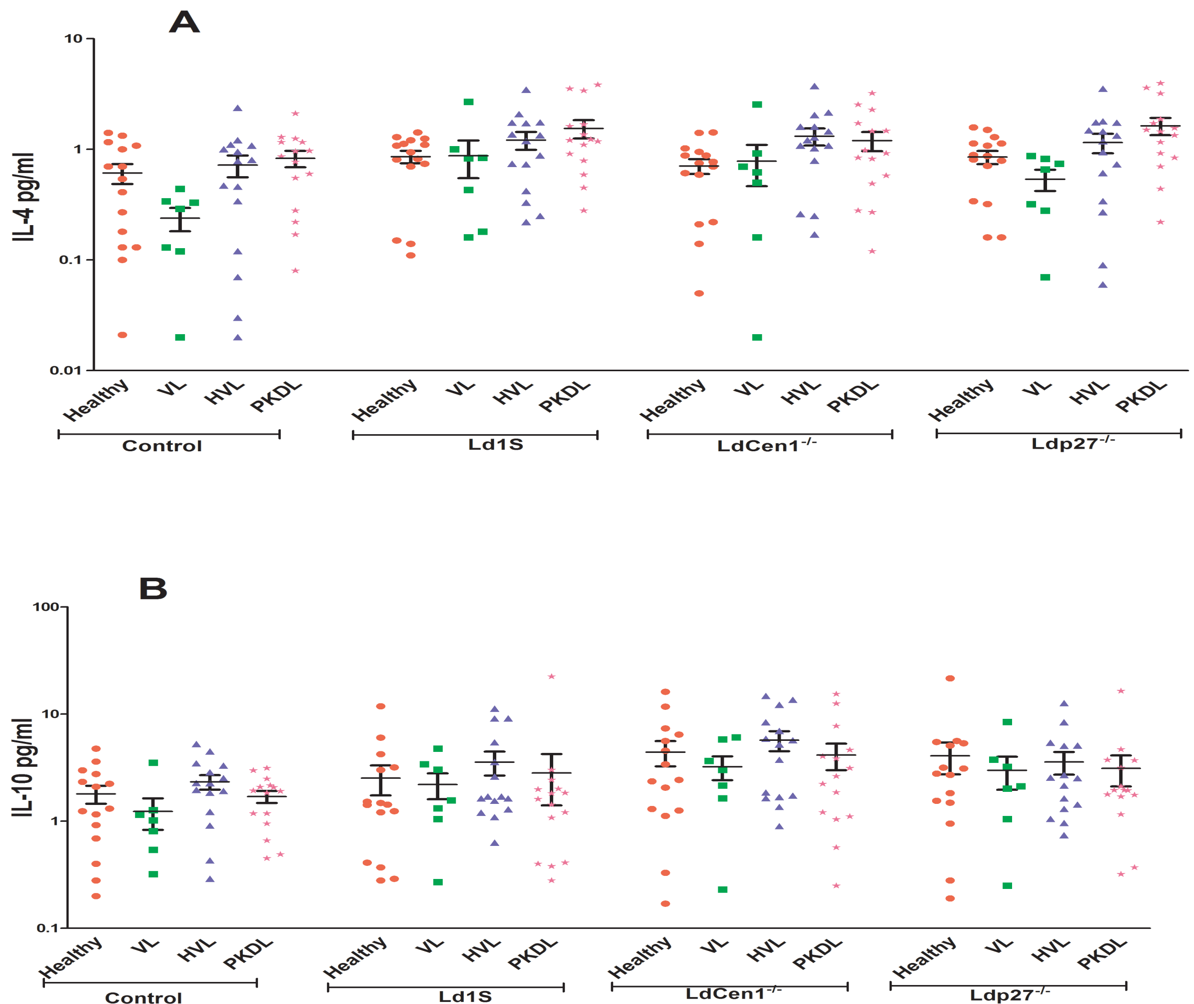


Figure 5.8: Level of anti-inflammatory cytokines stimulated in culture supernatant of PBMCs obtained from healthy, HVL, VL and PKDL groups by wild type (*Ld*1S), *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites. The results are expressed as scattering of individual values and data are given in Mean±SEM (pg/ml) of (A) IL-4 and (B) IL-10. Significance was determined by Mann-Whitney U test. P < 0.05 is considered statistically significant.

5.3.6 **Flow cytometry analysis for the determination of cellular source of IFN-γ, IL-10 and IL-17 cytokines upon stimulation with parasites**

We analyzed the phenotype of cytokine producing cells in response to parasite exposure in healthy and HVL PBMCs **(Figure 5.9 and 5.10)**. In HVL group, we observed that percentage of IFN-γ producing $CD4^+$ and $CD8^+$T cells was significantly increased (P<0.05) after stimulation with the live attenuated parasites as compared to the control unstimulated cells and the increase in $CD8^+$ T cells was more significant (P<0.01) as compared to the $CD4^+$T cells (P<0.05). No significant increase was found in the percentage of IFN-γ producing $CD4^+$ or $CD8^+$ T cells in the healthy group **(Figure 5.10A,B)**. We also found significant increase in the percentage of IL-17 producing $CD4^+$T cells after live parasite stimulation in cultures from HVL individuals, in comparison to non-stimulated culture (P<0.05) **(Figure 5.10 C)**.

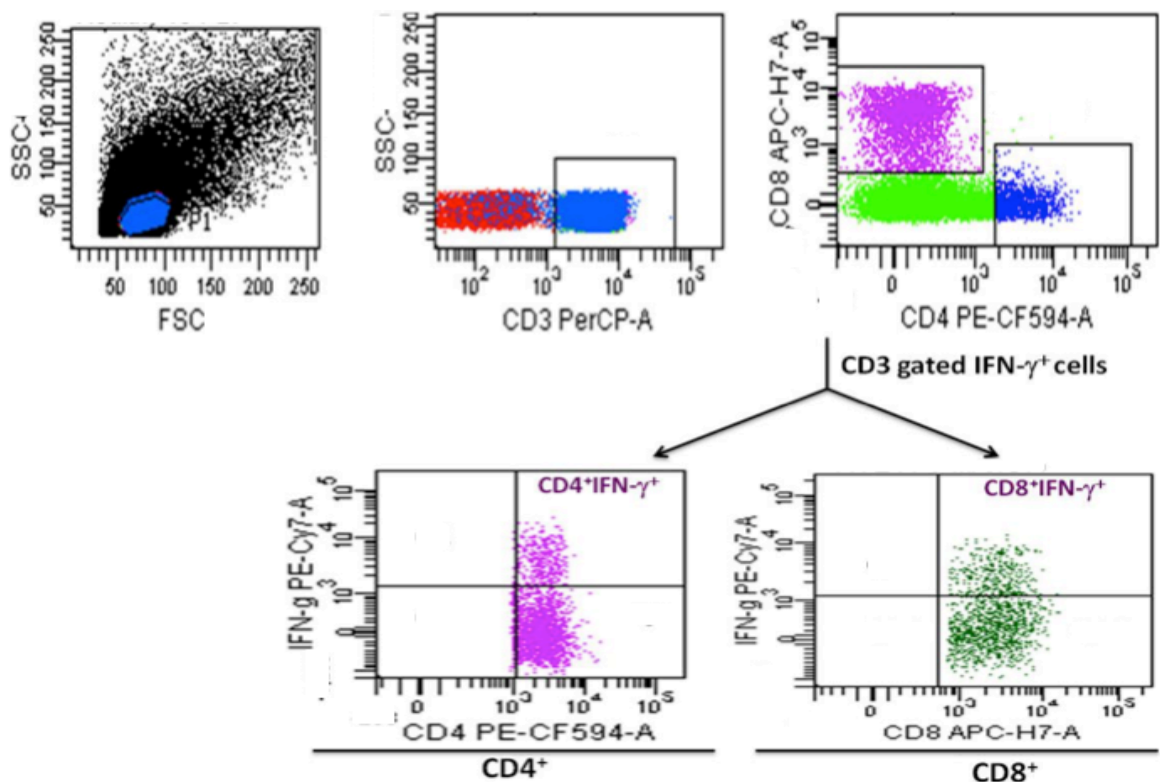


Figure 5.9: Flow cytometry analysis showing the gating strategy used to identify the phenotype of cytokine (ex:- here IFN-γ) producing cells.

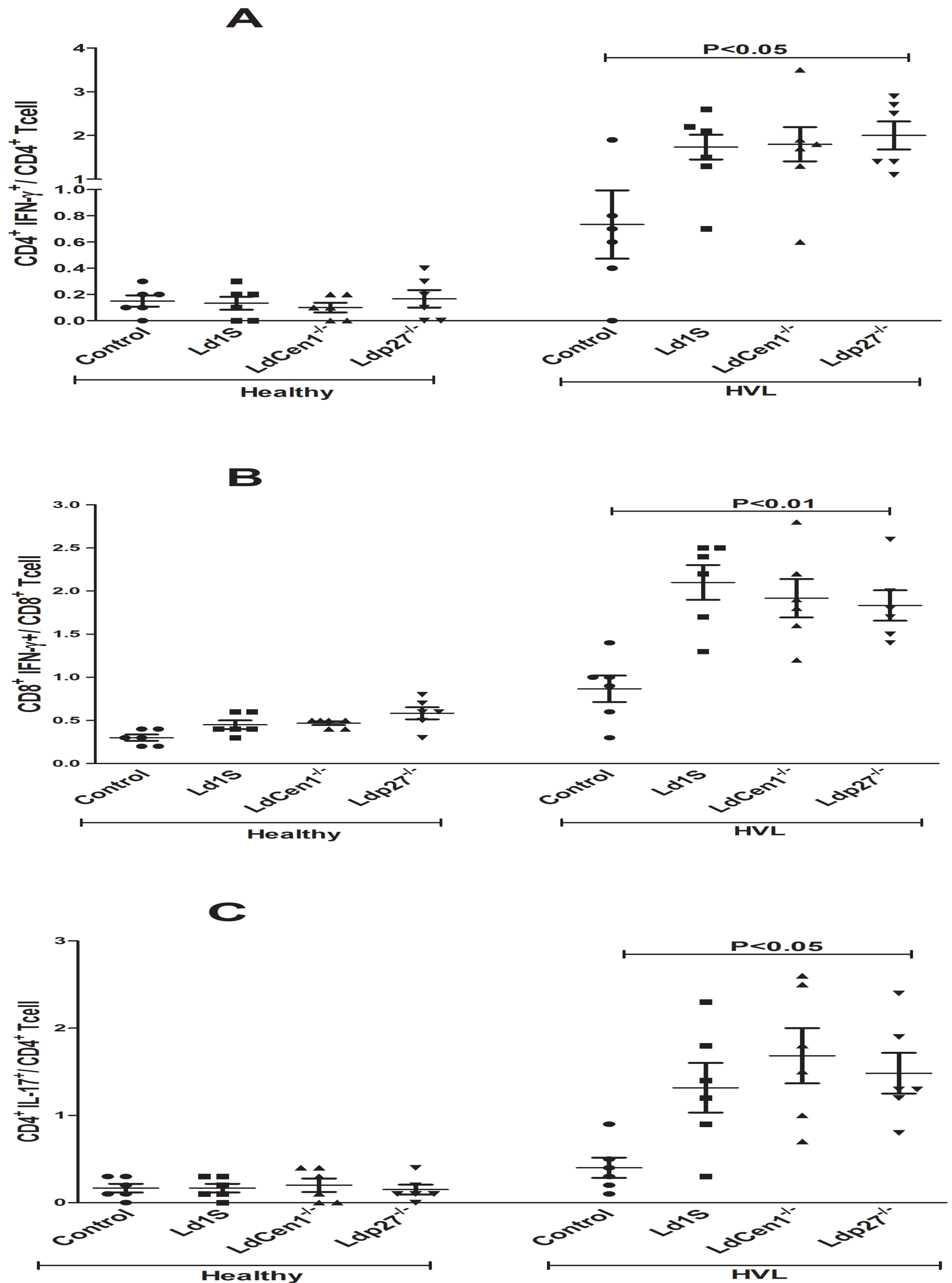


Figure 5.10: Percentage of IFN-γ and IL-17 -producing cells after stimulation of blood PBMCs obtained from healthy and HVL with wild type (*Ld*1S), *Ld*Cen1$^{-/-}$

and *Ld*p27$^{-/-}$ parasites. The results are expressed as scattering of individual values and data are given in Mean±SEM (pg/ml) of (A) % CD4^{+}IFN-γ^{+}/CD4^{+}cells, (B) % CD8^{+} IFN-γ^{+}/CD8^{+} cells and (C) % CD4^{+}IL-17^{+}/CD4^{+} cells.

Further, we evaluated the effect of parasite exposure on the percentage of IL-10 producing CD4^{+} and CD8^{+}T cells, and found no significant increase in the percentage of IL-10 producing CD4^{+} or CD8^{+}T cells in either healthy or HVL group (P>0.05) **(Figure 5.11)**.

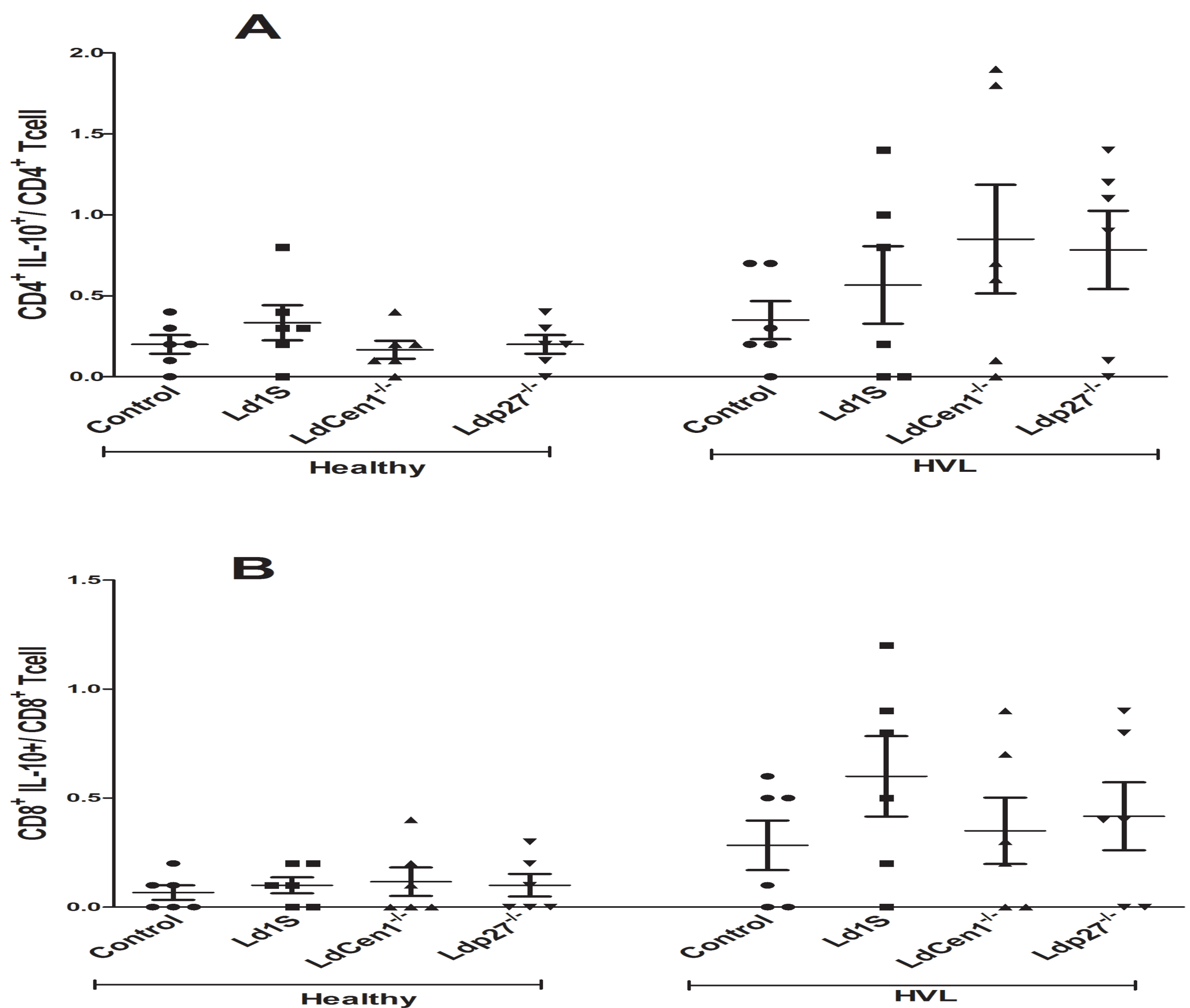


Figure 5.11: Percentage of IL-10 producing cells after stimulation of blood PBMCs obtained from healthy and HVL with wild type (*Ld*1S), *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites. The results are expressed as scattering of individual values and

data are given in Mean±SEM (pg/ml) of (A) % $CD4^+IL\text{-}10^+/CD4^+$ cells and (B) % $CD8^+IL\text{-}10^+/CD8^+$ cell.

5.3.7 **Ratio of IFN-γ/IL-10 producing cells**

An increased ratio of IFN-γ/IL-10 has been correlated with the parasite clearance in VL (Selvapandiyan.,*et al* 2009). Hence, we determined IFN-γ/IL-10 ratio for both $CD4^+$ and $CD8^+$ T-cells. Parasite exposed PBMCs of HVL group showed an elevated ratio of IFN-γ/IL-10 producing $CD4^+$ and $CD8^+$ T-cells **(Figure 5.12)**, suggesting that exposure of PBMCs to $LdCen1^{-/-}$ and $Ldp27^{-/-}$live attenuated parasites induced a host- protective cell-mediated pro-inflammatory cytokines producing $CD4^+$ and $CD8^+$ T cells.

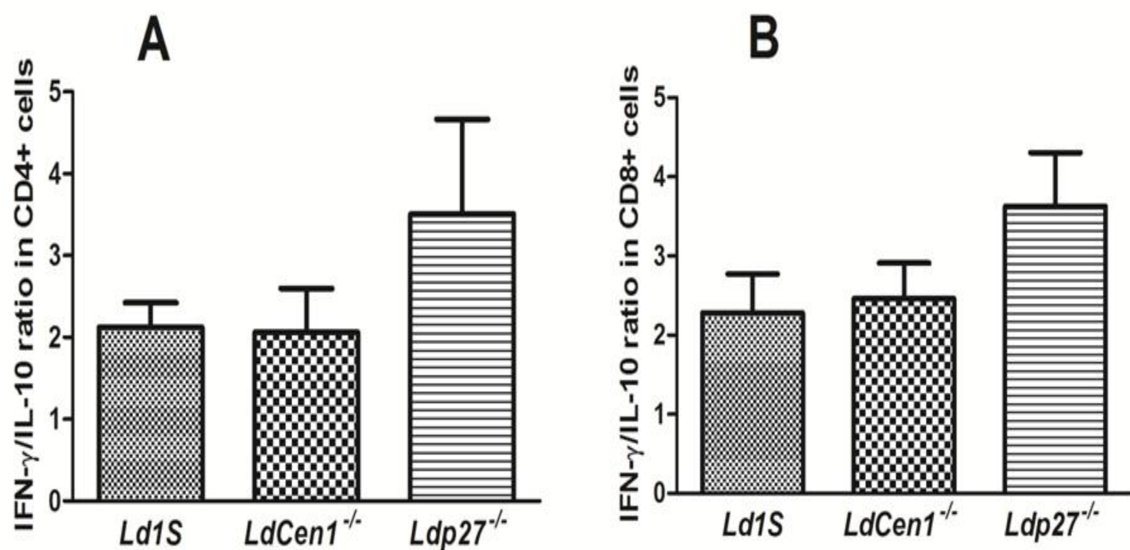


Figure 5.12. Ratio of IFN-γ/IL-10 producing cells after stimulation of blood PBMCs obtained from HVL with wild type (*Ld*1S), *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites. The results are expressed as bar graph and data are given in Mean±SEM (pg/ml) of(A) Ratio IFN-γ/IL-10 producing $CD4^+$ cells and (B) Ratio IFN-γ/IL-10producing $CD8^+$ cells.

5.4 **Discussion**

VL is one of the world's most neglected parasitic diseases second only to malaria (van Griensven *et al*., 2012), however there is no effective vaccine available for it. Previously, it has been shown that genetically modified live attenuated *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$parasites have great potential as live attenuated vaccines against VL (Selvapandiyan *et al*., 2009;Dey *et al*., 2010; Dey *et al*., 2013; Fiuza *et al*., 2013; Fiuza *et al*., 2015), mainly based on the data obtained from animal studies. It is important to understand the immune response of human host against the attenuated parasites for the development of an effective vaccine.In this study, we report the immune response generated by *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$in human PBMCs obtained from different clinical groups in comparison with the immune response generated by *ex vivo* infection with the wild type parasite.

Recent reports have shown the presence of *Leptomonas* in *Leishmania* parasite cultures. Their presence has raised some serious concern as it difficult to distinguish them from *Leishmania* because of morphological similarity further, they can also co-infect human and mice like *Leishmania* parasite (Srivastava*et al*., 2010; Selvapandiayan *et al*., 2015).Presence of *Leptomonas* in *Leishmania* culture can mislead the main interpretation of any study, hence; first we confirmed the absence of *Leptomonas* parasites in the cultures of *Ld*1S, *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ by PCR-RFLP. Molecular characterization of all parasite cultures confirmed that they all are completely free of *Leptomonas* contamination.

Having established that all *Leishmania* cultures are pure and completely free of *Leptomonas* contamination, we started our main experiment by evaluating infectivity and immunogenicity of wild type and live attenuated parasites. We found that

macrophage infectivity of both *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$was comparable to that of the wild type parasite, indicating that the attenuation did not limit the ability to infect human macrophages, consistent with the findings in animal models (Selvapandiyan *et al*., 2009; Bhattacharya *et al*.,2015; Dey *et al*., 2010). Infection with *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$parasites result in classical activation of murine macrophages (M1 phenotype) as reflected by increased production of pro-inflammatory cytokines, chemokines, reactive oxygen species, nitric oxide and reduced production of anti-inflammatory cytokines and arginase activity (Bhattacharya *et al*., 2015). In the present study, the observed increase in pro-inflammatory cytokines production by *Ld*cen1$^{-/-}$ and *Ld*p27$^{-/-}$ in PBMCs culture from pre-exposed group is consistent with that observed in classically activated murine macrophages.

Studies have shown that the leishmaniasis outcome is mainly determined by Th1/Th2 balance and Th1 response is known to provide protection by an induction of pro-inflammatory response leading to macrophage activation and elimination of intracellular parasite, whereas a Th2 response increases susceptibility to the disease(Kumar *et al*., 2010; Locksley *et al*., 1987; Scott *et al*., 1988). Live attenuated parasites that can predominantly elicit a Th1 response in individuals previously exposed to *Leishmania* infection would be good candidate for prophylactic and/or therapeutic vaccine, as was shown in similar studies with other vaccine formulations including recombinant antigen vaccines (Kumar *et al*., 2010). We evaluated pro-inflammatory response by measuring IL-12, IL-2, TNF-a, IFN-g, IL-6 and IL-17 cytokines and found that all were significantly elevated in HVL and PKDL groups, suggesting that the live attenuated parasite strains induced a Th1 biased immunity.

IL-12 is a critical cytokine that helps in differentiation of naive CD4^{+}T cells

into Th1 cells and plays a major immune-regulatory role in the development of cell-mediated immunity (CMI) during intracellular bacterial or parasitic infections by activating macrophages to produce IFN- γ and TNF-α (Trinchieri*et al*., 1994; Watford *et al*., 2003). Its critical importance in mediating a Th1 response and resistance to leishmaniasis was shown by IL-12 depletion experiments, which leads to susceptibility in naturally resistant mice (Mattner*et al*., 1996) and treatment with IL-12 resulted in conversion of susceptible BALB/c mice to a resistant type (Heinzel *et al*., 1993). It has been found that the PBMCs of *Ld*Cen1$^{-/-}$ immunized dogs (Fiuza*et al*., 2013) and *Ld*p27$^{-/-}$immunized mice (Dey*et al*., 2013) produced elevated level of IL-12 in response to *Leishmania* antigen. Here we found that both *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ stimulated significantly higher level of IL-12 in PBMCs from HVL and PKDL cases that are already exposed to *Leishmania* parasite and mimic a naturally protected individual. IL-12 is secreted by antigen presenting cells upon activation and dendritic cells serve as primary producer in initial phase of *Leishmania* infection (Gorak *et al*., 1998). The observed increase in production of IL-12 after infection with *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ indicates that the *de novo* antigen presenting function of APC may be fully functional in HVL and PKDL and that unlike virulent parasites, *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites do not induce immunesuppressive activities. In contrast, IL-12 production in active VL cases was low suggesting a poor function of dendritic cells in that group. Studies in mice have shown that the infection of macrophages with *Ld*Cen1$^{-/-}$ or *Ld*p27$^{-/-}$ does not alter the membrane architecture, hence does not affect the antigen presentation ability (Bhattacharya *et al*., 2015). However, wild type *Leishmania* parasite depletes membrane cholesterol of macrophages, resulting in defective antigen presentation to T cells (Subha*et al*., 2009). The robust production of IL-12 in our *ex vivo* studies with human PBMCs indicates that the attenuated parasites showed similar

macrophage/dendritic cells activation as observed in murine studies. IFN-γ is one of the most potent Th1 cytokine required for the control of *Leishmania* infection (Green *et al*., 1990) and can restrain pathogenic Th2 cell expansion. In previous studies, it has been shown that both *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$can induce IFN-γ production in animal models (Selvapandiyan *et al*., 2009; Bhattacharya *et al*., 2015; Dey *et al*., 2010). In the present stud, we observed significantly higher stimulation of IFN-γ in HVL and PKDL group as compared to the controluninfected cells and healthy group. Leishmanial antigens have previously been shown to induce stimulation of IFN-γ in PKDL and healed cases of VL, asymptomatic VL, cutaneous and mucocutaneous leishmaniasis (Kaushal *et al*., 2014; Kaushal *et al*., 2016; Burns *et al*., 1991; Russo *et al*., 1991; Dos Santos*et al*., 2016). In the present study, significantly higher stimulation of IFN-γ was also found in the VL group as compared to the healthy group. PBMCs from VL cases do not generally produce IFN-γ in response to *Leishmania* antigen (Kaushal *et al*., 2014; Saha S *et al*., 2009; Nylén *et al*., 2007) however, some studies have shown that human whole blood/PBMCs can indeed produce IFN-γ in active VL(Singh *et al*., 2012; Ansari *et al*., 2011; Gautam *et al*., 2014; Gidwani *et al*., 2011). It is important to note that the level of stimulation of IFN- γ observed in the active VL samples were much lower than in HVL and PKDL samples. It is also pertinent to highlight that unlike most previous studies where antigenic re-stimulation was done using soluble *Leishmania* antigen, our study used live parasites (virulent and attenuated) for this purpose suggesting that immunomodulatory activities of the live parasites could be responsible for the observed levels of IFN-γ.

Evaluation of defined *Leishmania* antigen vaccine candidates revealed that only those antigens, which were the most immunogenic and protective in murine

model, induced IFN-γ production in HVL cases, indicating that *Leishmania* antigens that are protective in experimental models, do not necessarily induce immune response in HVL (Kumar *et al*., 2010). Recognition of a single antigen by T cells from individuals with different immunologic and genetic background cannot be always expected, suggesting that the appropriately modified whole parasites would make a better vaccine (Kumar *et al*., 2010). IL-12 is a potent inducer of IFN- γ (Tripathi*et al*., 2006) and our findings also point towards apositive pair wise correlation between IL-12 and IFN-γ**.** Therefore, it is likely that the increased production of IL-12 observed here also induced production of IFN- γ, which further would activate macrophages for generation of ROS and NO for subsequent killing of intracellular *Leishmania* parasite after a virulent infection (Bhattacharya *et al*., 2015).We analyzed the capacity of *Ld*Cen1$^{-/-}$ and*Ld*p27$^{-/-}$ to stimulate TNF-a and found that similar to IL-12, significantly higher stimulation of TNF-a was seen only in the HVL and PKDL groups. The observed increase in production of Th1 cytokines (IFN-γ, TNF-α and IL-12) in *Leishmania* exposed group (HVL or PKDL) compared to naive or active VL individual in response to live attenuated or wild type *Leishmania* parasites, is in accordance with earlier studies carried out with *Leishmania* antigen in HVL and PKDL groups(Kaushal *et al*., 2014; Kaushal *et al*., 2016; Singh*et al*., 2012; Tripathi*et al*., 2006). Further, this increase in Th1 cytokine production in pre-exposed group suggested that the live attenuated parasites can induce protective effector response from memory response generated during resolution of infection in HVL individuals.

IL-6 is considered as a pro-inflammatory cytokine, however it also displays some anti-inflammatory properties by negatively regulating Th1 differentiation (Diehl *et al*., 2000). Previously it has been shown that the *Leishmania* antigen can induce IL-

6 production in blood PBMCs of healthy individuals also (Cenini*et al.*, 1993) similarly, in the present study we observed significantly higher stimulation of IL-6 in response to parasite exposure in healthy group along with the HVL and PKDL.

Th-17 cells play complementary role along with Th1 to provide protection against VL by producing IL-17 and IL-22 cytokines (Pitta *et al.*, 2009). IL-17 is a pro-inflammatory cytokine, although little is known (Pitta *et al.*, 2009) about its role during VL infection. Studies have shown that it provides protection against VL by activating innate immunity and inducing expression of innate inflammatory mediators, including IL-6, GM-CSF, IL-1, IL-8, TNF-α and inducible nitric oxide synthase (iNOS)(Kolls *et al.*, 2004). Further, it acts synergistically with IFN-γ to strengthen Th1 response and also prevents Treg and IL-10+ cell expansion, which helps in controlling parasite replication (Nascimento *et al.*, 2015). In our study, both live attenuated parasites significantly stimulated IL-17 from blood PBMCs of HVL and PKDL groups, which will further promote the leishmanicidal activity of macrophages and improve Th1 response. Anti-inflammatory response induced by *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$in PBMCs was evaluated by measuring IL-4 and IL-10. Both IL-4 and IL-10 increase susceptibility for *Leishmania* infection by inducing a Th2 response and are important for the prediction of vaccine success (Singh *et al.*, 2012). We observed no significant difference in stimulation of IL-4 and IL-10 between parasite exposed and control-uninfected cells in any study group. It has been previously shown that, production of IL-4 and IL-10 was not induced in animal models vaccinated with these live attenuated parasites after virulent challenge (Selvapandiyan *et al.*, 2009; Dey *et al.*, 2013; Fiuza *et al.*, 2015). Previous studies have also shownthat PBMCs from cured VL and naïve individual failed to stimulate IL-10 in response to *Leishmania* antigen

vaccine candidates (Himanshu *et al*., 2014; Singh *et al*., 2012). Importantly, lack of significant stimulation of IL-4 and IL-10 upon infection with *Ld*Cen1$^{-/-}$, *Ld*p27$^{-/-}$, and wild type infection suggests that the two attenuated parasites do not induce a disease promoting immune response significantly different than that of the wild type infection indicating the safety of these attenuated parasites.

In order to assess the intracellular cytokines producing cells after exposure to the parasites, we analyzed the production of IFN-γ, IL-17 and IL- 10 by CD4$^+$ and CD8$^+$ T cells. CD4$^+$ cells with the Th1 type cytokine profile such as IFN-γ provide protection against leishmaniasis and it is well established that CD8$^+$ T cells play a potential role in the cure of leishmaniasis, particularly VL by exerting its cytotoxic effect (Gautam *et al*., 2014; Himanshu *et al*., 2014). We found that PBMCs infected with live attenuated and wild type parasite displayed higher frequency of CD4$^+$ T cells expressing IL-17 and both CD4$^+$as well as CD8$^+$ T cells expressing IFN-γ in HVL, while no significant increase was seen in IL-10 secreting cells. This suggests that similar to a naturally occurring exposure to virulent parasites of the HVL individuals following cure in *Leishmania* endemic areas, *ex vivo* infection with *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ also produced an immune response consistent with a protection outcome. Similar results were observed in mice and dogs immunized with these live attenuated parasites where stimulation was found after exposure of *Leishmania* antigen in IFN-γ secreting effector CD4$^+$ and CD8$^+$ T cells but not in IL-10 secreting cells (Fiuza *et al*., 2013; Dey *et al*., 2013, Selvapandiyan *et al*., 2009). Previous studies with human subjects have also shown that theincrease in IFN- γ producing CD4$^+$ and CD8$^+$ T cells in response to *Leishmania* antigen was stimulated mainly in PBMCs of individuals cured of VL (Rym Chamakh-Ayari*et al*., 2014; Gautam *et al*., 2014) and CL (Ikbel *et*

al., 2014) infection. $CD8^+$ T-cells were shown to be exhausted in VL cases hence failed to produce IFN- γ in response to *Leishmania* antigen in whole blood cultures; however, the ability of $CD8^+$ T-cells to produce antigen specific IFN- γ was restored following clinical cure (HVL) (Gautam*et al.*, 2014). Another study with whole *Leishmania* parasite (live/dead) showed that proliferation of $CD4^+$ and $CD8^+$ T cells was significantly higher in cured CL individuals as compared to the healthy individuals. Further, the stimulation of IFN-γ secreting CD4+ and CD8+ T cells was higher with live attenuated parasites as compared to the killed parasites, providing another evidence that live attenuated *Leishmania* parasites are better in inducing protective immune response as compared to the killed parasites (Nateghi *et al.*, 2010).

The increase in percentage of pro-inflammatory cytokine producing $CD4^+$ and $CD8^+$ T cells upon stimulation with live attenuated parasites in HVL group corroborates our cytokine profile data observed in PBMCs culture supernatants. As IFN- γ and IL-10 are the two main regulatory cytokines governing the fate of the infection in VL, their ratio has been correlated to parasites elimination and indicator of vaccine success (Silvestre *et al.*, 2007). The increased ratio of IFN-γ: IL-10 producing CD4+ and CD8+ T cells in HVL after infection with *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ provide another correlate of protection. Previously, we have shown similar polarization to increased ratio of IFN-γ: IL-10 producing $CD4^+$ and $CD8^+$ T cells after parasite challenge in mice immunized with *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$(Selvapandiyan *et al.*, 2009; Dey *et al.*, 2013).

One of the major challenges for elimination of VL is the presence of asymptomatic carriers that are difficult to identify and might serve as a parasite reservoir (Singh *et al.*, 2014). Currently employed drugs for VL treatment are too toxic

to justify their use in asymptomatic cases which are otherwise healthy individuals, leaving vaccines as the only feasible option. Asymptomatic individuals reach a state of acquired protection against leishmaniasis due to low level parasite infection as suggested by earlier studies (McCall *et al*., 2013). The nature and durability of this protection is indeterminate, however, it is reasonable to argue that vaccination of asymptomatic carriers with live attenuated parasites would be beneficial in maintaining or perhaps adding to the protective immunity due to a favorable immune environment induced by the previous exposure to low level of virulent parasites. Previous studies have indicated that in the absence of drug treatment, vaccination of asymptomatic carriers presents the best approach to prevent and eliminate VL (Das *et al*., 2014; Lee *et al*., 2014).

5.5 **Conclusion**

In the present study, we have evaluated the vaccine potential of *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ live attenuated parasites by assessing their ability to induce cellular immune responses in human PBMCs *ex vivo*. Our results demonstrated that both parasites induce protective immune response in human PBMCs comparable to the wild type parasite, indicating that both parasites have great potential as live attenuated vaccine against human VL.

Conclusions and future scope

A key goal in the control of visceral leishmaniasis is development of vaccines with high protective capability to interrupt the transmission of parasite. However, no effective vaccine is available to prevent this potentially fatal infection. Development of a vaccine is highly desirable and it represents the most practical and efficient control tool. The complex life cycle of *Leishmania* parasite and its remarkable ability to subvert and modulate the host immune system makes it difficult to formulate vaccines. In the digenetic life cycle of *Leishmania* parasites, amastigote stage is adapted to survive and proliferate inside the phagolysosomal compartment of macrophages, which is controlled by differential expression of a variety of genes. Identification and functional characterization of such genes is an important step towards the understanding of parasite survival mechanisms and for development of new drugs or vaccines. One such gene is LdA1, which is highly expressed at the amastigote stage at both RNA and protein level. The present study evaluated the potential of LdA1 as a target gene to generate live attenuated parasite and further it was functionally characterized to elucidate biological roles. The study demonstrated that the LdA1 is unique to *Leishmania* genus, as its homologs are not present in any other organism. Sequence and structure level function prediction suggested that LdA1 gene might play an essential role in the *Leishmania* life cycle. We were able to generate stable overexpression and LdA1 single allele deleted parasites for functional studies. We observed that overexpression of LdA1 did not affect the growth and morphology of the parasites, indicating an unaffected metabolic rate. Deletion of single allele of LdA1 (LdA1$^{+/-}$) resulted in reduction in growth, motility and size of the parasites as compared to the wild type. Although, LdA1$^{+/-}$ was able to infect macrophages similar to the wild type parasites, they showed reduced survival as amastigotes inside the macrophages. Further, null mutants of LdA1 could not be

generated even after several attempts, suggesting the essentiality of gene for the parasite survival.

In the next part of the study, we evaluated vaccine potential of centrin 1 and p27 gene deleted live attenuated *Leishmania* parasites (*Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$) by evaluating their ability to induce protective immune response in the blood PBMCs of active cases of VL and PKDL, HVL and naïve individuals. We found that both *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$parasites infect macrophages similar to the wild type and elicit predominantly protective pro-inflammatory cytokines production including, IL-12, FN-γ, TNF-α, IL-2, IL-6 and IL-17 in the individuals pre-exposed to *Leishmania*parasites (HVL and PKDL); however, that response was dampened in the active VL patients. No significant stimulation was found in anti-inflammatory cytokines (IL-4 and IL-10). Further, significant increase was also found in IFN-γ secreting CD4^{+} and CD8^{+} T cells in PBMCs of HVL, with no increase in IL-10 secreting cells. Moreover, immune responses induced by these live attenuated parasites were similar to the wild type, mimicking a naturally acquired protection following clinical cure, which suggest that both *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ parasites have great potential as live vaccine for humans. The present study is the first of its kind and adds to the knowledge regarding vaccine development by evaluating immune responses induced by live attenuated vaccine candidates in different clinical groups of human population.

Future scope of work

Demonstration of attenuation of LdA1$^{+/-}$ offers possibilities to explore their mechanism of attenuation. We have determined the infectivity and survival of LdA1$^{+/-}$ parasites *in vitro* in mice peritoneal macrophages. However,*in vivo* studies in rodent

animal models are needed to evaluate safety and protective efficacy conferred by LdA1$^{+/-}$ against virulent *Leishmania* parasites challenge to explore its potential as a vaccine candidate. There is also scope for the evaluation of induced immune responses correlating with the protection. Further, we observed difference in size and motility of LdA1$^{+/-}$ parasites as compared to the wild type. At this time, we do not know what produces this difference hence, more information is needed to know the role and function of this protein and it is the subject of future studies.

The other objective of the study evaluated vaccine potential of *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ live attenuated parasites in the blood PBMCs of active cases of VL and PKDL, HVL and naïve individuals. However, future studies are needed to evaluate the immune responses induced in the PBMCs of asymptomatic carriers. Comparison of immune responses induced in asymptomatic carriers with those acquired protective immunity following clinical cure may provide new biomarkers. Furthermore, close follow-up of such asymptomatic cases can also help in the identification of biomarkers in asymptomatic carriers that gets converted to symptomatic VL from those that remain asymptomatic. Studies in animal models have shown that *Ld*Cen1$^{-/-}$ and *Ld*p27$^{-/-}$ are safe and effective as vaccine candidates against leishmaniasis. We have demonstrated here that LdCen1$^{-/-}$ and *Ld*p27$^{-/-}$ induce protective Th1 response in human PBMCs *ex vivo*. At this stage, to establish them as a vaccine for humans we need to perform toxicological studies followed by clinical trials.

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Appendix

Preparation of culture media

LB medium (Luria Broth)

10 gm of Tryptone, 5gm of Yeast, 5gm of Sodium chloride (Hi Media) were dissolved in 1liter of distilled water. Media were sterilized by autoclaving for 20 minutes at 15 lb/sq.in.

LB Agar

15gm of agar powder, 10gm of Tryptone, 5gm of Yeast, 5gm of Sodium chloride, (Hi media) were dissolved in dw. Media were sterilized by autoclaving for 20 minutes at 15lb/sq.in. LB agar was allowed to cool to 45°C and poured (~30 ml per plate) in 90 mm disposable petri plates (Tarsons) along with appropriate antibiotics and allowed to solidify.

M199 medium

9.50g of M199 powder and 2 pellets of NaOH were dissolved in 1 liter of autoclaved distill water. After adding 1ml of penicillin-streptomycin and 250μl of gentamycin antibiotics media was sterilized by filtration through 0.22 μm filter.

RPMI medium

10.4g of RPMI powder, 1.460g of L-glutamine, 2.3g of HEPES and 2.0g of $NaHCO_3$ were dissolved in 1 liter of autoclaved distilled water. After adding 1ml of penicillin-streptomycin and 250μl of gentamycin antibiotics media was sterilized by filtration through 0.22 μm filter.

Preparation of antibiotics

Ampicillin

100mg/ml ampicillin stock solution was prepared (Working: 100μg/ml) in autoclaved distill water and sterilized by filtration through 0.22 μm filter. Aliquots were stored at –20°C.

Kanamycin

50mg/ml kanamycin stock solution was prepared (Working: 100μg/ml) in autoclaved distill water and sterilized by filtration through 0.22 μm filter. Aliquots were stored at –20°C.

Neomycin

20mg/ml gentamycin stock was prepared (working: 40µg/ml) in autoclaved distill water and sterilized by filtration through 0.22 µm filter. Aliquots were stored at –20°C.

Hygromycin

20mg/ml gentamycin stock was prepared (working: 40µg/ml) in autoclaved distill water and sterilized by filtration through 0.22 µm filter. Aliquots were stored at –20°C.

Penicillin

1,00,000 I.U./ml penicillin stock solution was prepared (working: 100 I.U./ml) in autoclaved distill water and sterilized by filtration through 0.22 µm filter. Aliquots were stored at –20°C.

Streptomycin

10mg/ml streptomycin stock solution was prepared (Working: 100µg/ml) in autoclaved distill water and sterilized by filtration through 0.22 µm filter. Aliquots were stored at –20°C.

Penicillin-Streptomycin

10,000 I.U./mL penicillin with 10,000 µg/mL streptomycin stock solution was prepared (Working: 100I.U/ml of penicillin and 100µg/ml of streptomycin) in autoclaved distill water and sterilized by filtration through 0.22 µm filter. Aliquots were stored at –20°C.

Gentamycin

80mg/ml gentamycin stock was prepared (working: 20µg/ml) in autoclaved distill water and sterilized by filtration through 0.22 µm filter. Aliquots were stored at –20°C.

Preparation of reagents

1M Tris

121.1gm of Tris base was dissolved in 800ml of distilled water and pH set (6.8, 7.4, 8.0) with concentrated HCl. Volume was made up to 1liter and autoclaved.

0.5M EDTA

186.1gm of disodium EDTA.-$2H_2O$ was added in 800ml of distilled water, stirred vigorously on a stirrer, pH set to 8.0 with NaOH (~20 gm of NaOH pellets) and volume made up to1liter and autoclaved.

3M sodium acetate

204.5gm of $C_2H_3O_2Na$. $3H_2O$ was dissolved in 400ml of distilled water, pH set to 5.3 with glacial acetic acid, volume made up to 500 ml and autoclaved.

10% SDS

10gm of electrophoresis grade SDS was dissolved in 70ml of autoclaved distill water, heated at 60°C to dissolve and the volume made up to 100ml.

Ethidium Bromide (10 mg/ml)

10mg of ethidium bromide was dissolved in 1ml distilled water, stored in a opaque bottle.

30% Acrylamide Stock

29.2gm of acrylamide and 0.8gm of bis-acrylamide were dissolved in 50ml of autoclaved distill water. Volume was made up to 100ml, the solution filtered through Whatmann no. 1 paper, degassed and stored in an opaque bottle.

Calcium Chloride (0.1 M)

1.47gm of $CaCl_2.2H_2O$ was dissolved in 100ml of distilled water and sterilized by autoclaving.

IPTG (1M)

238mg of IPTG was dissolved in 1ml of autoclaved distill water, filter sterilized and stored at -20°C in 50μl aliquots.

Sodium Phosphate (1M)

- **Monobasic**

  138gm of $NaH_2HPO_4.H_2O$ was dissolved in 800ml of dw and volume made up to 1liter.

- **Dibasic**

  268gm of $Na_2HPO_4.7H_2O$ was dissolved in 700ml of dw and volume made up to 1liter.

Ammonium persulfate (10%)

To 1gm of ammonium persulfate, 10ml of autoclaved distill water was added and the solution stored for several weeks at 4°C.

10 X TAE buffer (Tris acetate, EDTA)

4.84gm of Tris base in 80ml of dw was dissolved and 1.2ml of glacial acetic acid and 2ml of 0.5 EDTA pH 8.0 were added. Final volume was made up to 100ml.

Phosphate Buffer Saline (PBS)

8gm of NaCl, 2gm of KCl, 1.44gm of Na_2HPO_4 and 0.2gm of KH_2PO_4 were dissolved in 800ml of dw. pH was set to 7.4 with HCl. Final volume was made up to 1 liter and sterilized by autoclaving at 15lb/ sq.in for 20 minutes and stored at room temperature.

DNA loading dye (6X)

0.2gm bromophenol blue, 0.2gm of xylene cyanol and 30ml of glycerol were dissolved and volume set to 100 by autoclaved dw.

Electroporation Buffer

0. 25gms HEPES, 0.403gms NaCl, 186mg KCl, 6.2mg Na_2HPO_4, 54 mg glucose, pH7.4 added to 100 ml dw and sterilized by filtration.

Reagents mainly used in SDS-PAGE and western blotting

SDS-PAGE electrophoresis buffer

3gm of Tris base, 14.4gm of glycine and 1gm of SDS were dissolved in 1 liter of dw.

2X SDS-PAGE sample buffer

The composition of sample buffer is as follows

Tris-Cl (pH6.8)	100mM
DTT	200mM
SDS	4%
Bromophenol blue	0.2%
Glycerol	20%
β-mercaptoethanol	10%

Composition of resolving gel (12%) 10 ml

30% acrlyamide solution	4.0 ml

1.5M Tris-Cl pH 8.8	2.5 ml
dw	3.3ml
10% SDS	100μl
10% APS	100μl
TEMED	10μl

Composition of stacking gel (5%) (5.0 ml)

30% acrylamide solution	0.83ml
1.0M Tris.Cl pH 6.8	0.68 ml
dw	3.4ml
10% SDS	50μl
10% APS	50μl
TEMED	5μl

Coomassie Brilliant Blue R250 staining solution

1gm of coomassie blue (R250) was dissolved in 450ml of methanol. 100ml of glacial acetic acid was added and the volume made up to 1liter by double distilled water, filtered through Whatman no.1 filter and stored at room temperature.

Destaining solution

Methanol: water: acetic Acid were mixed in the ratio of 45:45:10 and stored at room temperature.

Transfer buffer

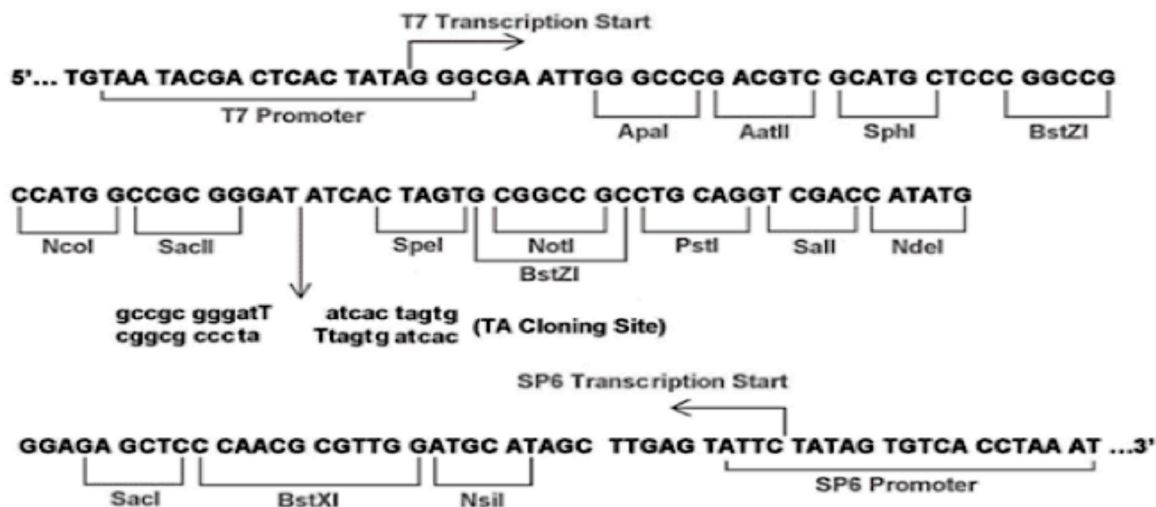
14.4g of glycine, 3.04g of Tris-HCl and 100ml of methanol were added. Final volume was made up to 1 liter with autoclaved distilled water.

Ponceau staining solution

220mg of ponceaue was added in 2ml of glacial acetic acid. Final volume was made up to 1 liter with autoclaved distilled water.

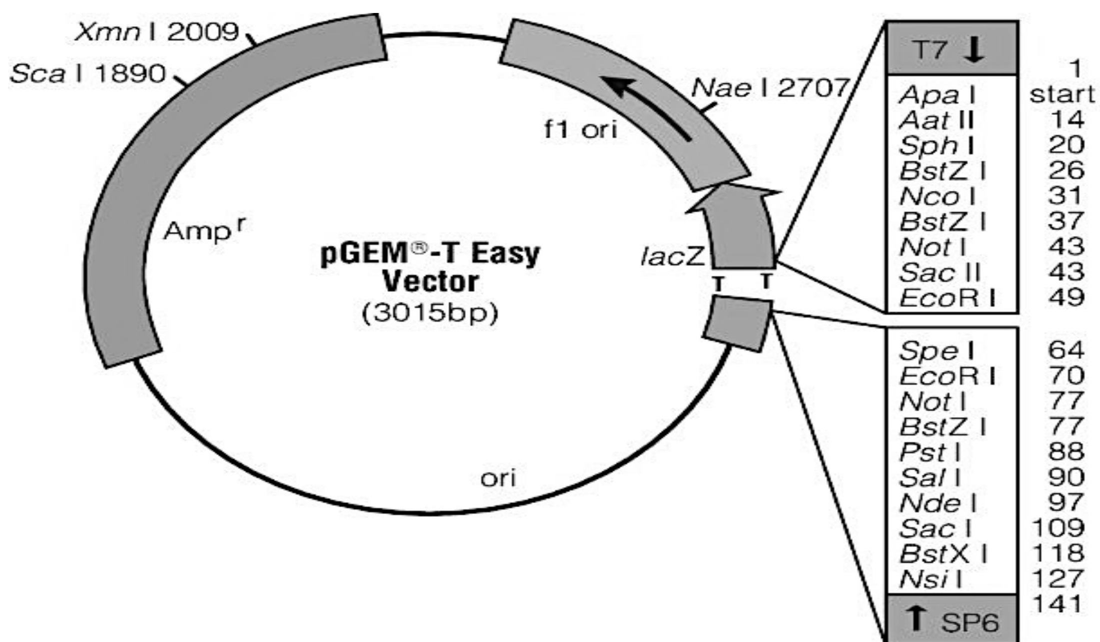
Vectors used in the study

pGEM®-T Easy TA cloning Vector



The promoter and multiple cloning sequence of the pGEM®-T Easy Vector. The top strand shown corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase.

pGEM®-T Easy Vector Map and Sequence Reference Points



T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

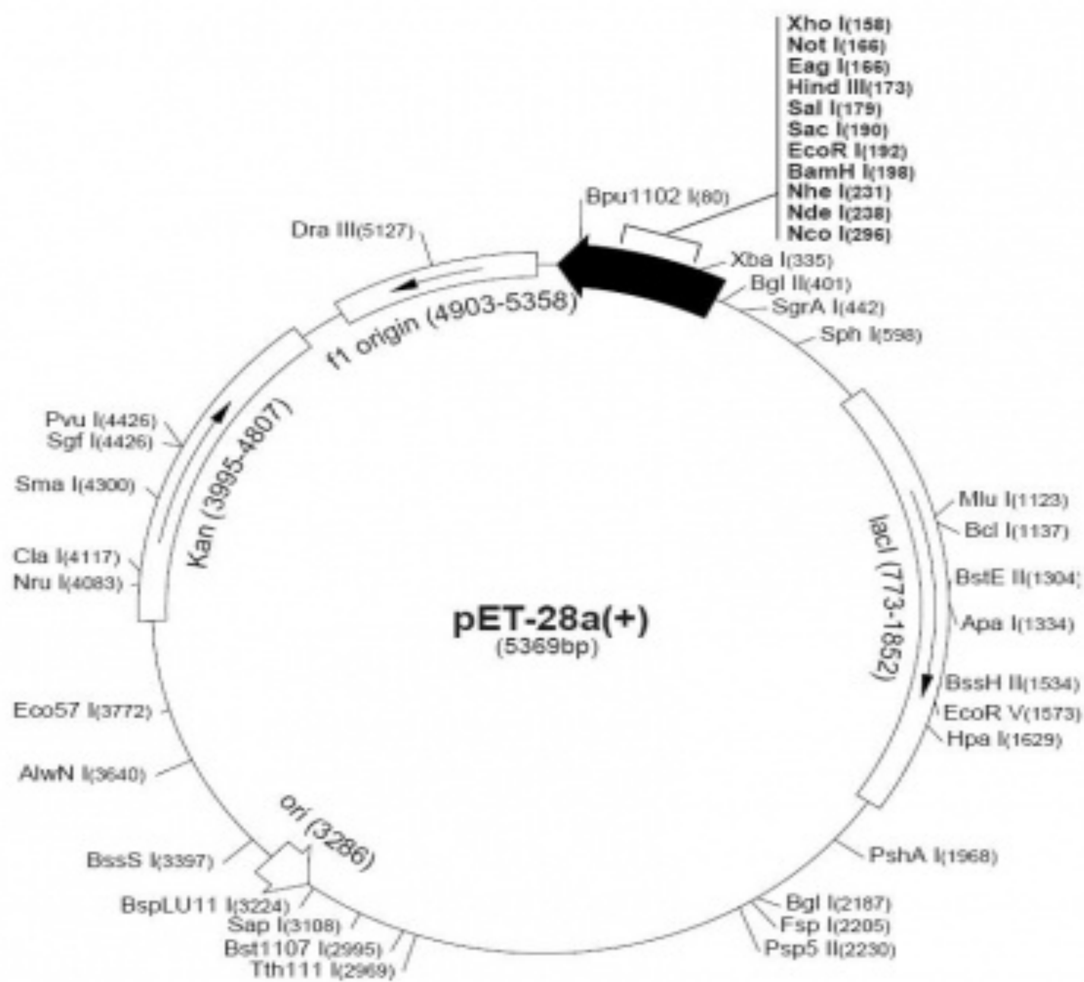
To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation. ng of vector × kb size of insert × insert:vector molar ratio = ng of insert

Multiple cloning sequence of the pKSNEO Vector

GTCAGCATCGCGCCCCGCGGCATGGGTGCTCGCACCTTTCCATTCTCTCCGTACGG
CGTTCGCCACTCTGCTCACTCTTCTCCCCTTTCCTCTCTCCGCATCTGCGCCTTGTG
TTCTGTGTGTGCGCCCCACACGTACTCTACACGCCTTCTCTGCATGCGCTCCTCGCC
TGCCTGAAACGATGACGTGCGCCGCCACTCGCTCAGCCGCTCCCATCCACATGCAT
TCCTGCACTCTACAGCGACTCTAAGCCGTATAGTCCATCTCCTCAACACCCACTCG
CTTCCTTCTGCTCTCACCTATTACTTCGCCAGCCCACATATCTGCTATAACCTGCCC
TCCCCCACCCGCTTCCCACACATCCGCCACCGCTACGCAGCGTCGACTCTAGA**ACT**
AGTGGATCCCCCGGGCTGCAGGAATGCGATCGATGATCTAGATGCGCCTCTCCAC
GACATGGCCGGAGGCGGGAGATGAAGGCAGCGACCCCTTT

Spe1 restriction site (in bold) is used to clone the target gene.

pET28a (+) Vector Map



Nucleotide sequence of genes used in the study

LdA1 gene

ATGGACGCCGCCAGGAAACGTCACAGAGGGAACGCGTCAGAAGCGGCAGCCAGA
GGTGATGAAGATGACTCGGCCGCGATCCCCGGACGTGAGGCACGGCACCAACTGC
ACAGCAGCAAAAGCGCCATCCCTTTCACGTCTACTCCCGAGGAAGCGTGTGGGGA
TGAAAGCGACTACGCTGTCCTGGTGAAACGGAGCACGGCACTGTTGGCAAAGCTC
GGCCAGCGCAATGTACTCGACAACTGCGCAGCCCAAGAAACCAGCAGCACGCAA
AAGGAGGCAACACAACCAATGACACTTCCGTGTAGGTTTGAGAATCACAAGTTCA
GCTTGTACGGTGTTTCTTGCTCCGCTGGCTCTCTTGTTTTACCCTTTGCCACGGCTG
CGTGCATACAGCCCACAGAGCTGGCTCATCGCTCCACCAAGTGCACACAAACGGA
GTCATCGACCACTCTAGAAGCCCGCAAGAAGCTGCTAGCTACGGAGTTGAGTGAG
CTCTACACACTGTTGCATGTCTTTTTCTCGTGA

Neomycin^R gene

ATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGC
TATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTC
CGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGC
CCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGC
GTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGC
TATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAG
AAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTA
CCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGAT
GGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCG
CCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCG
TCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTT
TCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAG
CGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTT
CCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCC
TTCTTGACGAGTTCTTCTGA

Hygromycin^R gene

ATGAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGT
TCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTC
AGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATG
GTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATT
CCGGAAGTGCTTGACATTGGGGAATTCAGCGAGAGCCTGACCTATTGCATCTCCC

GCCGTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGT
TCTGCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAG
ACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGC
GTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATG
GACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGG
CCGAGGACTGCCCCGAAGTCCGGCACCTCGTGCACGCGGATTTCGGCTCCAACAA
TGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATG
TTCGGGGATTCCCAATACGAGGTCGCCAACATCTTCTTCTGGAGGCCGTGGTTGGC
TTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGA
TCGCCGCGGCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAG
CTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCA
ATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCG
CGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACG
CCCCAGCACTCGTCCGAGGGCAAAGGAATAG

<u>5’ Flanking region of LdA1 gene</u>

AGTCCCTGCCGCTGCTCTGCACGCATCGAAACATTGCACTCGAGTATACGTAAAG
GGATGGAACGCCGACAAAGCGCTTGCCTTTTCAATTTGCGAATACGTGAAGCGAA
ACAACGCTCGCAGGCGCTTTCGCCCCCTTCCTTTTGCTTACACCAAATGACTCCTT
GATGCCGTCATGCATCTGACCGCGAAACAGGGGGGGGCGATGGCGAGTGTGCCAC
AGGGCCATCACAATCGATGCGTGGTGTTTCGCACTTCTCCTCGCTTATCCAAGTAT
GCATGTTGTCCAAACCATTTGCCGTGGTGCATCCACTTCATTTTTTTTTCGTTTTT
CTCGACACCTATTCTGCTTCGCGTACTTCTTTTCTTCATAGAGGTCACTGTTACTTG
TTCGTCATCTGGGATCCCCACTGCTTCTGCAAAGGCTCAAGGTTTCATTGACAAGA
ACCCATTGTTTCCCAGGAGGGGCGCACGCTACCCACTGTCAGAGCACTCGTTGCCC
CTCCGCAGAAAAAAAAGGGCCAGCGGTGCAGAA

<u>3’ Flanking region of LdA1 gene</u>

ATACGTTCGCGTGCTCGCGAGTTGCTTTTTTCCCCTTGCTCTTTTCTTTTCTTCACGG
ACATGCGCGCTGATGGAAGGTTCTGCTGTACTGCAAGCACCACTCTCTCTGCTCAT
TGATTTCACTGCTGCTGCTTACGGCTCAGACACTGCATCGATTCCTCGAACTCACA
GTTAGCGATTCAAGCCGTGCCTGCGTGCATCCTCGCACGGCACCTGGCGTTGTTGT
GCTCCTTCACCTCGGGACGCTTTCTCGAGATGTCAGGAATGTTCTTTCCAGCACAC
GTTAACTGGTGGTGGCTTTATAATAATATATGTATATATATATTTGCTAGCAAGT
ACCGCAGATCTTACGCCCGCGCCATTGGTTCTCCTTGGACCTCTGCTCATGCTTTTC
CTCTCTCGTCCCTGTCTCTTTGCCTCTGCATCGCTGTCGTTTTTCCGCCAGGCTCTCT
CTTCTGCGCGATGCCTTCGCCCTACTCAGTAGTCGTTTGTCTTCCGCTACCCTGTCG
CATTTTTTTTCTTTGAAAGCTTACGACAGGAATGAAAGAGGGCGGGATGATCTAA

GTAATAAAAAAGACGATAGGATGATGAGGAGTGAGTCAAGGCACTTTCCACGATT
CCAATGACATGCTTCACCACACACGTATCTTTTCTTCATTTGTTCTTCTGCTCATTT
CAGATCTCGAGGAATGGAGTCATGACAAGCGTTTGCTTTTTCCCTCCACCCGCCCT
CTTGATTACTCACCATGACAGGGGGATCCTTGGGTGACGGAAAAGGGGGATACGT
AAGTCTACCTATTCGTGGGTAGGGTTACCCGATGGGGACCTGAAAATAACAGAAT
TCTCACTGATAGCTGTGCCAGCTCCAGAGGTATCGAGGTCGCGAGCAAAGGGATA
CCAAATCGGTGTACTCGTCCACGGCAATGGGAAACTCACCAACCAGGAAGAGTTG
GAAACACATGGATCGCAATGCATCATGCCTATCTTGTGACATCGTTCTCTCTCTCC
TTTTATTCTTCTCTCAGTTTCCCTTTCTCACTCAGGTCGAGGGAGCTTTCTTTTTTGC
GTTTTCCTTTCTTCGTGCAGCCTAATACTTACCTTTTTTACTGTGGATTTTACTTTCC
GTGCACCCTGAAGTACAAGTGGTTGGGTGTGTGCTCTGTCTTTTGGTAAAATTCGT
CCTATTTCGGAGTAAGCTTCGAATACATATATATATATATTAGTTCATTTTCTTTTT
GCTGCACGGACTTGTATTGTTTTCTGGAACCAGAGACACAA

Publications and Presentations

Publications and Presentations

Research Publications

1. **Avishek K** and Salotra P. Characterization of amastigote specific gene of *Leishmania donovani:* role in growth, phenotype and infectivity of parasite **(Manuscript under preparation).**
2. **Avishek K,** Kaushal H, Gannavaram S, Dey R, Selvapandiyan A, Ramesh V, Negi NS, Dubey US, Nakhasi HL, Salotra P. Gene deleted live attenuated Leishmania vaccine candidates against visceral leishmaniasis elicit pro-inflammatory cytokines response in human PBMCs. **Sci Rep. 2016;** 14:33059
3. Kaushal H, Bras-Gonçalves R, **Avishek K**, Deep DK, Petitdidier E, Lemesre JL, Papierok G, Kumar S, Ramesh V, Salotra P. Evaluation of cellular immunological responses in mono- and polymorphic clinical forms of Post Kala-Azar Dermal Leishmaniasis in India. **Clin Exp Immunol. 2016; 185, 50-60**
4. Ramesh V, Singh R, **Avishek K**, Verma A, Deep DK, Verma S, Salotra P. Decline in Clinical Efficacy of Oral Miltefosine in Treatment of Post Kala-azar Dermal Leishmaniasis (PKDL) in India. **PLoS Negl Trop Dis. 2015**; 9 (10): e0004093.
5. Ramesh V, **Avishek K**, Salotra P. Post-Kala-Azar Dermal Leishmaniasis in HIV-co infected individuals: Problems in diagnosis and treatment. **Int J Dermatol. 2015**; 54 (1): 116-20.
6. Gannavaram S, Dey R, **Avishek K**, Selvapandiyan A, Salotra P, Nakhasi HL. Biomarkers of safety and immune protection for genetically modified live attenuated leishmania vaccines against visceral leishmaniasis - discovery and implications. **Front Immunol. 2014**;5:241.
7. Ramesh V, **Avishek K**, Sharma V, Salotra P. Combination therapy with Amphotericin-B and Miltefosine for Post Kala-azar Dermal Leishmaniasis(PKDL).**Acta Derm Venereol. 2014**; 94(2):242-3.
8. Katara GK, Raj A, Kumar R, **Avishek K**, Kaushal H, Ansari NA, Bumb RA, Salotra P. Analysis of localized immune responsesreveals presence of Th17 and Treg cells in cutaneous leishmaniasis due to *Leishmania tropica*. **BMC**

Immunol. 2013; 14 (1): 52.

9. Verma S, Bhandari V, **Avishek K**, Ramesh V, Salotra P. Reliable diagnosis of Post Kala-azar Dermal Leishmaniasis (PKDL) using slit aspirate specimen to avoid invasive sampling procedures. **Trop Med Int Health. 2013**; 18(3):268-75.
10. Verma S, **Avishek K**, Sharma V, Ramesh V, NegiNS and Salotra P. Application of loop-mediated isothermal amplification assay for the sensitive and rapid diagnosis of visceral leishmaniasis and post-kala-azar dermal leishmaniasis. **Diagn Microbiol Infect Dis. 2013**; 75(4):390-5.

Chapter in book

1. Sreenivas Gannavaram, Parna Bhattacharya, RanadhirDey, Nevien Ismail, **Kumar Avishek,** Poonam Salotra, Angamuthu Selvapandiyan, Abhay Satoskar, Hira L Nakhasi. Methods to Evaluate the Preclinical Safety and Immunogenicity of Genetically Modified Live-Attenuated *Leishmania* Parasite Vaccines. Edited by John M. Walker. Published by Springer Protocols; Humana Press. Methods Mol Biol. 1403:623-638 (2016).

Presentations/Abstracts published in proceedings

1. **Kumar Avishek**, V Ramesh, A. Selvapandiyan, Sreenivas Gannavaram, Hira L. Nakhasi and Poonam Salotra. Development of genetically modified live attenuated *Leishmania* parasites as potential vaccine candidate against visceral leishmaniasis. **Oral presentation** at "6th World Congress on Leishmaniasis (WorldLeish 6) ", will be held at Toledo, Spain from16th-20th May 2017.
2. **Kumar Avishek.** Preclinical Studies to Assess the Protective immunogenicity of Centrin1 Gene Deleted Live Attenuated *Leishmania* vaccine Candidate in Human PBMCs. **Poster presentation** at "17th Annual Conference on Vaccine Research 2014", April 28-30, 2014, held at Bethesda, Maryland, USA.
3. **Kumar Avishek,** V Ramesh, A. Selvapandiyan, Randhir Dey, Hira L. Nakhasi and Poonam Salotra. *Ex vivo* evaluation of cellular immune responses elicited by genetically modified live attenuated *Leishmania* parasite vaccine candidates

in human PBMCs. **Oral presentation** at " World Congress of Infectious Diseases (WCID)2013" held at Chennai, India from 18th-22nd December 2013.

4. **Kumar Avishek**, A. Selvapandiyan, V Ramesh, Hira L. Nakhasi and Poonam Salotra. **Poster presentation** at *Ex vivo* evaluation of immunogenicity of Centrin1 gene deleted live attenuated *Leishmania* vaccine candidate. "Immunocon 2013" held at Delhi, India from 15th -17th Nov 2013.
5. **Kumar Avishek**, Sandeep Verma, Vasundhra Bhandari, V Ramesh and Poonam Salotra. Use of Slit aspirate specimens to diagnose Post Kala-azar Dermal Leishmaniasis (PKDL):A non- invasive strategy. **Poster presentation** at "Microcon 2012",held at Delhi, India from 22nd-25th Nov, 2012.
6. P. Salotra, **Kumar Avishek**, Kavita Pahuja, A. Selvapandiyan. Development of genetically modified live attenuated parasites as potential vaccines against visceral leishmaniasis. Presented at 3rd International Conference on Clinical Microbiology & Microbial Genomics, held at Valencia, Spain in Sep, 2014.
7. Hira L. Nakhasi, Ranadhir Dey, Jacqueline Fiuza, Parna Bhattacharya, Sreenivas Gannavaram, InèsElakhal Naouar, G Natarajan, Robert Duncan, A. Selvapandiyan, **Kumar Avishek,** Poonam Salotra, Ricardo Fujiwara, Hamide Aslan, Shaden Kamhawi, Jesus Valenzuela. Advances in genetically modified live attenuated *Leishmania donovani* vaccine candidates. "5th World *Leishmania* Congress (Worldleish5)" held at Pernambuco**,** Brazil from 13th-17thMay 2013.
8. Poonam Salotra, Sandeep Verma, **Kumar Avishek,** Vasundhra Bhandari, N.S. Negi, V. Ramesh. New tools for molecular diagnosis with simultaneous measurement of parasite burden in Kala Azar and Post Kala-azar Dermal Leishmaniasis. "PGI Golden Jubilee Workshop on Molecular Diagnosis for Parasitic Diseases: Conventional and Real Time PCR Techniques 2012", held at Chandigarh, India from 10th-11th Sep, 2012.

Brief Biography of Candidate

Kumar Avishek joined his masters in Biotechnology at Madurai Kamaraj University, Madurai after securing all India Rank of 49 in Combined Entrance Examination for Biotechnology (CEEB)-2007, conducted by Jawaharlal Nehru University (JNU), New Delhi. During masters, he did 6 months dissertation on "Cloning of plant drought resistance genes in expression vector for transformation". He qualified various National level examinations including, National Eligibility Test for JRF/ Lectureship twice by CSIR, India, in June 2009 and December 2009, Indian Council of Medical Research (ICMR) Junior Research Fellowship (JRF)-2009, GATE (Graduate Aptitude Test for Engineering) – 2009. He joined PhD in Molecular Parasitology Lab at the National Institute of Pathology in 2010 and got registered at Birla Institute of Science and Technology, Pilani. His research interest is vaccine development, diagnosis and Immunology of leishmaniasis. His research work was mainly focused on development of genetically defined live attenuated *Leishmania* parasite as vaccine candidates against leishmaniasis. He has presented his research work in various national and international conferences. He was Selected amongst the 11 participants for Travel Grant Award to attend "17th Annual Conference on Vaccine Research- 2014", held at Bethesda, Maryland, USA. He also received Travel grant award from Department of Science and Technology, Govt. of India to attend "6th World Congress on Leishmaniasis -2017", held at Toledo, Spain. He has authored 9 articles in peer reviewed international journals and 1 book chapter.

Brief Biography of the Supervisor

Dr. Poonam Salotra worked at V.P. Chest Institute, Delhi University for PhD and at Roche Institute of Molecular Biology, New Jersey, U.S.A., for postdoctoral research. She is currently the Director In-charge at the National Institute of Pathology (ICMR), New Delhi.

Her pioneering research for the last 20 years has been in the fields of genomics, vaccines, diagnostics and mechanism of drug resistance in visceral leishmaniasis (VL) and Post Kala-azar Dermal Leishmaniasis (PKDL). Attenuated gene knock-out mutants developed as vaccine candidates are under pre-clinical trials. Drug resistance studies with antimony and miltefosine yielded new insights into mechanisms operative in Indian field isolates. She established molecular tests for non-invasive diagnosis and assessment of cure of VL and PKDL. Her studies yielded fundamental understanding of the immunopathogenesis of VL, PKDL and cutaneous leishmaniasis. She has authored more than 110 research articles and 10 book chapters and has 4 patents to her credit.

Dr Salotra serves as a member of WHO Expert Committee on Leishmaniasis since 2009. Dr Salotra has won several National awards, notably the JC Bose fellowship, Drs Kunti and Om Prakash Oration award, ICMR, Basanti Devi Amir Chand Award, ICMR, BK Aikat Award and Kshanika Oration Award for Eminent Woman Scientist. She received Silver Jubilee award by Indian Association of Medical Microbiology. She won numerous International fellowships including Bill & Melinda Gates Global Health Series award , Courtesy Fellowship by CBER, FDA, USA, National Foundation of Infectious Diseases Fellowship, USA. She is an elected Fellow of the National Academy of Sciences, India (NASI), the Indian National Science Academy (INSA), The World Academy of Sciences (TWAS) and the National Academy of Medical Sciences (NAMS).

Brief Biography of the Co-Supervisor

Dr Uma S. Dubey is Associate Professor in the Department of Biological Sciences at BITS Pilani, Pilani Campus. She did her Masters in Life Sciences from the Institute of Life Sciences at Kanpur. After this she was a graduate student and conducted research in the Department of Plant Sciences, University of Alberta, Edmonton, Canada. Later she did her Ph.D in Immunology from Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow under the guidance of Prof. S.S. Agarwal (Ex Director SGPGI, Lucknow). Later at Kanpur, she was also involved in teaching and program development in the departments of Biotechnology, Microbiology and Environmental Sciences at the Institute of Life Sciences, Kanpur University. Her broad area of research interest is Cancer Biology and Immunology. She is also interested in interdisciplinary research on mathematical modeling of immunological systems, cancer cells and their interactions. She has been involved in teaching various courses and guiding students in research related projects. She has coordinated the course restructuring changes at M.Sc. and ME level in biosciences department at BITS, Pilani as the DCA Convenor (2012-2014). She has published 12 original research articles, 3 book chapters and 1 lab manual. She is a member of Departmental committee of Academics, Ex- Coordinator of Departmental Committee of Academics, Coordination and organization of Science, Imagination and discovery (SID) BITS, Judge of various events APOGEE from 2006-2010 in Biological Sciences and Medical Sciences categories, Life member of Indian Immunological Society and Member of My India Team, BITS, Pilani.