ROLE OF META 1 GENE IN HOST PATHOGEN INTERACTION; *Leishmania donovani* AS MODEL ORGANISM

M. Sc. Thesis
(2015)

Submitted to
CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Tribhuvan University
Institute of Science and Technology
Kirtipur, Kathmandu, Nepal

For partial fulfillment of the requirement for the Master of Science in Biotechnology

Submitted by

Gomati Pant

Roll No: BT 104/069

TU Regd no: 5-1-61-51-2004

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Dedicated

to

our dearest parents

Chandra dev Pant and Durga devi pant who encouraged us

and

our respected

TEACHERS, who enabled us

ACKNOWLEDGEMENT

The completion of this thesis on CCMB gives me much pleasure. Foremost; I would like to express my sincere gratitude to my supervisor, Krishna das Manandhar central deparment of Biotechnology Tribhuwan University, who was abundantly helpful and offered invaluable assistance, support and guidance and helped me IN thesis writing time , stastical analysis, after coming from CCMB. He is one of the better advisor and mentor for my Master study. His continuous support helps to compete my thesis work. The appreciable guidance and support provided by him have made it possible for me to complete my writing part.

I am deeply indebted to Dr.Tushar Vaidya who encouraged me a lot during my working time in the CSIR-Centre for Cellular and Molecular Biology, CCMB. And for the continuous support of my Master thesis study and research, for his motivation, enthusiasm, and immense knowledge guidance helped me in all the time of research and writing of this thesis. He not only provided me help whenever needed, but also the resources required to complete this project report on time. I could not have imagined having a better advisor and mentor for my Master study.

Besides my supervisor, I would like to thanks, Snehal Jamalpure for her great assistance, guidance and supports in teaching me a lot and encouraging me a lot during my working time in Infection assay, lending her support whenever required so it would not have been possible to perform experiment without her help. My thanks goes to Nitin Tupperwar, who helped me a lot in real time PCR, Pradyumna Paranjape, V.Devi Prasad, loka Ram Prasad, kaneez E Rabab, Bhushan chougule, Venkateshwarlu for their help and support. My sincere thanks to Parasitology lab of Tushar Vaidya of Centre for Cellular and Molecular Biology,CCMB which is the part of CSIR Opening their laboratories to my research and provide me with all resources needed.

My sincere admiration goes to Head of the Department Dr. Rajani Malla for providing every kind of facilities, encouragement, support during my tenure at CCMB.

I would also like to expand my deepest gratitude to Dr. Tilak Shretha, from central department of Biotechnology, Nepal, who gave me many information and ideas about CCMB;

I am indebted to my academic institution, Central Department of Biotechnology, TU,Nepal. I would like to express my sincere gratitude to my respected teachers:,Mohan Kharel, Dr.Tribikram Bhattrai, , Mrs. Jarina Joshi, Smitha shretha,Balhari Paudel. I would also like to thank Om dai for helping me in stastical analysis.

I would also like to thank all the Teacher and staff members of Central Department of Biotechnology. I would like to say thanks to my friends Aarati Paudel, Santosh Dahal, MItesh Shretha and Rajindra Napit for their help and support at CCMB. I take this opportunity to express my profound gratitude to my dearest parent, Mom you are the best, Dad thank you for being there for me and my brother who support me a lot and encourage me in every moment of my study to achieve my goal, I am indebted to my devoted husband Bhoj, who encouraged me in every step of my work and sister for helping around, Sister in law and brother in law as well.

.

LIST OF ABBREVIATIONS

IL	Interleukin		
CL	Cutaneous Leishmaniasis	MOI	Mode of infection
PB	Phosphate buffer	WHO	World Health Organization
PBS	Phosphate buffer saline	MCA	Mucocutaneous leishmaniasis
GFP	Green fluorescent protein	MOI	mode of infection
VL	Visceral Leishmaniasis	TH2	helper T-cell 2
et al.	And other	TH1	helper T-cell 1
FCS	Fetal calf serum	MØ	Macrophage
µl	microlitre		
mM	milli Molar		
Mg	milli gram		
Min	minutes		
Fig	Figure		
%	percentage		
^{o}C	Celsius		
LD	Leishmaniadonovani		
PV	parasitophorous vacuole		
LPG	lipophosphoglycan		
PKC	protein kinase C		
CPs	Cystein Proteases		
GIPL	Glycoionositol phospholipids		

Cht1chitinase 1

ABSTRACT

Leishmaniasis is a vector-borne disease causing significant morbidity and mortality in Africa, Asia, Latin America and Mediterranean regions of the world. Viseral leishmaniasis, the Kala azar, disease is a deadly one caused by an obligate intra cellular protozoan parasite *Leishmania donovani* transmitted by a tiny sandfly, *Phlebotomus argentipes* of the family Trypanosomatidae, which is characterized by the possession of a kinetoplast that has unique form of mitochondrial DNA inside it. There is also expression of Meta1 gene in the parasite which could have playing efficient role for the enhancement in its virulence. Therefore, experiments were carried out by overexpressing Meta1 gene (test parasite) in the control GFP tagged parasite. Over expression of Meta1 protein was identified by polymerase chain reaction. Macrophage Infection Assay (MIA) was carried out by infecting the J774 cell line with metacyclic promastigotes (both test and control parasites)at the ratio of 1:10 in late log/stationary phase on 7^{th} day and analyzed quantitatively as well as qualitatively studying the capability to attach, internalize and multiply within the macrophage with respect to each groups at four time points 1hr, 4hr, 12hr and 24 hours. After infection at a particular time points, the cells were stained by giemsa to visualize the infected parasite on bright field of phase contrast microscopy. There was significant difference between two strains, at early and later time point (0.0001***) in Meta1 and GFP. In case of attachment category, both the *Ld*Meta1 & *Ld*GFP shared subtle differences at earlier time points but at later time point Meta 1 showed significantly higher infective rate than *Ld*GFP,(93%to 94% and 95% to 86%). In reference to macrophage infected in internalized category, both the *Ld*Meta1 & *Ld*GFP showed significant difference (p value, 0.0005 in 1h,<0.0001 in 24h) at earlier and later time points. The exclusively external infected parasites were found higher in *Ld*GFP (Pvalue,<0.0001***). The exclusively internal parasitic infection showed significant increase by *Ld*Meta1 at early and later time points,5.7% and 0.22%, 77% and 41% in Meta1 and Ld GFP respectively, p value-0.001** at 1h and 0.0001**** at 12 hr). In reference to the number of infecting parasites in all categories, greater than five promastigotes were significantly higher in case of LdMeta1 over expressed than Ld GFP. However in less than five category, the percentage were higher in *Ld*GFP. Hence, parasites load were higher in Meta1 to be claimed that Meta1 plays crucial role to infect host cells though both strain used were virulent. Still the exact function of meta1 protein is yet to know underlying mechanism remained to be explored. By this research work the role of Meta1 gene is explored as the parasite load and total infectivity of host cells were significantly higher in over expressed Meta1 than LdGFP. Overall the studies of Meta1 contribution to virulence will lead us in generating a more target specific drug or vaccine which will help in eradication of Leishmaniasis.

Key words: *Leishmania donovani,* Visceral leishmaniasis, Meta 1 protein, Macrophage infection assay,Attachment, exclusively external, internalization, exclusively internal

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CHAPTER I

INTRODUCTION

1.1 Background: *Leishmania* and Leishmaniasis

Leishmania spp. is the obligate intracellular parasites (Chang, 1990; Croft and Coombs, 2003) which is the etiological agent of leishmaniasis. The parasite is a haemoflagelate protozoan parasite (Schuster and Sullivan, 2002). *Leishmania* belongs to the order Kinetoplastida and the family Trypanosomatidae, and can be further divided into subgenera – *Leishmania* and *Leishmania (viannia)* on the differences in the site of development of parasite within the female sand fly vector(Fig. 1.1)(Sacks and Kamhawi, 2001) The *Leishmania* protozoans are classified into following taxonomic systemic position in animal group.

Systemic Position

Kingdom	**Protista**
Subkingdom	**Protozoa**
Phylum	**Sarcomastigophora**
Subphylum	**Mastigophora**
Class	**Zoomastigophora**
Order	**Kinetoplastida**
Family	**Trypanosomatidae**
Genus	***Leishmania***
Species	***donovani, tropica, mexicana, braziliensis***

(Source: https://www.msu.edu/course/zol/316/lspptax.htm)

Leishmaniasis is a spectrum of complex diseases, *viz.* visceral, cutaneous, and mucocutaneos leishmaniasis. The disease is a vector born disease transmitted by insect, sand fly of genus like *Phlebotomus, Lutzomia etc.*(Lainson and Shaw, 1973).

There are two main life cycle stages of *Leishmania* parasites. Amastigotes stage found in vertebrate host is the disease causing stage of the parasite, which is 2-4µm, oval or round non-flagellated and non-motile that live permanently and replicate intracellularly, within the host macrophages(Richard et al., 1981). The next is promastigotes having the measurement of 3µm-4µm by 15-20µm, spindle shaped, flagellated, motile which

develops in the mid gut of infected sand flies and are ready to transmit to humans (Richard et al., 1981).

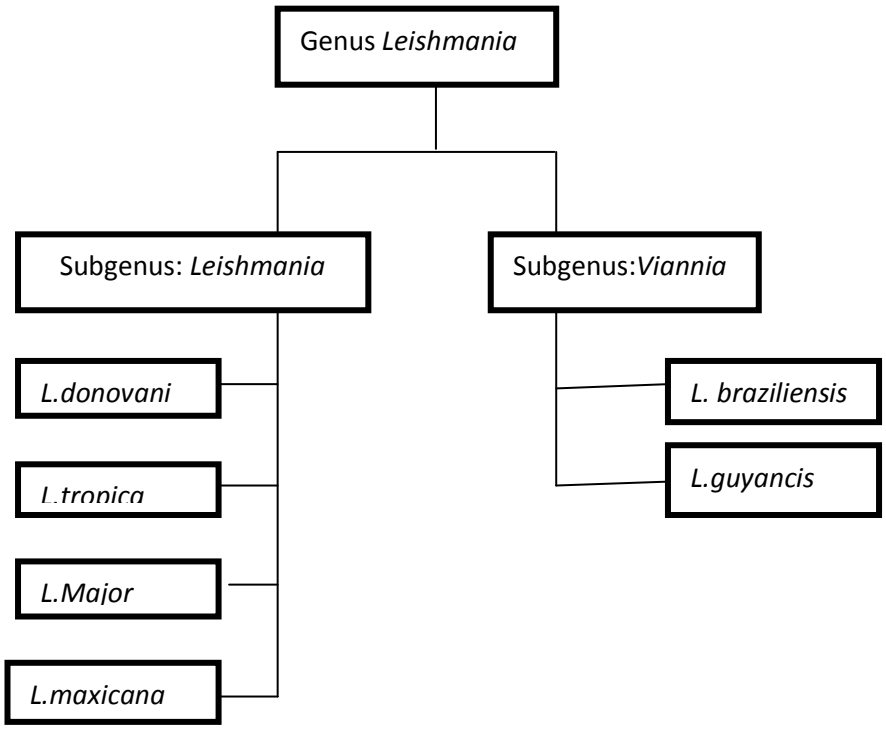

Fig 1.1: Modified form of classification of genus *Leishmania* (Attar, 1997)

Specific parasites are common responsible for the different forms of leishmaniasis disease, however there are some rare parasites which have played role to cause the specific form of leishmaniasis (Table 1.1).

Table1.1 The various species of *Leishmania* causing wide spectrum of symptoms leading to three different forms of ,leishmaniasis.

Visceral leishmaniasis	Common Rare	*L. donovani* *L. infantum* *L. chagasi* *L. tropica*
Cutaneous leishmaniasis	Common Rare	*L. major* *L. tropica* *L. amzonensis* *L. maxicana* *L. braziliensis* *L. infantum* *L. donovani*
Mucocutaneous leishmaniasis	Common	*L. braziliensis* *L. panamensis*

The disease includes a complex of vector borne diseases, caused by more than 20 *Leishmania* species creating symptoms ranging from cutaneous lesion at the site of sand fly bite to systemic visceral leishmaniasis(McCall et al., 2013) *Leishmania donovani* is obligate intracellular parasites(Chang, 1990; Croft and Coombs, 2003) which are the main causative agents of visceral leishmaniasis.

Leishmania exists as extracellular, flagellated, less infective procyclic promastigotes within the mid gut of the sandfly. These procyclic promastigotes undergo developmental changes to metacyclic promastigotes and become highly virulent. Thereafter, they migrate to the mouthparts of the sandfly. This process of differentiation is called as metacyclogenesis. When the sandfly takes the blood meal, metacyclic promastigotes get transferred to the bloodstream of mammalian host where they are phagocytosed by macrophages.

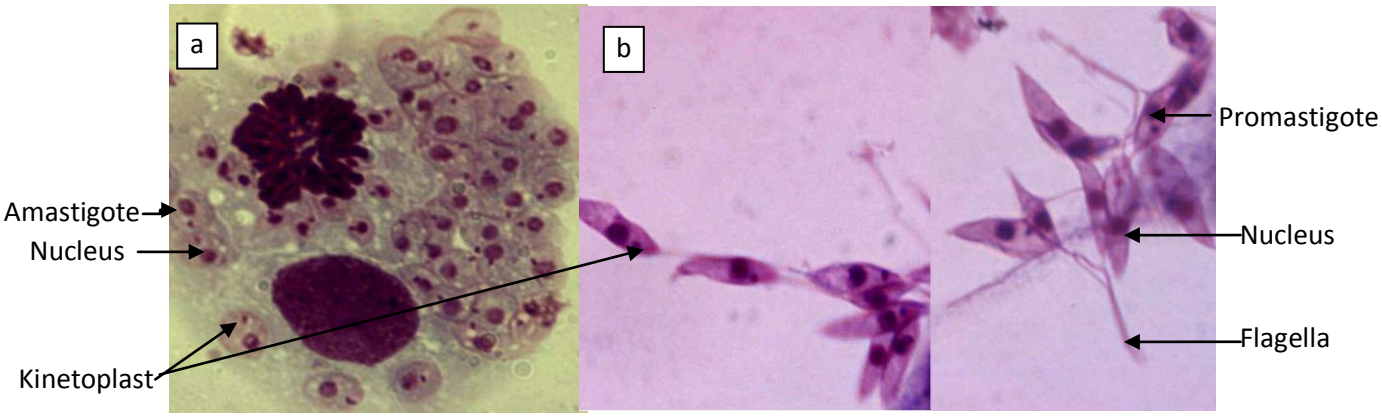

Fig 1.2 *Leishmania* cells stained with giemsa stain (a) Amastigotes with nucleus and kinetoplast but without flagella (b) Promastigote showing nucleus, flagella and kinetoplast.

Vaccines of potential interest are live *Leishmania* (leishmanization) (Bates and Rogers, 2004)**,** killed and attenuated *Leishmania made after 20 passage and* mutants of its Promastigotes differentiate into non-motile amastigotes within the phagolysosomes of the macrophage. Amastigotes multiply in the macrophage, rupture it and infect the surrounding macrophages. The life cycle of the parasite completes when released amastigotes are taken up by sandfly vector in a subsequent blood meal and differentiate into flagellated promastigotes in the sandfly mid gut. During the digenetic life cycle of the parasite, it has to survive and multiply successfully in biologically two disparate environments. This is accomplished by profound biochemical and developmental changes during the parasite's life cycle. The parasite adopts various mechanisms by which it is able to survive in such hostile environments. For successful survival and evasion of host immune response, the parasite expresses several 'virulence factors'

1.2 Prevalence of the disease

1.2.1 Global report

Nowadays, Leishmaniasis is prevalent in large land masses of world, except Australia and Antarctica Leishmaniasis is found in parts of Asia, the Middle East, Africa, and Southern Europe in case of the Eastern Hemisphere(CDC, 2010)**,(http://www.who.int/en/)** The visceral leishmaniasis have got the most severe form of Leishmaniasis, which is the second largest parasitic killer in the world (after malaria) responsible for an estimated 500,000 infections each year worldwide (global health report;2010). An estimated 500,000 new visceral Leishmaniasis cases and 60,000 deaths from visceral Leishmaniasis occur each year(Ready et al., 2008)). Visceral leishmaniasis (VL) caused by *Leishmania donovani* or kala-azar is a fatal systemic disease if left untreated(Chappuis et al., 2007; Hailu et al., 2006)There is high incidence of kala-azar in East Africa.

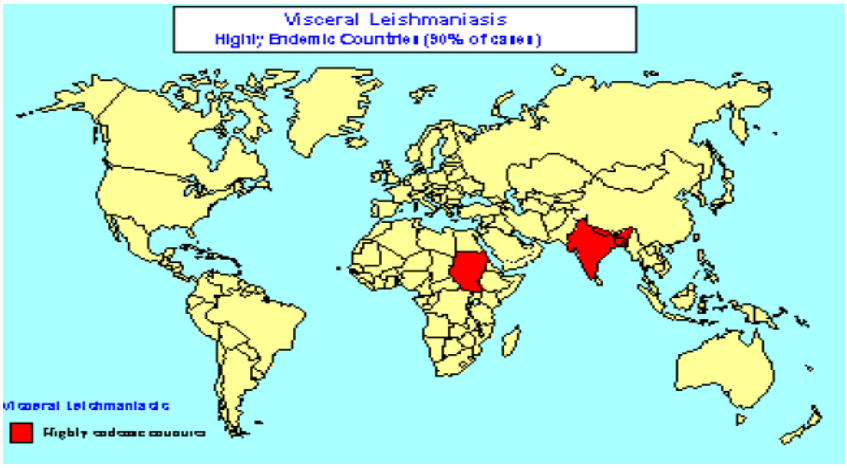

Figure: 1.3 Global distribution of Leishmaniasis A) countries highly endemic (90% of cases) for cutaneous Leishmaniasis) countries highly endemic (90% of cases) for visceral Leishmaniasis
Source (http//www.who.int/Leishmaniasis/Leishmaniasismaps/en/index2.html)

(CDC, 2010),the second leading in annual incidence in the world, next to the Indian subcontinent. (Schaefer et al., 1995; Zijlstra and EI-Hassan, 1993) The distribution of Kala-azar and incidence in East Africa are greatly influenced by environmental, behavioral and socioeconomic factors in addition to the HIV co-infection and genetic susceptibility(Alvar et al., 1989), In case of East Africa and the Indian subcontinent, VL is caused by the *L. donovani* complex, where as in case of Europe, North Africa and Latin America where the agent is *L. infantum* .The country like Ethiopia, it is the second largest number of annual VL cases (4000–7000) in Africa, next to Sudan(Kolaczinski et al., 2008; Seaman et al., . 1996).

In case of India, kala-azar (visceral leishmaniasis) has been endemic for much longer time with outbreaks was well known since the late 19th century (Hailu et al., 2009)**.** In contrast, the previous report of the disease being endemic in Nepal was in the mid-20th

century (Peters and Pasvol, 2002). Kala-azar has been always known to be a disease of the poorest of the poor, as proved in a recent study from Bihar, India (Shrestha and Pant, 1994). Housing conditions in the poor communities (mud plastered house) and the environment (damp soil and organic debris) provide an excellent breeding site for sandflies (Rijal et al; 2010). The relation between poverty and leishmaniasis is complex and involves multiple factors (Belaerti et al., 2009)**,** but lightening the leishmaniasis burden would help to alleviate poverty In recent study it has been shown that, malnutrition had no association with the infection rate, though they cannot yet conclude that it has no association with clinical VL. Malnutrition diminishes cell-mediated immunity and is a risk factor for VL in *L. infantum* endemic areas. (Alvar et al., 1989)Follow-up of these infected individuals to determine the proportion of those who develop disease may provide a clearer picture in the future. In endemic areas of VL, *L. donovani* infection does not necessarily mean clinical illness (Cerf et al., 1987).

1.2.2 Visceral Leishmaniasis in NEPAL and INDIA

In case of Nepal, Vector born disease caused by the genus *Leishmania donovani* is called the visceral leishmaniasis (VL), which was first officially identified in Nepal in 1980 from the Dhanusha district(Chappuis et al., 2007).It is now endemic in 12 lowland districts in the central and eastern Nepal bordering North Bihar and Uttar Pradesh of India, where, estimated 8.5 million population is at risk (Bista, 1998) and is transmitted by *Phlebotomus argentipes*, and hence, its transmission cycle is considered to be anthroponotic in invertebrate with humans being the only known reservoir (Rijal et al., 2010)**.** In India and Nepal, the disease has been spreading very rapidly in the past few years and has assumed alarming. The developing country like Nepal and india (where tuberculosis is more prevalent) cutaneous leishmaniasis is very likely to be mistreated as cutaneous tuberculosis It is a serious problem in Bihar, West Bengal and Uttar Pradesh where, 40% of all the world's cases of visceral leishmaniasis are found in northern Bihar alone ((Lawyer and Young, 1992).

Geographically, Nepal is divided into three areas: the Mountain, Hills, and Terai regions where, VL transmission generally occurs in plain Terai region, with an altitude of a maximum of 305 meter (World Health organissation, 2004). It has been found that , lack of surveillance, cross border migration from Bihar, inadequate treatment of cases and an increasing vector density are contributing to an increasing load of infection and disease hence Koirala et al.; 1998; described the factors that contributed to the rising trend of kala-azar in Nepal. In case of India, they have observed that, VL has been found in the hilly regions above the altitude of 1,000 m(Pandey, 2011) which could be due to the improved monitor system, seasonal climate changes over the past years, and potential changes of geographical distribution of the vectors(Mahajan et al., 2004). However, they could not justify if this patient was suffered from VL in this region because of the history of traveling to India (Pandey, 2011). It has been suggested that a higher infection

rate in men, and similar ratio has been observed in relation to VL cases. Women in these communities wear long dresses which could prove to be protective to some degree from sand fly biting (Pandey, 2011).

1.3 *Leishmaniasis* Vector and Reservoirs

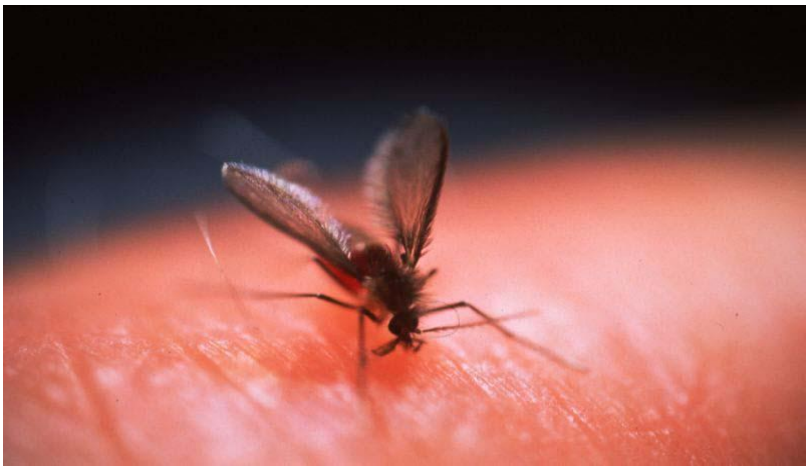

Fig 1.4 sand fly feeding blood from human host

Different species of female sandfly are the vector host of *Leishmania* parasites(Rijal et al., 2010)). The disease is transmitted by female sandflies (*Phlebotomus or Lutzomya*) that feed on the blood of an animal or human host (Cheesbrough, 1998). Most of sandfly species feed at night or dawn, usually on plant juices, they require blood meal for egg development. Species *Phlebotomus* is the main vector host of leishmaniasis in Europe, Asia and Africa, while *Lutzomyia* is the *leishmaniasis* vector in the Americas (Neva and Brown, 1994).

1.4 Major clinical symptoms

The major clinical symptoms, after, multiplication of *Leishmania* inside the macrophage of the host body, whose immune system failed to destroy the parasite (Cheesbrough, 1998) According to its symptoms, four major clinical forms of Leishmaniasis are distinguished(Berman and Neva, 1981.) they are -

- Cutaneous Leishmaniasis
- Diffuse cutaneous Leishmaniasis (DCL)
- Visceral Leishmaniasis (VL),
- Mucocutaneous Leishmaniasis (MCL)

1.4.1 Cutaneous Leishmaniasis

The identification, of cutaneous Leishmaniasis had been first reported by Alexander Russell following an examination of a Turkish patient (Zhang et al., 2003)In cutaneous leishmaniasis, The patient skin has lesion which ulcerate later & hence disfigurement

scar after healing was observed(WHO, 2007).The Parasites metastasizes through the lymphatic to the lymph node, resulting into the enlargement of the region lymph nodes and subcutaneous nodules and hence complication in some patients occur (Cheesbrough, 1998).After infection by *Leishmania* in cutaneous Leishmaniasis, Due to the host response ulceration in patient occur (Kubba and Gindan, 1989).

1.4.2 Mucocutaneous Leishmaniasis (MCL)

It is usually caused by New World *Leishmania* species such as *L. panamensis* and *L. guyanensis (Wilson and Pearson, 1990)*. Those patients who are immune compromised can show MCL symptoms by other *Leishmania* species including *L. major*, *L. infantum* and *L. donovani*. MCL starts as lesions that ulcerate and it become large and long-lasting that involve of human mucousal system (Peake et al., 1996). The parasites attacks the nasal (nasopharynx) or the buccal cavity of human body and slowly degenerate the cartilaginous and soft tissues to cause disfiguration and destruction of the nasal septum, lips and larynx (Neva and Brown, 1994).

1.4.3 Visceral Leishmaniasis

It the characteristic symptoms include bouts of fever, malaise and substantial weight loss(Cheesbrough, 1998; Cunningham, 1885; Peake et al., 1996). The clinical signs commonly seen are splenomegaly, heptomegaly, lymphatic adenopathy, wasting and pallor of mucous membranes and Visceral Leishmaniasis is accompanied by reticulo endothelial hyperplasia affecting spleen, liver, mucosa of the small intestine, bone marrow, lymph nodes and other lymphatic tissues(El Hag. et al., 1994). In VL case macrophages in all the tissues are severely parasitized and Hematopoiesis is repressed and life span of blood cells is reduced leading to anemia (El Hag. et al., 1994).

Fig: 1.5 A child with Symptoms of visceral leishmaniasis

Diarrhea may occur due to intestinal parasitism and ulceration. During advanced stages of visceral Leishmaniasis, it gets complicated by serious secondary bacterial infections such as pneumonia, dysentery and pulmonary tuberculosis (El Shoura 1994) which are generally the cause of death in case of visceral Leishmaniasis patients. Other complications that can be fatal include hemolytic anemia, renal damage and severe mucosal hemorrhage which is characterized by bouts of fever, malaise and substantial weight loss (WHO, 1991

)

The deadly visceral leishmaniasis disease shows many symtoms which are listed below-

- Anemia
- Cachexia
- Cirrhosis of liver
- Cough
- Diarrhoea
- Hepatic failure
- Hepatocellular jaundice
- Hepatomegaly
- Hyperpigmentation
- Hypoglycaemia
- Immune deficiency
- Immunoglobulin levels raised (plasma or serum)
- Lymphadenopathy
- Monocytosis
- Neutropenia
- Pyrexia of unknown origin
- Splenomegaly
- Thrombocytopenia, Fever, Weight loss

1.3.4 Diffuse Cutaneous Leishmaniasis(DCL)

Fig: 1.6 A adult with symptoms of diffuse cutaneous leishmaniasis

It occurs both in New World and Old World. Characterized by a wide, firm and smooth skin lesion which become scaly and rough later DCL in the New world caused by *L .amazonensis which* is resistant to medication, while Old world DCL caused by *L .aethiopica* relapse after treatment (Cheesbrough, 1998; Alrajhi , 2003).

1.4 Diagnosis and Treatment of Leishmaniasis

For diagnosis of Leishmaniasis microscopy of clinical specimens is generally followed. Amastigotes are identified from biopsies, scrapings or impression smears (for CL, MCL) and splenic, bone marrow and lymph node aspirate smears in case of visceral Leishmaniasis (Cheesbrough, 1998). Combining of culture and microscopy both increase the sensitivity for diagnosis and DNA analysis allows species identification (Vega-lopez,2003). Anti – Leishmanial immunoglobulin G titer determination is also commonly used for visceral Leishmaniasis (Sundar et al., 2000).Rapid detection by anti- K39 antibody with finger stick blood in an immune-chromatographic strip in 90-100% sensitive in symptomatic patients (Veeken *et al*.; 2003). Serology based diagnosis show a high degree of cross reactivity, unable to discriminate between a past and present infection and cannot be used to quantify parasite burden (Collin et al. 2004).

Among the immunoassays, Leishmanin skin test (LST) and direct agglutination test (DAT) are widely used in kala-azar endemic areas to determine *L. donovani* infection rates for epidemiological studies((Strauss- Ayali et al., 2004). But, the problem is that, kala-azar patients will not show LST positive result until 3–6 months incubation phase and less useful during VL outbreaks(Zijlstra and EI-Hassan, 1993) . Out of the several serological tests, DAT appears to be a simple and economical test with high sensitivity and specificity (Zijlstra and EI-Hassan, 1993). Although, it cannot differentiate among past kala-azar, subclinical infection, and active disease(Harith et al., 1988) In a previous study in the same region, Schenkel et al. 2006)it has been found that a DAT seroprevalence of 7.5% and LST positivity of 13.2% However, The rate of infection observed by different study, in Nepal is much lower than that in the neighboring districts in Bihar State, India (Zijlstra and EI-Hassan, 1993).

PCR based methods have been developed now for diagnosing Leishmaniasis (Chappuis et al., 2007; Sunder, 2009). *Leishmania* DNA from biopsies, peripheral blood and serum can be readily detectable by PCR testing. (Strauss- Ayali et al., 2004)**.** These methods vary in their sensitivity and are unable to quantify parasite load. More recently, quantitative real-time PCR based diagnostic methods have gained popularity due to its potential to accurately identify target DNA and determination of parasite loads in clinical samples (Osman et al., 2000).

For Visceral Leishmaniasis antimony has been used most commonly all over the world but it was found to be ineffective in Bihar, India(Bell and Ranford- Cartwright, 2002; Tupperwar et al., 2008) . Pentamidine also proved to be ineffective in India (Jha, 1983).Lipid formulations of amphotericin B have been successful in India and cures 90% of the patients ((Sundar et al., 2000). Paramomycin has completed phase IV clinical trial in India and now is being tested in East Africa. Miltefosine is the first effective oral treatment for visceral Leishmaniasis and has also been effective for antimony resistant infection (Thakur and Narayan, 2004)**.** All of the above drugs have been used extensively for curing Leishmaniasis, but they have at the same time severe limitations too such as unaffordable cost, toxicity, drug resistance, duration of treatment etc. For example - Miltefosine is teratogenic, antimonial drugs have side effects like renal injury and not user friendly administration, lipid formulation of Amphotericin B have less side effects but very expensive, pentamidine causes hypoglycemia and hyperglycemia during prolonged treatment and paramomycin being aminoglycoside causes renal toxicity and eigth cranial nerve toxicity(Sundar et al., 2000).

1.5 Vaccines for Leishmaniasis

virulence factors such as HSP100-/-, recombinant antigens such as flagellar pocket antigen, cystein protease, GP63, HSP80 and DNA vaccines such as GP63,GP46,LACK, A2**.** Many of these candidate vaccines have shown to be effective in preliminary experiments in the laboratory and their potential to be developed as therapeutics are currently being explored.(Berman and Neva, 1981.)

1.6 Life cycle of *Leishmania* parasites

1.6.1 Procyclic Promastigotes

Natural source of food for adults and flies is sugar present in the plant sap and Uptake of blood meal by a female sand fly from an infected host introduces the parasite back into the sand fly and protein content in the blood is required for the development of sandflys eggs (Desjeux, 2001) *Leishmania* have a digenetic life cycle during which the parasite differentiates into two forms. In the sandfly midgut, *Leishmania* exist as extracellular flagellated motile form called promastigotes (reviewed in (Kellick-kendrick et al., 1997) which are long, slender form of 1.5-4 µm by 14-20 µm and a motile with anterior flagellum 15-28 µm that have function in locomotion and attachment to the insect gut wall(Sacks, 2001).

1.6.2 Metacyclic promastigote

The second stage is the non-dividing infective stage found within the vector (Neva and Brown, 1994). The *leishmania* slender shape body is differentiated from non infective procyclic promastigotes by elongated flagellum, shorter, narrower body and increased expression of some surface molecules such as Lipophosphoglycan (LPG) and Glycoprotein of 63KD (g63), resulting into the formation of the parasite that is adapted to infect macrophage cells (Neva and Brown, 1994)**.** Metacyclic promastigotes are highly virulent; they migrate into and block the pharynx, mouth and proboscis of the sandfly and the development in sequence of the promastigotes from an actively dividing, non infective stage to a non dividing, highly infectious stage in the sand fly is known as metacyclogenesis (Liu and Chang, 1992)

1.6.3 Amastigotes

Amastigotes are non motile ,it does not have flagella, rounded, about 2-5 um diameter established within macrophages Phagolysosomes of mammalian hosts or any other mononuclear phagocytes (Sacks and Perkins, 1984). looking at the phase contrast light microscopy by giemsa stain *Leishmania* has a rod shaped kinetoplast (a specialized portion of the highly extended single mitochondrion), nucleus at the central and sometimes a basal body that contains the centriole construction (Chang, 1990; Neva and Brown, 1994).

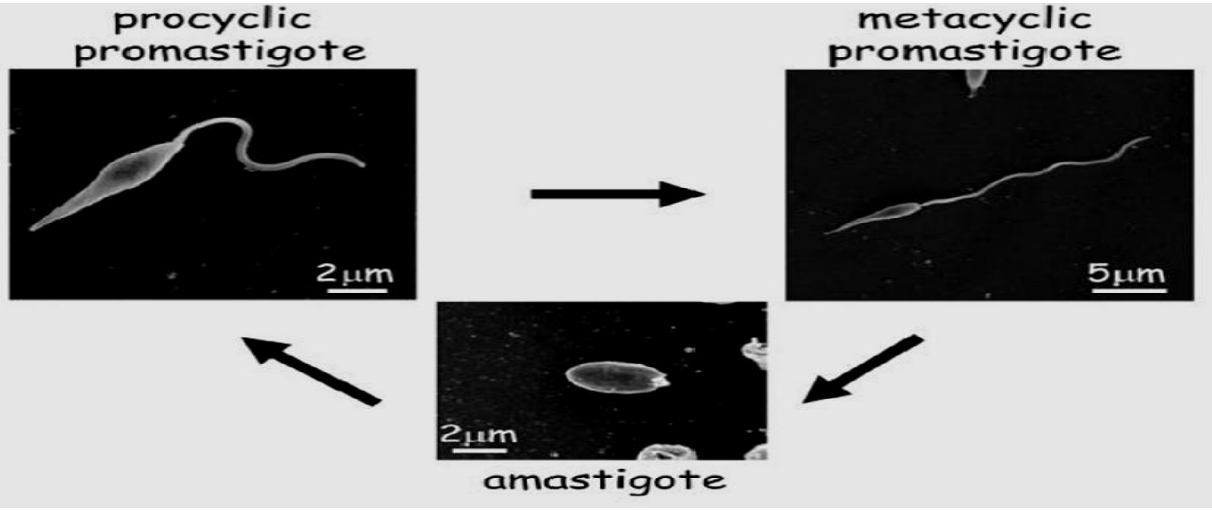

fig :1.7 The Leishmania life cycle taken from by scanning electron microscope images of the main Leishmania major life cycle stages, the first one is the , procyclic and second one is the metacyclic promastigotes were grown in culture, the virulent form of *Leishmania* ,amastigote was isolated from an infected macrophages isolated from mouse.

1.6.4 Life cycle in human

Infected female Sandfly uptake a blood meal from a healthy person for their feed and injects the infective metacyclic promastigotes into the host blood stream (Chang, 1990; Neva and Brown, 1994).The host cell Macrophages will be attracted to the infection site to up take the pathogen (Peters and Pasvol, 2002), which immediately, enters a sac-like organelle known as parasitophorous vacuole(Zer et al., 2001). Multiple functions of host cell macrophage are recognized in *Leishmania* life cycle. They serve as host cells for *Leishmania* replication and as a source of cytokines produced after infection by parasites. Which modulate the T cell-mediated response of healing mechanism that may kill the parasite (Ali and Bahador, 2005). The promastigotes stage change shapes inside the macrophages, lose the flagellum and become round amastigote as an adapting mechanisms which helps to the parasite survival within the macrophages (Teixeira et al., 2006). The mechanisms through which the parasite tolerate killing within the toxic environment of the phagolysosome of host cell remain incompletely defined.

Infected female Sandfly takes a blood meal from a healthy person and injects the metacyclic promastigotes parasitess into the host blood stream (Chang, 1990; Neva and Brown, 1994). Macrophages will be attracted to the infection site to up take the pathogen (Peters and Pasvol, 2002), which enters a sac-like organelle known as parasitophorous vacuole(Zer et al., 2001). Multiple functions of macrophage are recognized in *Leishmania* life cycle. They serve as host cells for *Leishmania* replication and as a source of cytokines.

The location of *Leishmania*-infected host cell macrophage differs according to the parasite species, effectiveness of the immune system, and the host cell temperature. For example, *L. donovani* can live at the high body temperature at 37 degree within deep organs such as spleen, liver, lymph glands, bone marrow and other tissue of the reticuloendothelial system, but in case of *L. major which* remains in the external tissue macrophages (Ali and Bahador, 2005) . Amastigotes start multiplication by asexual binary fission and accumulate in the macrophage until it ruptured to release the amastigotes. Then amastigotes will be picked up by new circulating or other local macrophages, this cycle continues and resulting into the one of the clinical symptoms of *Leishmaniasis* with different species having different tropism for macrophages in particular organ in the host (Cheesbrough, 1998; Neva and Brown, 1994; Peters and Pasvol, 2002). Finally, the infection can be transmitted among people *via* sandflies feeding on macrophages containing amastigotes (Chang, 1990).

1.6.5Life cycle in sandfly

Intracellular and free amastigotes after released from parasitophorous vacuole are ingested by female Sandfly while feeding blood from an infected individual. In the midgut, these

round amastigotes change back into elongated and flagellated promastigotes forms (Peters and Pasvol, 2002) . These promastigotes attach themselves to either midgut or hindgut wall of the sandfly, where they multiply, mainly by asexual binary fission (Cheesbrough, 1998; Peake et al., 1996). Then *Leishmania* promastigotes develops into metacyclic promastigotes by several biochemical and slight morphological changes of *Leishmania* surface molecules (Peters and Pasvol, 2002). Finally, metacyclic promastigotes migrate forward into the pharynx and proboscis- of sand fly remains there- until they will be injected into a new host when infected female sandfly is ready for another blood meal(Ali and Bahador, 2005; Neva and Brown, 1994) . However, salivary glands do not become parasitized due *Leishmania* life cycle, some studies demostrated that female sandfly saliva may play an important role in the infection (Neva and Brown, 1994) . According to Zer and his colleagues, Low dose of *Leishmania* promastigote unable to initiate the infection of susceptible mice strain, while similar dose flourish the infection if inoculated by female sandfly. Saliva of female sandfly might explain this phenomenon *via* its effect on T-lymphocytes, which secrets cytokines like, Inter leukin-4 (IL-4) that attracts the host macrophage to the spot of the infection (Cheesbrough, 1998; Zer et al., 2001).whereas, other people, suggested that the saliva contains specific peptide which inhibits macrophage production of TNF-α and decreases their ability to make nitric oxide in order to destroy the parasite (Zer et al., 2001).

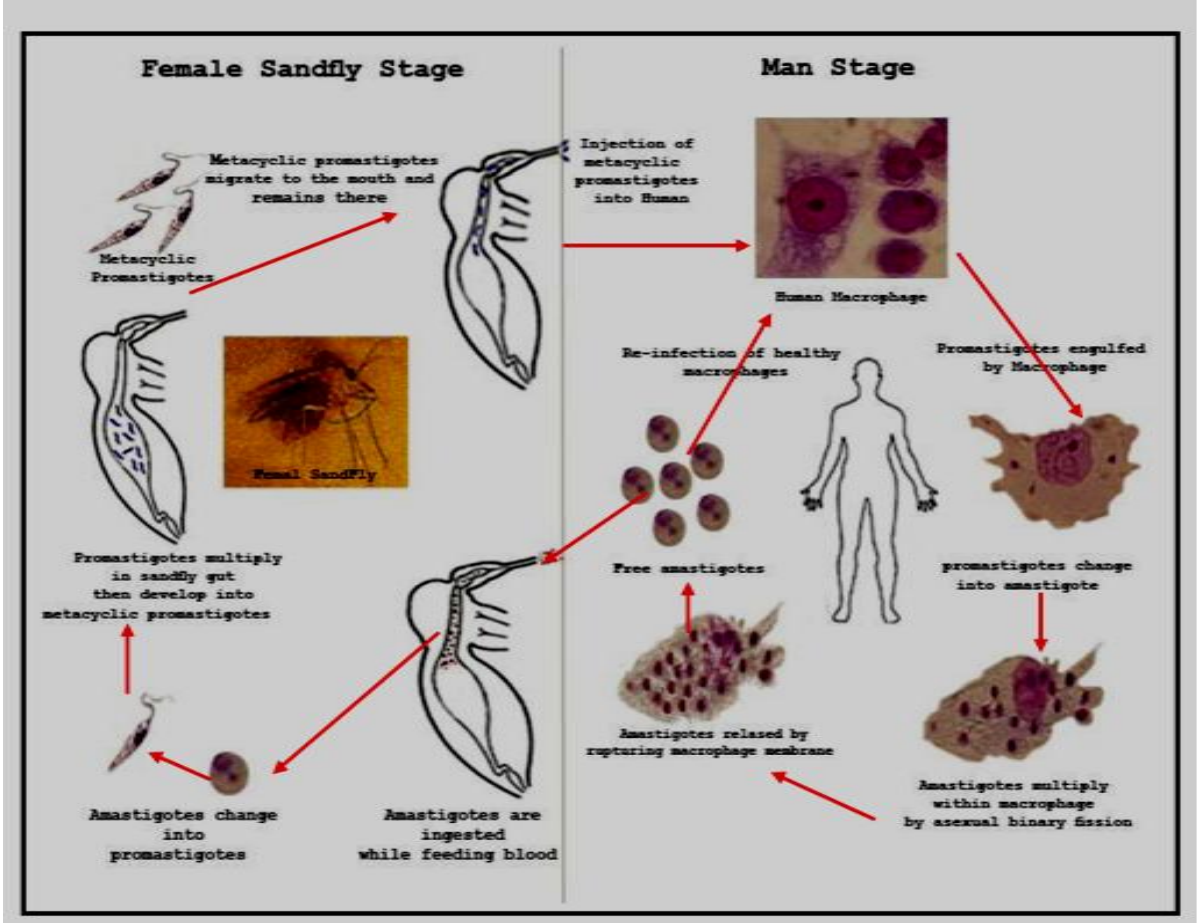

Fig:1.8 An illustration showing Leishmania life cycle.female sandfly image and, sandfly diagrams were adapted and modified form (Cunningham, 1885)

1.6.6 Navigation from skin to Viscera

The sand fly while feeding lacerates the blood vessels so that the *Leishmania* are introduced into the blood stream (Neva and Brown, 1994). In the blood stream free amastigotes have been detected so there is a possibility that they are directly delivered to the organs such as spleen and liver(Bates, 2007) . It is also possible that the spread to the visceral organs is due to the movement of infected cells. Neutrophils are the earliest cells that are found at the site of sand fly bite and thus represent as the first infected cell population (McCall et al., 2013). Neutrophils that are infected along with free parasites present in the blood are then taken up by dendritic cells and macrophages, which migrate away from the site of sand fly bite (Ribeiro-Gomes et al., 2012).

1.7 Genomic study of *Leishmania*

Previously, the *Leishmania* genome network (LGN) was set up in 1994 with the support of the World Health Organization (WHO).The reference strain *Leishmania major* MHOM/IL/81/Friedlin (LmjF) was selected for genome mapping and sequencing studies (Ribeiro-Gomes et al., 2012)(Ravel et al., 1998). Old World *Leishmania* (*L. donovani* and *L. major* groups) have 36 chromosome pairs (0.28 to 2.8 Mb)(Ravel et al., 1998), whereas New World species have 34 or 35, with chromosomes 8^{th} & 29^{th} and 20^{th} & 36^{th} fused in the *L. mexicana* group and 20^{th} & 34^{th} in the *L. braziliensis* group(Wincker et al., 1996).

The genome sequence of *Leishmania major* (the causative agent of cutaneous leishmaniasis) (Britto et al., 1998) was completed along with the genome sequence of two other kinetoplastid parasitic protozoan, *Trypanosoma brucei* (the causative agent of African trypanosomiasis) (Ivens et al., 2005) and *Trypanosoma cruzi* (the causative agent of Chagas disease) (Berriman et al., 2005.). The sequencing of *L. infantum* clone JPCM5 (MCAN/ES/98/LLM-877) and *L. braziliensis* clone MHOM/BR/75M2904 are also completed and sequencing of *L. mexicana* clone MHOM/GT/2001/U1103 is already started (http://www.genedb.org). Sequencing projects of tritryps suggested that they have ~6200 trypanosomatid specific genes.

Most of the *Leishmania* genes have ortholog in the other *T. brucei* and *T. cruzi*. Still 910 genes in *L. major* don't have their ortholog in tritryps and supposed to be "*Leishmania*-restricted" genes. These genes are randomly distributed in the genome and most of them (~ 68%) have unknown functions (El-Sayed and Blandin, 2005). A comparative study of three genome of *Leishmania* spp revealed conserved gene content, synteny and architecture of genome but also found ~ 200 disparities in the gene or pseudo gene content**)**. Pathologies of *L. infantum* and *L. donovani* are very similar therefore genome sequences and organization could also be similar.

1.8 *Leishmania donovani* with Meta1 protein

Macrophages, the host cells for the intracellular parasite *Leishmania*, are crucial for the outcome of disease. Assays with macrophage-promastigotes models are considered closer to the pathophysiological conditions of *Leishmaniasis*, and are therefore the most appropriate for in vitro screening. The gene *Ld*Meta1 is derived from metacyclogenesis where *Ld*Meta1 gene is upregulated in metacyclic promastigotes of *leishmania* species. People suggested that, the mechanism that trigger metacyclogenesis in vivo is poorly known where as the in vitro is induced by low ph and anaerobic condition(Ivens et al., 2005) and a decline in levels of tetrahydrobiopterin (a cofactor essential for the catalytic activity of nitric oxide synthase and a byproduct of biopterin reduction by pteridine reductase 1) (Mendez, 1999) high temperature 37°c. Several genes have been identified which is specific to or upregulated in the metacyclic stage, but their function remains poorly defined, such as the Meta 1 gene cluster, have been associated with increased virulence in the vertebrate host (Cunningham, 2002)

Among the stage-regulated genes investigated in *Leishmania major*, the *Ld* Meta1 gene is present and conserved in sequence in all *Leishmania* species analyzed**.**(Ramos et al., 2004,) However, recent study suggested that there is no correlation between *Meta 1* expression and cell infectivity or the number of metacyclic cells present in culture. Although, *Meta 1* did overlap with the phase in which the metacyclic percentage in culture started to increase. Hence it has been suggested that *Meta 1* might be involved in cell differentiation from the procyclic form into the metacyclic form, preparing the cells for the infective form in the insect host, and that it could be an indicator of the differentiation rate in the culture.

1.9 Hypothesis

- *In vitro* model of *Leishmania* infection allows one to learn about the virulent genes involved in infection & to determine its function coherently. There are still many unanswered question about the virulence mechanism of *Leishmania*. It has been known that gene expression pattern of *Ld*Meta1 GFP is promastigote specific and it is upregulated in stationary phase of metacyclic promastigotes. So our hypothesis is; the over expressed *Ld*Meta1 GFP gene play crucial role in virulence.

- The infectivity rate of *Ld*Meta1 GFP is different from the control *Ld*GFP promastigotes in *in vitro* model. Similarly, the subsequent process of attachment, internalization, multiplicity, exit of *Leishmania* for **stationary phase** at **temp 37^{o}C** by host cell lysis is also different.

- There is difference between *LdMeta1* GFP and *Ld* GFP infectivity at early & later time points. Their rate and quantum during their interaction with j774 macrophage host

cell and host response after interaction with parasites having different gene expression profiling express differently.

1.10 Objectives

General objective

To identify the role of *Ld*Meta1 GFP protein in *Leishmania in vitro* by macrophage infection assay

Specific objective

1. Identification of the *Leishmania* with overexpreesed Meta1 gene by Polymerase chain reaction
2. *In vitro* kinetic study, quantification & elucidation of different strains of *Leishmania* that infect macrophages during attachment, internalization, proliferation & exit from the macrophages.
3. Identification of the total infectivity of host cell by macrophage infection assay.
4. Evaluation of the parasite load on host cell by infection assay.
5. Observation of infected macrophages at different time points by phase contrast microscopy.

1.11 Rationale

The assay has been working on *L. donovani* which belong to the kinetoplastida order that, cause the deadly form of the disease that is visceral leishmaniasis (VL). VL is the major burdens on human health in developing countries, so it ranked as one of the fourteen "most neglected tropical diseases". It is the most severe form of the disease, confirm fatal if left untreated. In the poor population, people are uneducated therefore, need to carry preventive as well as the control measures. Due to the lack of knowledge on virulence mechanism, scientist is unable to develop the effective drug, vaccine and prophylactics.

Nepal is one of the endemic country for visceral leishmaniasis. About 8.5 million people are in threat of the disease and many people are living with the least econom,ic profile. They have daily income which barely covers their food to survive. Being neglected disease, world has yet not expected the easy remedies for its' control, though many scientist are working hard to understand the molecular, immunological, biomarker, signaling pathways, biochemistry etc of the parasite as well as host. Macrophage infection assay is the one of the simple reliable and low cost taking technique to identify how the *Leishmania* interact with mammalian body after suffering from these diseases. The answer is very useful for the

human resources engaging to unveil the factual cause of infectivity in order to control VL. Hence, this research work might help to identify the role of different virulence associated gene and their mechanism on mammalian host. Based on the outcome of the work, it might help in the development of new targeted drug and vaccine candidate for prevention. So this work is justifiable to carry.

Following two findings independently implicate *Ld*Meta1 GFP associates with virulence of *Leishmania*. However, exact function of Meta1 protein is still not understood. Because of association of Meta1 gene with virulence and its developmental regulation, we decided to work on the following questions-

1. How does META 1 influence / contribute to virulence of the parasite in an in –vitro infection assay?
2. Is there any difference between expression profiles of host macrophages when infected with *Ld* Meta1 GFP and the *Ld* GFP?

The ability to perceive clearly, Meta1 function will provide us a better understanding of virulence mechanisms of *Leishmania* on host cell. It will also help us to understand *Leishmania* biology better with respect to *Ld*Meta1 GFP. Definitely, after knowing host pathogen interaction *in vitro*, we would get some idea and clue about how pathogen interacts with host cell *in vivo.*

1.12 Scope

It will help to explain about the virulent antigenic protein which will ultimately useful for vaccine development, drug development, production of synthetic epitope for antibody production in animal model etc.

Chapter II

LITERATURE REVIEW

2.1 *Leishmania* parasite

The Infectious diseases are caused by pathogenic microorganism such as bacteria, viruses, parasites and fungi etc. which can be spread directly or indirectly from one person to other person. Most of these diseases are known as “neglected” in the absence of effective, affordable or ease of treatment. The concept of “neglected disease” has been derived from the requirements in the development of new drugs to combat from the infectious diseases which have been ignored by public and private sector such as cholera, Buruli ulcer, Lymphatic Filariasis, chagas disease and African trypanosomiasis etc. Approximately 1 billion people in the world suffer from one or more “neglected tropical diseases” (NTDs) primarily to the poor population living in tropical and subtropical regions of the world (WHO Fact Sheet, 2008). Among the classical neglected diseases, leishmaniasis has a major impact on the mortality, morbidity and its geographical distribution and remains one of the major burdens on human health in developing countries (Uliana et al., 1996,). Hence, leishmaniasis is ranked as one of the fourteen “most neglected tropical diseases” (Hotez et al.). *Leishmania* are the microscopic, unicellular protozoan parasites that are causative agents of a variety of diseases in humans and animals. The etiological agents causing leishmaniasis was first discovered by Sir William Leishman in1900, and later it was independently confirmed by Charles Donovan in 1903. The term "*Leishmania*", was first introduced by Sir Ronald Ross in 1903. The spectrum of disease caused by the different species of *Leishmania* is collectively known as leishmaniasis(Yamey and Torreele, 2002) Different species of *Leishmania* cause different clinical manifestations that differ in severity. The General status of health and genetic makeup of the host also contribute in the severity and clinical manifestation of disease (Lainson and Shaw, 1973). Leishmaniasis is classified as one of the “most neglected diseases” based on the limited resources invested in diagnosis, treatment and control, and its strong association with poverty. Leishmaniasis being one of the opportunistic infections can also infect hosts who are immune compromised such as those who are infected with the human immune deficiency (HIV) virus or those who are taking immune- suppressive drugs , such as in the case of cancer therapy or organ transplantation

There are two main life cycle stages of *Leishmania. Leishmania* exists as extracellular, flagellated, less infective procyclic promastigotes within the mid gut of the sandfly.

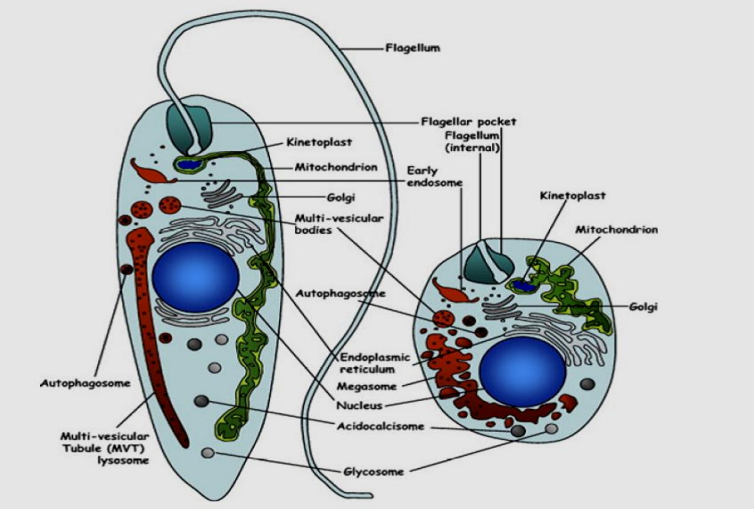

Fig:2.1 Diagrammatic, representation of the main intracellular organelles from *Leishmania* promastigote (left) or Amastigote (Blackwell et al., 1985) forms, the flagellar pocket marks the anterior end of the cell [Source(Besteiro et al., 2006.)

These procyclic promastigotes undergo developmental changes and become highly virulent or metacyclic, and thereafter, migrate to the mouthparts of the sandfly. This process of differentiation is called as metacyclogenesis. When the sandfly takes the blood meal, metacyclic promastigotes get transferred to the bloodstream of mammalian host where they are phagocytosed by macrophages. Promastigotes differentiate into non-motile amastigotes within the phagolysosomes of the macrophage. Amastigotes multiply in the macrophage, rupture it and infect the surrounding macrophages. The life cycle of the parasite completes when released amastigotes are taken up by sandfly vector in a subsequent blood meal and differentiate into flagellated promastigotes in the sandfly mid gut. During the digenetic life cycle of the parasite, it has to survive and multiply successfully in biologically two disparate environments. This is accomplished by profound biochemical and developmental changes during the parasite's life cycle. The parasite adopts various mechanisms by which it is able to survive in such hostile environments. For successful survival and evasion of host immune response, the parasite expresses several 'virulence factors'.

2.2 Current studies

Leishmania exhibits many peculiar features in their genome organization and gene expression. In *Leishmania,* genes are organized in a large clusters compromising up to hundreds of genes, in the same 5'-3' direction along the chromosome DNA strand called Directional gene clusters (DGCs) (Besteiro et al., 2006.). The *L. major genome* is categorized into 133 DGCs of tens to hundreds of protein coding genes with unrelated predicted

function. The cluster size can be up to 1260 kb and separated by divergent or convergent strand switch regions of 0.9-14 kb (Ivens et al., 2005). None of the known sequences responsible for initiation and regulation of eukaryotic gene expression have been observed in *Leishmania* (Myler et al., 1999; Worthey et al.). Generally transcription undergoes polycistronic transcription which initiates in both direction from a divergent strand switch region .(Cruz and Tosi, 1996) and ends with convergent strand switch regions which often include tRNA, rRNA and/or snRNA genes.

people produce high-quality reference genomes and then, compare them with the genomes of related strains of unknown or other species to provide evidence of drug selection, identify genes involved in host–pathogen interactions, or uncover other biologically interesting features. As a follow-up to the African trypanosome (Trypanosoma brucei), people have sequenced the genome of the subspecies responsible for most human deaths in Africa. (http://www.genedb.org).

2.3 Virulence Mechanisms in *Leishmania*

Leishmania virulence certainly is modified by the environmental and genetic factors of its mammalian host (Martinez-Calvillo et al., 2004; Martinez-Calvillo S. et al., 2003)and sand fly vector(Blackwell, 1996).Amastigotes that are released into the gut of the sandfly from infected macrophages (after a blood meal) transform into procyclic promastigotes, which multiply rapidly and does not infect the vertebrate host (Titus et al., 1995). The promastigotes, unlike the amastigotes, express on their surface lipophosphoglycan (LPG) in large quantities and also they express metalloprotease gp63 (Sacks and Perkins, 1984). Both of the above glycoconjugates are thought to play a role in protecting promastigotes from hydrolytic enzymes in the gut of the sandfly, (Davies et al., 1990) whereas LPG mainly facilitates attachment to the insect gut epithelium. Transformation from non-infective dividing procyclics to infective non-dividing metacyclics can involve changes to the LPG structure(Sacks and Perkins, 1984); up regulation of gp63 expression (McConville and Blackwell, 1991; Turco and Descoteaux, 1992) and also changes in enzyme content (Alexander and Russell, 1992; Kweider et al., 1987) varying with the strain.

2.4 *Leishmania* - macrophages cellular interaction

There are two types of Immune response one is the nonspecific (innate) and another is the specific (adaptive) (Mallinson and Coombs, 1989). Innate response are those that includes defense in a non- specific manner by the activity of the complement system and some leukocytes, such as monocytes, resident macrophage, basophils, eosinophils, neutrophils, dendritic cells, mast cells and natural killer cells (Sherwood, 2004). In order to develop a

successful parasitic relationship of *Leishmania* with its host, the *Leishmania* must evade both the non specific and specific immune responses (Sherwood, 2004; Wilson and Pearson, 1990)where they are obligated to parasitize, survive and multiply inside the phagolysosomes. Macrophages play a crucial role in *Leishmania* infection so that *the* promastigotes form of Leishmania enter a parasitophorous vacuole after phagocytosis by macrophages in which the macrophage can provide a safe place for the parasite to change into amastigotes and proliferate in a naive host(Chang, 1990; Croft and Coombs, 2003). Amastigotes that are released into the gut of the sandfly from infected macrophages (of vertebrate host (Alexander and Russell, 1992) The surface layer of *Leishmania* promastigotes is coated by a dense glycocalyx (literally "sugar coat") which is rich in glycosylated phosphatidylinositol (GPI) glycolipids and that covers the entire promastigotes and it is unable to see by electron microscopy (Sacks and Perkins, 1984). This dense glycocalyx contains lipophosphoglycan (LPG), proteophosphoglycans (PPGs), low molecular weight glycoinositol phospholipids (GIPLs), and GPI anchored proteins such as the metalloproteinase of abundant 63-kDa surface proteinase, gp63,(Pimenta et al., 1991).In contrast to promastigotes stages, the infective stage of *Leishmania* amastigotes downregulate the expression of LPG and other surface macromolecules and have a much reduced surface coat, where as a glycocalyx of parasite derived GIPLS and host derived sphingolipids is retained(McConville and Blackwell, 1991).

At the time point to infect the vertebrate host, the transformation from procyclic to metacyclic brings changes of LPG structure and enzyme content, and upregulation of gp63 expression (McConville and Blackwell, 1991), up regulation of gp63 expression (Sacks and Perkins, 1984; Saraiva et al., 2005; Turco and Descoteaux, 1992)and (Alexander and Russell, 1992; Kweider et al., 1987; Ramamoorthy, 1992) varying with the strain.

Furthermore, *Leishmania* try to escape from the defence system of macrophages by inhibiting the macrophage ability to produce the parasite antigen to other components of immune system (Mallinson and Coombs, 1989) Due to these reasons, *Leishmania* manages to survive, differentiate into amastigotes, and multiply by binary fission within those cells (Olivier et al., 2005).

2.5 Role of Host macrophage associated gene and their regulation (TH1 and TH2 Response)

It has been shown that, in mice and man the multiple genetic loci influence the success of infection, affecting both acquired and innate immune responses against the parasite (for useful overviews see (Chang, 1990). Both Th1 and Th2 effectors differentiate from naïve CD4 T cells depending on the type of cytokines in the environment and the stimulating antigen (Blackwell, 1996).Previous studies have shown that animals with paucibacillary lesions are likely to express a cell mediated, Th1-type, immune response that is protective

against intracellular bacteria, while a Th2-type response is generally detected in animals with multi-bacillary lesions (OŠ, 2000). Nevertheless, it is widely known that the events responsible for resistance or susceptibility occur early in infection and appear to involve elements of the innate immune response that precede the development of specific Th1 and Th2 cells (Begg et al., 2010).

People found that, NK cells activated by IL-12 from macrophages (or dendritic cells) are the primary source of early IFN-γ, which not only plays an important role in controlling early resistance to *L. major* but is also influential in initiating Th1 differentiation which is the healing mechanism in resistant mice (Chatelain et al., 1992). IL-12 activity from macrophages appears also to be augmented by IL-18: simultaneous administration of IL-18 and small quantities of IL-12 enhances the protection of BALB/c mice against cutaneous *L. major* infectio(Scharton-Kersten et al., 1995). Although previous studies found that IFN-γ-producing gama delta T cells play important role in the development of protective immunity against *L. major (Yoshimoto et al., 1998)*, Recently, studies have demonstrated that a Th1 response develops, and healing takes place, in the absence of such cells in genetically resistant mice (Rosat et al., 1993). Several immunological mechanisms described that the non-healing response observed following infection with *Leishmania* have been observed, These include an IL-4-driven Th2 response that down regulates Th1 development(Satoskar and Alexander, 1995) , the presence of a Th2 response along with a Th1 response, the absence of a Th1 response irrespective of the presence of a Th2 response (Chatelain et al., 1992; Leal et al., 1993), and unable to produce or respond to IL-12 .

These apparently different observations may reflect the experimental systems being used and differences in both the species of parasite and the mouse strains examined. Nevertheless, people observed many experiment and have got results where it is generally accepted that early IL-4 synthesis is essential for the initiation of Th2 development. IL-4 not only downregulates IL-12 and IFN-ɣ production and IL-12R expression, but also inhibits macrophage NO production, which is crucial for macrophage leishmanicidal activity(Satoskar and Alexander, 1995). In the murine cell line, *L. major* model, CD4+ T cells are a primary source of early IL-4 that renders T cells unresponsive to IL-12 in BALB/c mice and induces the development of a Th2 response (Chatelain et al., 1992; Macatonia et al., 1993)

2.6 Entry of *Leishmania* in Macrophages

The host macrophage is said to be phagocytic cell which plays important role in the infection. The attachment of *Leishmania* into host macrophage is carried out by receptor-mediated phagocytosis (Launois et al., 1997). The parasite promastigotes/amastigote must

bind to the surface of the macrophage in order to be engulfed and The *Leishmania* attached to macrophage receptors can be achieved through interaction between several native surface molecules of *Leishmania(Chang, 1990; Kane and Mosser, 2000)* or through complement system of the host cells (Chang, 1990; Neva and Brown, 1994). The primary method of *Leishmania* attached is the interaction between phagocytic macrophage complement receptors and surface molecule glycoprotein of Leishmania 63KD (gp63), Hence it is the most crucial surface molecule of this process (Chang, 1990).

2.7 Intracellular survival of *Leishmania* in Macrophage

Leishmania itself adopt from the unfavorable environment inside the phagolysosomes by several mechanisms (Chang, 1990) *Leishmani*a has several defensive enzymes and a functional molecular surface system, both can survival inside the phagocytic cells(Cunningham, 2002) the achievement is due to down regulation of host phagosome-endosome fusion, hydrolytic enzymes, cytokines production, nitric oxide production and manipulating cell signaling pathways (Chang, 1990).

2.8. Invasive/evasive determinants

These are the determinants which are crucial for infection but are absolutely not responsible for pathology in the host (Cunningham, 2002). These determinants help in following events of intracellular parasitism:

a) Avoiding of humoral lytic factors like complement system

b) The Attachment and internalization of *Leishmania* parasite into macrophages

c) Then, Survival of the parasite into host cell macrophage

d) Differentiation of Promastigotes into infective amastigote stage of the *Leishmania*

e) Replication of infective amastigote form into host cell macrophage

These determinants help intracellular amastigotes maintain continuous infection by growing at a slow rate in the parasitophorous vacuoles of host macrophages. These determinants proposed to be evolutionarily selected to become immunologically 'invisible', hence facilitating pathogen invasion into the hosts by evading their immune response (Chang et al., 2003). There are several invasive/evasive molecular determinants which are well characterized and studied. Some are lipophosphogylcan (LPG), Leishmanolysin (GP63), phosphoglycans (Alexander and Russell, 1992; Chang, 1990), Proteophosphoglycans (PPG), Cystein Proteases (CPs), Glycosylphospholipid (GIPL), Glycosylphosphotidylinositol (GPI) and

others. These molecules by some means help *Leishmania* in one of above events during infection.

Phagolysosomes of the macrophages are the final destination of the promastigote. Earlier it was thought that macrophages are the first cells that encounter the promastigotes. Promastigotes reaching the dermis at the wound site rapidly adhere to resident recruited macrophages(Lawyer and Young) . The binding of the parasite to the macrophage is mediated through a number of receptors of which complement receptor I (CRI) and complement receptor III (CRIII) are the most important (Alexander and Russell, 1992). Components of the complement system that bind to various molecules on the parasite membrane help in docking the parasite to the receptors on the macrophage cell surface. Now a day's, evidences are increasing that PMNs are the first cells that first encounter the promastigotes as they enters into host. In both type of cells adhesion of the parasite is followed by rapid internalization. Phagocytosis by the host cell is the basic mechanism involved in the uptake of the parasites (Brittingham and Mosser, 1996). Immediately following phagocytosis, *Leishmania* are located in endocytic compartments limited by membrane derived from the plasma lemma of the macrophage(Alvar et al., 1989) . These phagosomes undergo maturation and subsequently fuse with the lysosome, giving rise to the parasitophorous vacuoles (Antoine et al., 1998). Within the PV, the promastigotes converts into the amastigote form in about 2-5 days after the infectious bite. *Leishmania* amastigotes remain tightly bound to the PV membrane via the posterior pole (Lang, 1994).

2.9 The Parasitophorous Vacuole

When, macrophages are attracted to the infection site to uptake the pathogen(Alexander and Russell, 1992) , which enters a sac-like organelle known as parasitophorous vacuole (Zer et al., 2001).After phagocytosis, *Leishmania* promastigotes enter a parasitophorous vacuole within which the macrophage can provide a safe haven for the parasite to transform into amastigotes and proliferate in a naive host (Ali and Bahador, 2005).Immediately after phagocytosis, the infective metacyclic promastigotes located within macrophage derived phagosomes fuse with endocytic organelles resulting in the formation of an acidic compartment known as the parasitophorous vacuole (Alexander and Russell, 1992). Within the PV the infective metacyclic promastigotes differentiate into replicative amastigotes that periodically escape from the host cell to reinfect other phagocytic cells (macrophages or dendritic cells) or non-phagocytic cells (fibroblasts) (Antoine et al., 1998).

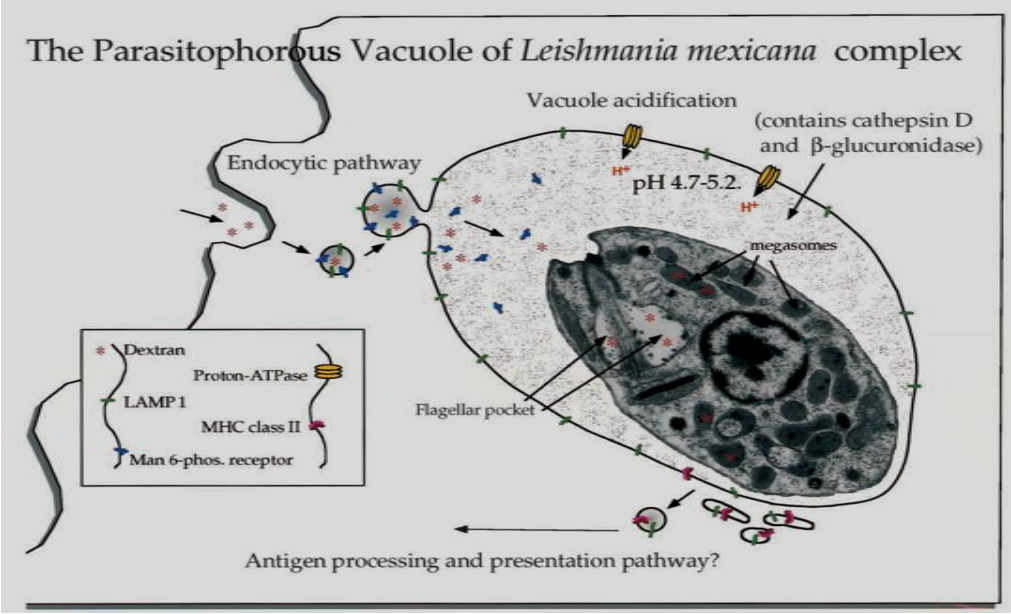

Fig.2.2 The amastigotes of *Leishmania mexicana* reside within a membrane-bound vacuole inside the host macrophage. The vacuole is acidic and appears to contain a full complement of active hydrolases. Nonetheless, the parasites thrive and multiply in this environment. The interaction is an extremely stable one: in culture, parasitised macrophages are capable of persisting for weeks until the parasite burden eventually causes the host cell to degenerate and die. It is only if the macrophage is activated that the parasites are killed and then 'absorbed' by the host cell, then returns to its normal appearance ,modified from(Ali et al., 2013) .

Previous investigation on the parasitophorous vacuole of *Leishmania* suggested that it can fuse with compartments containing endocytosed electron-dense colloids (McConville et al., 2007). Recently, studies on *L. mexicana* and *L. amazonensis* have shown that PV vacuoles are late endosomal in nature so they have a pH of 4.7-5.2(Alexander and Vickerman, 1975) which, contain the lysosomal hydrolases cathepsins D, B, H and L and the lysosomal-membrane markers LAMP1 and LAMP2, the proton ATPase and MHC class II molecules (Antoine et al., 1990). When, the vacuole matures, it acquires the cationic-independent mannose 6-phosphate receptor (Russell, 1994), and it has ability to fuse with early endosomes and hence, enhanced,

2.10 The Role of *Leishmania* Surface Molecular components

There are different types surface molecules which helps to protect the parasite within the phagolysosome of the human host cells such as gp63 and LPG by monitoring lysosomal enzyme and inhibiting the oxidative burst.(Lang, 1994).

2.10.1 Leishmanolysin (GP63)

GP63 is a zinc dependent metallo-proteinase attached to the parasite membrane by a GPI anchor (Cunningham, 2002; Olivier et al., 2005). Expression of GP63 is found to be up regulated during metacyclogenesis and plays a role in protecting the parasite from complement-mediated lysis (Wilson and Pearson, 1990). GP63 is the only ecto-proteases expressed by all pathogenic *Leishmania* and serve as ligands for binding macrophage complement by cleaving C3b to C3bi which binds to CR1 and CR3 fibronectin receptors on the macrophage surface (Turco and Descoteaux, 1992). GP63 also plays a role in suppressing the oxidative burst (Brittingham, 1995; Brittingham and Mosser, 1996) and its proteolytic activity protects the parasite from cytolysis and degradation by lysosomal enzymes (Sorensen et al., 1994). Hydrolytic enzyme of host i.e lysosomes are destroyed by protease enzyme gp63 and at the same time some of the Leishmania species discharge ammonium ions which, act upon one another with lysosomal hydrolyti enzymes activity directly or by enhancing the phagolysosomal ph (Seay et al., 1996).

2.10.2 Phosphoglycans

These are most abundant molecules of *Leishmania* surface and consist of polymer of disaccharide phosphate repeat back bones [Galα1, 4Manα1-PO_4]. When they are attached to the membrane through GPI (Glycosylphosphotidylinositol) they are LPG and when they are attached through proteins they are PPGs (Proteophosphoglycans) (Chang, 1990). LPG also transiently inhibits the fusion of the phagosome with endosomes (Dermine et al, 2000), scavenges oxygen radicals produced during the oxidative burst (Iigoutz and McConville, 2001), inhibits protein kinase C (PKC) activity (Chang, 1990), inhibits macrophage IL-12 synthesis and suppresses macrophage nitrogen oxide synthase 2 expressions and NO production . Loss of LPG through the alteration in *LPG1* galectofurnosyl transferase gene in *L. major* results in the loss of ability to survive in the sand fly vector and to establish the infection in macrophages. This is due to the increased susceptibility to complement system and oxidative stress(Giorgione and J-Turco, 1996). However LPG synthesis is very much down regulated in amastigote form and seems to have no role in virulence for amastigote(Spath, 2003).

The abundant infectious cell surface glycolipid lipophosphoglycan (LPG) is highly anionic in nature, might act as degradation obstacle which promote to destroys the macrophages hydrolytic enzymes (Spath, 2003), It also helps to inhibit protein kinase macrophage Nitric Oxide (NO).

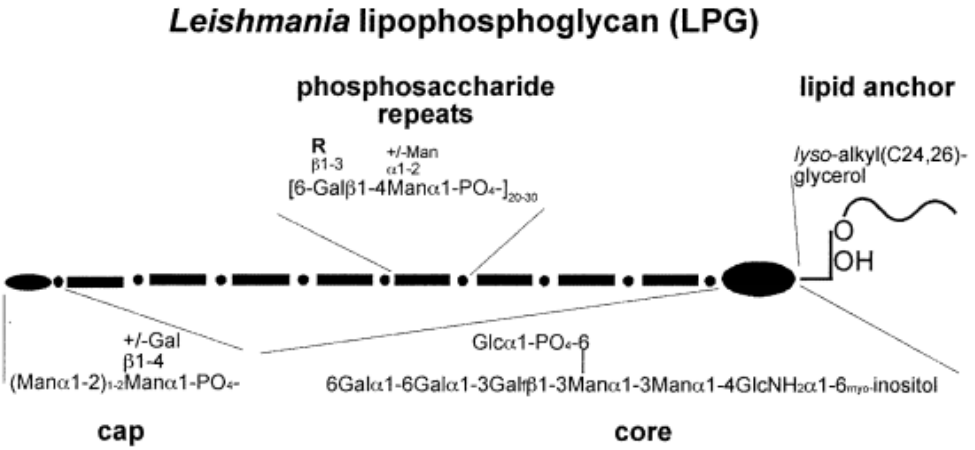

Figure: 2.3 schematic showing a generic structure of LPG in *Leishmania.*

2.10.3 Cystein Proteases (CPs)

Leishmania contains multiple CPs in which some are stage regulated (Cunningham, 2002; Olivier et al., 2005). These enzymes serve to be as degradative role for nutrient benefit of amastigotes to modulate lysosomal activity for their survival. Targeted gene deletion of CP-B gene array in *L. mexicana* cause the attenuation of the parasite and parasite lost the ability to infect the macrophages *in-vitro* and to survive in BALB/c mice *in-vivo* .

2.10.4 Glycoionositol phospholipids (GIPL)

Containing glycosphingo lipids constitutes a dense glycocalyx that remains closely associated with the parasite surface and protects it from the hostile environment of the parasitophorous vacuole (Sacks and Kamhawi, 2001). Membrane bound and secretory acid phosphatases produced by the parasite are non-specific monoesterases that hydrolyse a variety of phosphorylated substrates at low pH. This provides protection to the parasite from enzymatic degradation in the PV(McConville and Blackwell, 1991).

2.10.5 Cht1 gene

Chitinases are important for the successful development of *Leishmania* in the sand fly. Within 48–72 hours of blood feeding, the chitinous PM is degraded by chitinases enzyme,

and then expelled from the sand fly along with the undigested bloodmeal where, both *Leishmania* and sand flies secrete chitinases). The activity of sand fly chitinases peaks (reach at extreme condition) at 48 hours after blood feeding, which corresponds to the time nectomonads escape the confinement of the PM so Previous data demonstrated that inhibition of chitinase activity leads to loss of parasites, even in a competent vector, presumably this is due to their inability to escape the PM before its defecation(Glew et al., 1988).It has been found that out of several strain, the strains that escaped more rapidly from the PM of P. papatasi developed more successfully in the fly (Bates, 2004; Sacks and Kamhawi, 2001). The relative contribution of parasite and sand fly chitinases to the degradation of the PM and their significance to the release of nectomonads remains to be fully elucidated. Later on, in mature infections of 5–7 days, *Leishmania* chitinases, probably produced by haptomonads attached to the cuticular lining of the stomodeal valve, which are responsible for degeneration of the chitinous lining of the valve (Cihakova, 1997). Valve degeneration impairs the feeding dynamics of the sand fly, resulting in more probing and longer feeding periods, which in turn contribute to increased transmission efficiency.

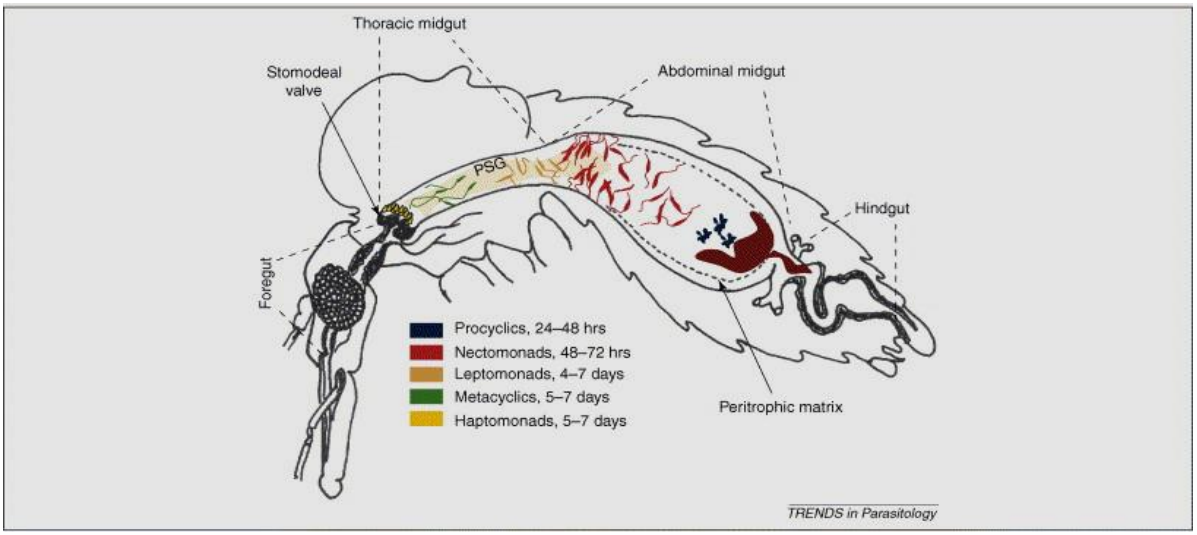

Figure: 2.4 the life cycle of *Leishmania* in a competent vector illustrating the time-dependent appearance of distinct morphological forms of promastigotes within the sand fly midgut (Shaden kamhawi, phlebotomine sandfly and Leishmania parasite frien or foes, parasitology, 2006)

2.10.6. Amastigote specific gene A2

The *Leishmania donovani* A2 genes have been characterized previously as amastigote-specific (Schlein, 1992) hence, amastigotes specific gene of A2 family, which is present in leishmania species that causes visceral leishmaniasis not present in species that causes cutaneous infection(Charest, 1994) Hence A2 protein has been shown to be important for

visceral infection and not for cutaneous infection(Charest, 1994) Previous study have been suggested that the expression of the A2 transcripts could be induced experimentally in cultured promastigotes by a combination of temperature and pH shifts, conditions that mimic the passage from the insect vector to the phagolysosomal compartment of the macrophage cell (Zhang et al., 2003; Zhang W and Matlashewski, 1997). The ability of *Leishmania* to induce A2 expression *in vitro*, together with the ability to transform and express exogenous DNA in *Leishmania* cells(Charest, 1994).

2.11 *Leishmania donovani* with over-expressed Meta1 protein

The mRNA of *Ld* Meta1 gene is upregulated in metacyclic promastigotes and encodes a 12 kDa cytoplasmic protein that is gathered in vacuoles around the flagellar pocket. The gene has been observed to be essential and over expression of *Ld*Meta1 protein in *L. amazonensis* results in increased virulence of the mutant parasites in susceptible mice (Cruz and Tosi, 1996) also, it has been observed that the stage-independent over-expression of the *Meta 1* gene enhances the infectivity of both promastigotes and amastigotes. The *Ld*Meta1 protein also behaves as a strong inducer of a non healing mechanism that is Th-2 type immune response in the mammalian host (Serezani et al.2002) The *L. amazonensis Ld* Meta1 gene was initially isolated from a cosmid genomic library, together with overlapping *Ld* Meta1 clones corresponding to a 60-kb contig (Uliana and Silvia, 1999)in addition to that,A virulence-related function has been previously demonstrated for the Ld Meta1 gene which, when overexpressed, resulting into the hyper virulence in mice. The nearly12 kb *L. amazonensis* genomic sequence in the *Ld Meta1* region is only 53.27% GC-rich as opposed thethe overall 63%GC-content calculated for the *L.major* genome (http://www.sanger.ac.uk/Projects/L_major). People investigated whether *Meta 1* expression correlates with the ability of the parasites to infect macrophages in vitro for this, they infected murine peritoneal macrophages with *L. amazonensis* at different growth stages. The infectivity of the LA remained low during the log and early stationary phases and increased significantly during the stationary phase this is due to the peak of Meta *1* expression appeared at the late-log and early stationary phases, this results revealed that no direct correlation between *Meta 1* expression and cell infectivity, demonstrated that *Meta 1* is expressed at a higher level when the cells are gaining infectivity, compared to when they are already infective (Uliana and Silvia, 1999) People suggested that the L-Major *Ld*Meta 1 protein was down regulated in tissue amastigotes compared to promastigotes *Ld* Meta1 protein expression varies between the two species. *Ld*Meta1 is expressed maximally in axenic amastigotes where as in *L. Major Ld* Meta 1 is down regulated in amastigotes and expressed maximally in metacyclic promastogotes(Santos et al., 2011). This suggests a principle role in *Ld* Meta1 in the mammalian host rather than the insect vector in visceralising species. In *Leishmania* genus, it has been investigated that, gene *Ld* Meta1 is

conserved (Uliana and Silvia, 1999), which is essential for the parasite's viability. (Nourbakhsh, 1996).

First time, the protein is found in the *L. major* which is localized in the region surrounding the flagellar pocket in stationary phase of promastigotes (Uliana et al., 1996,). In case of amazonensis, the virulence capacity is increased when the *Ld* Meta1 protein is overexpressed later another protein was identified in the same genetic locus which is called the Meta2 it contains 3 Meta domains and a c-terminal calpain like domain at the carboxyterminal (Nourbakhsh, 1996).

Previously in our lab work had been done on *Ld* Meta1which showed that the *Ld* Meta1 GFP mutant has similar structural fold with bacterial HSIj Meta 1 gene is upregulated in axenic amastigotes compared to promastigotes stage of the *Leishmania donovani*. It has been found that, the infective metacyclic stage of promastigotes of Leishmania, the transcript and protein was upregulated. The over expression of *Ld* Meta1 gene in *L. amazonensis* parasites were found to be more virulent than wild type *Leishmania*. After investigation on of *Ld* Meta1 It has been investigated that, the heat shock inducible protein of bacterial gene is homolog with Ld Meta1gene of *Leishmania* which was transferred by an ancient lateral gene transfer mechanism between bacteria and a trypanosomatid ancestor. They, were similar to their pathogenecity or virulence, heat inducible expression and also they have shown, that they are not evolutionary linked but structurally similar as well. Furthermore, their homology modeling identified yet another structural homolog MxiM, a secretin pilot protein of Shigella flexneri, which is involved in that pathogen's type III secretion system. Based on structural homology between *Ld* meta1 & MxiM and subsequent mutagenesis, they have identified a putative hydrophobic cavity in Leishmania *Ld* Meta1 that seems to be important for function of *Ld* Meta and indicates a role for *Ld* Meta1 GFP in *Leishmania* secretory processes(Ramos et al., 2004).

2.12 Murine cell line J774

Murine cell line were derived from solid forms of lymphomas without the use of reducing agents, it can be also derived by repeated cycles of culture and mouse passaging. Cell is grown in RPMI1640 with 10% FCS. A *murine* macrophage cell line isolated from BALB/c mouse which is established from a tumor cell lines. The growth of this cell lines is inhibited by dextran sulfate, purified protein derivative, and bacterial lipopolysaccharides. The murine cell lines; J774 cells have been used for numerous biochemical studies and host pathogen interaction aimed at understanding the physiology of monocytes-macrophages and its interaction with pathogen. It's doubling time range from 10 to 24 hour and is adherent and maintained at plastic T25 and T75 flask. The murine cell lines produces large amounts of lysozyme and exhibits minor cytolysis but predominantly antibody-dependent

phagocytosis. These cells have been shown to express cell-bound receptors for immunoglobulin and complement. These cells express high levels of the enzyme nitric oxide synthase and produce large amounts of nitric oxide when activated with IFN-Gamma and a low concentration of bacterial lipopolysacharide (10ng/mL). The Pretreatment of the J774 cell lines with IL10 inhibits this process in a dose-dependent manner while addition of IL-10 at the same time or after IFN-γ activation is without effect. Treatment of J774 cell lines with IFN-gamma induces synthesis of IL-12 mRNA.

(http://www.copewithcytokines.de/cope.cgi?key=J774)

CHAPTER III

MATERIALS AND METHODS

3.1 Source and materials

3.1.1 *Leishmania* strains

The wild type strain of *Leishmania donovani* (MHOM/IN/11983/AG883) was a kind gift from Dr H. Majumdar, IICB Kolkata. Promastigotes were taken from the spleen of golden hamsters which was infected with AG83 and named AG83Re. They were used for subsequent experiments. For this study*, L. donovani* transfectants were generated: *Ld*GFP, *Ld*Meta1 GFP.

3.1.2 Macrophage J774 Cells

In this infection assay study, the macrophage cell line was available in Dr Tushar Vaidya's Lab.

3.1.3 Complete HOMEM media (1X)

Modified Eagles Medium (Designated as HOMEM) media was prepared by dissolving 11g sodium pyruvate; 1.5g D-glucose; 2.2g $NaHCO_3$; 0.1 mg Biotin; 1mg para-aminobenzoic acid(PABA) in 1 litre of MEM containing Hanks salts, L-glutamate and non essential amino acids; 25ml of 1 M HEPES buffer pH 7.5 was added to it; pH was adjusted to 7.4. The media was filter sterilized using a 0.2µm bottle top filter apparatus (Nalgene Nunc International) and stored at -20^{0}C. Foetal calf serum (FCS) (100ml) was decomplemented by heating at 56^{o}C for 30 min and stored at -20^0 C. A 1000X stock of Hemin was prepared by dissolving 60mg Hemin in 10ml of 1 N NaOH, filter sterilized using a 0.2µm syringe filter (Nalgene Nunc International) and stored at -20^{0}C .Hundred milliliter of FCS and 1ml of Hemin stock solution was added to 900ml of HOMEM media to make complete HOMEM media and was stored at 4^0 C.

3.1.4 RPMI 1640 media and buffer

RPMI 1640 was used to make 1 L of medium. It was added to 700ml of MilliQ water and to it 3.6gm of HEPES sodium salt, 2gm of sodium bicarbonate, 1ml gentamycin and 10ml of penicillin-streptomycin were added. The pH was adjusted to 7.2 and the volume was made up to 1L.Trypsin-:(Tris HCL and EDTA) was used for trypsinization.

Phosphate buffer saline was made with 10 X stock solution initially in which 137 mM Nacl was mixed on it. Nearly, 2.7mM KCl was added into the solution. Both sodium and potassium biphosphate of 4.3mM and 1.4mM (Na_2HPO_4, KH2PO4) were mixed and added into the solution. Finally 7.0pH was adjusted by adding HCL to make working solution 1X PB buffer.

In addition, phosphate buffer for stain was also made to make final1 M stock solution of PB buffer in which 20.41gm and 26.13 gm of KH_2PO_4 K2HPO4 was mixed simultaneously. The pH was adjusted 7.4 by adding NaOH to make final working solution of 0.1M.

3.2 Cell Culture

3.2.1 *Leishmania donovani*

Revival of *Leishmania* parasites from cryopreserve

For maintaining culture, *Leishmania* flask was taken out from liquid nitrogen and it was thawed slowly at ice and then it was pelleted by spinning and immediately cultured at 26^0Cin HOMEM media containing 10% FCS with additional 20mg/ml Geneticin/Gentamycine (G418) added to it. For all experiments, cell numbers were estimated by direct counting in a hemocytometer under a phase contrast microscope.

The promastigotes were routinely inoculated at a starting density of 10^6cells/ml; typically logarithmic growth was observed between day 2-4 and stationary phase between day 5 and 7. In case of transgene promastigotes the shape change was observed maximum on 10^{th} - 13^{th} day hence, the cultures were taken on particular days accordingly to the experiment.

Passaging of *Leishmania* Parasites

The *leshmania* cells were passaged every 7-8^{th} days to maintain it at stationary phase for infection assay. If culture is considered in good condition and to be in mid-late stationary phase, passage 100 µL of this culture to a new flask with 5 ml fresh medium (HOMEM+20% HIFCS) in a sterile condition. Close cap of flask of new culture, and shake carefully to mix parasite inoculum with fresh medium. The new culture now be grown for 6-8 days (depends on strain) at 26°C, till mid-late stationary phase is carried out. The *Leshmania* cells were passaged every 7-8^{th} days to maintain it at stationary phase for infection assay.

Cryopreservation of *Leishmania* parasties

Promastigotes of *L. donovani* (AG83Re) a *sub*-clone of MHOM/IN/1983/AG83 both virulent *Ld* Meta1 GFP and *Ld* GFP strains were first freezed down into the liquid nitrogen for maintaining the *Leishmania* culture. To freeze down the *Leishmania* strain of required

density in late log phase or stationary phase was put into the vials DNA which was mixed with 7.5% DMSO and HOMEM medium into the liquid nitrogen tank.

3.3 DNA extraction method

The parasites were washed thoroughly in PBS inside biosafety cabinet 2 and pelleted it out by centrifugation at 3000 rpm for 10 minutes at room temperature. The pellate was then resuspended in an appropriate volume of lysis buffer (150mM nacl,10m MEDTA,10mM Tris-HCL pH 7.5,0.4% SDS,200ug/ml protinaseK) vortex it hard and incubated at 37^0C till pellet dissolved completely.DNA was then extracted with phenol-chloroform with equal volume. It was precipitated overnight with 100% ethanol at -20^0c and was washed with 70% ethanol and resuspended in sterile water containing 25ug/ml RNAse. A concentration of DNA was estimated by using nanodrop reading. Dissolved air dried pellet in 100µl TE Buffer (100mM Tis-HCI pH 8.0 and 1mM EDTA) or in sterile H_2O.Then by using NanoDrop, quality and quantity of DNA were checked. A good DNA shows a 260/280 ratio >2Visualization, 0.25ug/ml of ethidium bromide was added to the gel while preparing 1X- was used as running buffer.

3.4. PCR (Polymerase Chain Reaction) amplification of DNA fragments

A standard PCR mixture (25 µl volume) with following components were prepareds

Tab: 3.1 reaction mixture for the polymerase chaina reaction

	Reagents	volume
i.	10X PCR reaction buffer	2.5 µl
ii.	MgCl2 (50 mM stock solution)	1 µl
iii.	dNTP mixture (stock of 2 mM each)	2.5 µl
iv.	Primers mixture (forward and reverse, 1 pM)	2µl
v.	DNA template	1ul parasite DNA
vi.	Taq (Pfu) polymerase (5U/ µl stock)	1µl
vii.	Sterile H_2O	15 µl

PCR amplification of DNA fragments was done using Taq Polymerase.

Tab: 3.2 A standard protocol for a PCR reaction of *Leishmania donovani*

Condition	Time period
1. Initial denaturation	95°C for 3 min
2. Denaturation	95°C for 45 seconds
3. Annealing	60°C for 45 seconds
4. Primer extension	72°C for 40 seconds to 2 minutes 5.
5.Steps 2 to 4 were repeated for	35 cycles
6. Final extension	72°C for 10 minutes.
7.Hold	4^0C

The time for the denaturation and extension steps, and the temperature and time for annealing step were changed depending on the length of the amplicons and the melting temperature of the primers.

Finally, the PCR product was loaded into the 0.8% agarose gel for 1hour and it was put into the UV light for visualization.

These are the primer pair used for LdMeta1 GFP GFP and Ld GFP in PCR

5-TAGATACACGCGCCCTGTCTTATT3 Ld LdMeta1 GFP-F

5CTGTTGATGATGGCCATGCGCGT3LdLdMeta1 GFP-R

FORWARD PRIMER	GAGGAGTTCCCGCTTGTGTG-DRG1
REVERSE PRIMER	CCGCTCGAGCGCACAGCATCTCTTCACTAAATGGGT-DRG

3.5. J774 cell line

At first cells were harvested by spinning the cells as gently as possible at 1200 rpm for 5 minutes. Then it was Resuspend in the growth medium at room temperature to a concentration of 2 x 10^6 cells per ml. It was stained by trypan blue dye for viability check. Then, cryoprotectant was added to the growth medium in which the cells are to be frozen. DMSO (dimethyl sulphoxide) in 7.5% concentration was addeded to the cells to make final

concentration of 7.5 and were mixed with cryoprotectant at room temperature to final concentration of viable cells in the range between 10^6 cells per ml cells were collected into one vial and was properly mixed, before aliquoting , finally it was stored into CryoTube vials onto the liquid nitrogen with internal thread and silicone gasket use for protection. Finally, all tubes were closed properly

Thawing of cells

For next passage, cells, in the cryotube vials, were removed from the liquid nitrogen tank, the cryotube vials were soaked in 70% ethanol before they are transferred to the laminar flow cabinet and it was placed directly into a 37°C water bath and was shaken until it is completely thawed and was left untouched in incubator with RPMI medium for 16 hour of 10 ml. Finally, cells were ready for experimental purpose.

Passaging of Macrophage (J774) Cell line

Murine macrophage cells were cultured in RPMI 1640 media prepared 1 Sachet of RPMI 1640 was used to make 1 L of medium. Then MilliQ water of 700ml, 3.6gm of HEPES sodium salt, 2gm of sodium bicarbonate, 1ml gentamycin and 10ml of penicillin-streptomycin was added on it . The pH was adjusted to 7.2 and the volume was made up to 1L. Cells were seeded in the flask at a starting density of 10^6 cells/ ml and were maintained under 95% humidity at 37^0 C in 5% CO_2 atmosphere. The media was changed every day to make cells healthy. The cells were observed everyday under inverted microscope it was passaged every 7 days. The J774 cells were passaged into the fresh media by using following procedure.

Before infection assay of *Leishmania* experiment, the adherent cells were observed microscopically; it should be 70 to 80% confluent to the flask so was shaked gently onthe platform to access adherence. The RPMI 1640 medium was discarded from the flask by gently pipetting out the medium from the flask and it was washed twice with 10 ml PBS and then it was discarded after each wash. The 1 ml trypsin–EDTA solution was added over the entire surface of flask for 1 minute for detachment of macrophage from the wall of flask.

It was observed under inverted microscope to see whether the cells were detached or not. To observe cell detachment the flask was banged by hand several times. After 50 % cells were detached the reaction was stopped by adding 10 ml of RPMI 1640 medium containing alfa- antitrypsin FBS which inhibit trypsin. The flask was flushed with medium several times to deatch the remaining cells. The detached cells were Aspirated and transferred into the falcon tube to be centrifuged at 1200rpm at 25^0Cfor 5 min, the supernatend was discared and then pellete was washed with PBS by spinning.

Viability Evaluation by Trypan blue staining

After trypsinisation of J774 cells it was stained with trypan blue dye as the dilution was 1:30 after dilution, 10µl of cells were placed it over the haemocytometer. Then, cells were counted under the phase contrast microscope. The total cells in four square of haemocytometer with white cells were taken except blue for counting by average and it was multiplied by 30 dilutions and $1x10^6$ cells were taken for 1ml medium. Finally the required density of cells was suspended into the PBS and it was seeded into the T75 flask containing 10 ml RPMI 1640 with 20% FCS. It was observed under inverted microscope and was maintained under 95% humidity at 37^oC in 5% CO_2 atmosphere. It was passaged after every 2-3 days for maintain the cells.

Freez down of J774 cell line

At first cells were harvested by spinning the cells as gently as possible at 1200 rpm for 5 minutes. Then it was Resuspend in the growth medium at room temperature to a concentration of 2 x 10^6– cells per mL. It was counted by trypan blue dye for viability check. Then, cryoprotectant was added to the growth medium in which the cells are to be frozen. . The cryoprotectant DMSO in 7.5% (dimethyl sulphoxide) at room temperature was addeded to the cells to final concentration of viable cells in the range between 10^6 cells per ml and final concentration of cryoprotectant was of 7.5%. And cells were collected into one vial and it was properly mixed, before aliquoting is performed, then it was stored into CryoTube vials onto the liquid nitrogen with internal thread and silicone gasket use for protection finally, all tubes were closed properly.

3.5 Giemsa Staining stock preparation

Giemsa stain powder based preparation was prepared for infection assay. First the 3.8gm powder of giemsa was mixed into 250ml of methanol (absolute acetone free) then the solution was heated for overnight from step 1 to 60^0C in water bath and 250ml of glycerin was added to the solution next day & kept in the hot water bath at 60^0C for 3 hours. Solution needs to stand for a period of 3 to 4 months prior to use. The stain was allowed to stand for few days prior to use and mixed 3-4 times a day for a few days. For working 20% giemsa stain was made from the stock.

3.6 Macrophage infection assay

3.6.1 Infection assay

The macrophage seeded flask were trypsinized and washed rigorously with 1X PBS and transferred to a falcon tube. Then the cells were pelleted down and concentrated. To observe the viability of the cells, trypan blue staining was done. The cell density was counted in hemocytometer and accordingly the volume was decided for seeding 0.5 x 10^6 cells per slide.

For each group of *Leishmania* and its respective time points 3 slides were seeded at a cell density of 0.5 x 10^6 cells and kept in a petridish with 10ml of RPMI 1640 media. The petridish was then incubated overnight in a 5% $C0_2$ incubator at 37^0 C temperature so that the cells adhere to glass slides properly. Next day *Leishmania* cells at stationary phases from respective groups were counted in hemocytometer and volume was counted accordingly for 1x 10^7 cells (Since the Mode Of Infection is 1:10).
The petridish with the slides were taken out and the media was sucked. The volume of media containing 1X 10^7 Parasites were added over each slides and then incubated for respective time points in CO_2 incubator at 37^0C. After incubating respectively according to the time points (1 hour, 4hour, 12hour and 24 hour) the slides were washed with 1xPBS to remove unattached *Leishmania* parasites and the PBS was sucked out. Then the slides were taken for Giemsa staining.

3.6.2 Giemsa Staining

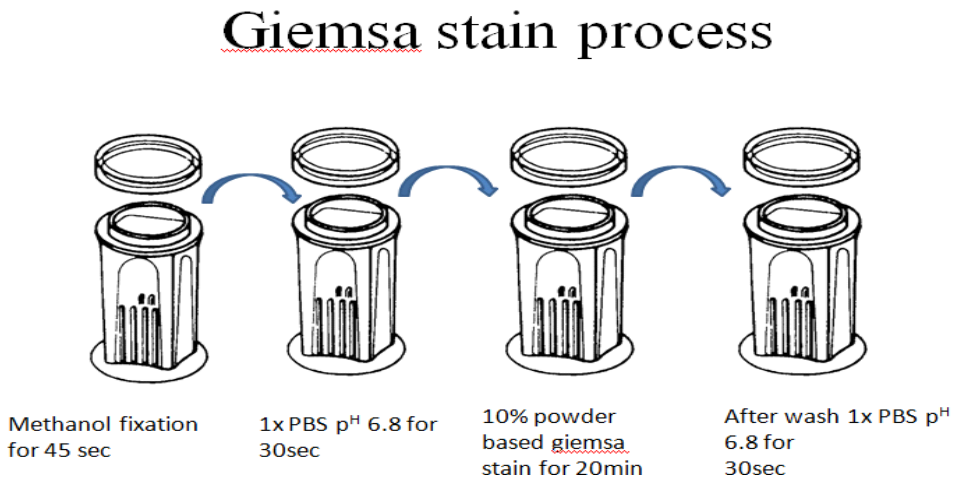

Fig 3.1 procedure for lab made giemsa staining

The slides on which infection assay is being done , after their incubation period the slides were washed with PBS to remove free parasites and were fixed in methanol for 45 seconds then washed with 1X PBS for 30 seconds followed by keeping it immersed in 20% giemsa stain in a coupling jar for 30 minutes. After that a quick wash in PBS and allowed to dry. It was then observed in 100X under bright field of phase contrast microscope.

3.7 Bright field Phase contrast Microscopy for counting

Randomly 300 macrophages were counted per slide by using phase contrast microscopy at 100x under oil immersion and were split into the following distribution for data interpretation.

Fig3: 2 Bright field of phase contrast microscope

3.7.1 *Leishmania* attachment score

The counts were distributed into the following categories

M+1, M+2, M+3, M+4, M+5, M+5>

Where M+1 comprises of macrophage population having only one *Leishmania* attached and/**or** internalized, M+2 having 2 *Leishmania* attached or internalized & so on for the other groups

3.7.2 *Leishmania* internalization score

The counts were distributed into the following categories

M+1N, M+2N, M+3N, M+4N, M+5N, M+6N,M+7N,M+8N.M+9N,M+10N,M+10N>

Where M+1N comprises of macrophage population having only one *Leishmania* internalized, M+2 having 2 *Leishmania* internalized & so on for the other groups. There sub categories were made for the macrophages that showed internalized *Leishmania*

- Attachment = consist of macrophages that have attached and ointernalized *Leishmania* to the macrophage
- Internalization = consist of macrophages that have internalized *Leishmania* irrespective of the no of *leishmania* attached outside the macrophages
- Only external = consist of macrophages that exclusively have *Leishmania* attached externally to the macrophage & not internalized
- Only internal = consist of macrophages that have *Leishmania* internalized & non attached
- Randomly 300 macrophages containing parasites which contained, attached, internalized, only internalized and only external parasites were counted per slide so slides in triplicate were counted for one time point(1hr) and respectively for other time points,(4hr,12hr,24hr)

Tab:3.3 Catagorisation of infected macrophages and parasites

The infected macrophages were scored & were categorized into four categories

Category	**Description**
ATTACHED	All the infected MØ are scored in this category
'ONLY EXTERNAL	Only those MØ which have promastigotes attached externally are scored in this category
INTERNALIZED	MØ which has internalized promastigotes with/without promastigote attached externally
ONLY INTERNAL	Only those MØ which has promastigotes internalized are scored in this category

- Macrophages with one *Leishmania* attached were categorized in the group M+1
- Macrophages with two *Leishmania* attached werer categorized in the group M+2

- Macrophages with three *Leishmania* attached were categorized in the group M+3
- Macrophages with four *Leishmania* attached were categorized in the group M+4
- Macrophages with five *Leishmania* attached were categorized in the group M+5
- Macrophages having more than 5 attached *Leishmania* was in group M+5<

The analysis of the same data has also been carried out by summing up the categories less than five for instance the categories were M+1, M+2, M+3, M+4, M+5 & then were compared with M+5<

Similarly, the macrophages with internalized *Leishmania* were scored in similar & categorized into following groups.

- Macrophage with one *Leishmania* internalized were grouped into M+1N
- Macrophage with two *Leishmania* internalized were grouped into M+2N
- Macrophage with three *Leishmania* internalized were grouped into M+3N
- Macrophage with four *Leishmania* internalized were grouped into M+4N
- Macrophage with five *Leishmania* internalized were grouped into M+5N
- Macrophage with six *Leishmania* internalized were grouped into M+6N
- Macrophage with seven *Leishmania* internalized were grouped into M+7N
- Macrophage with eight *Leishmania* internalized were grouped into M+8N
- Macrophage with nine *Leishmania* internalized were grouped into M+9N
- Macrophage with ten *Leishmania* internalized were grouped into M+10N
- Macrophage with more than ten *Leishmania* internalized were grouped into M+10N<

In this way manually number of *Leishmania* per macrophages of both Ld Meta1 GFP and Ld GFP were counted and added on excel sheet for each time points under oil immersion at 100X at bright field, phase contrast microscope for first round.

We had repeated same process for both strain of Leishmania that is Ld LdMeta1 GFP and Ld GFP at1 h, 4h,12h,24h time points where each time points contained 3 slides till 4^{th} round, Then it was subjected to data interpretation for analysis.

Data interpretation was done by using Microsoft excel 2007 filter tool.

Statistical Analysis

All the infection assay studies, were performed in triplicates where for four time points three slides were infected for different category ie, attached, internalization, and same experiment were repeated four times to get robust dada. We had taken 300

macrophages per slides for each time point till 4th round because it is statically significant. Then it was subjected for stastical analysis to calculate the percentages of macrophages, standard deviation and error bar of respective time points by using Microsoft excel 2007 filter software. And finally to confi.rm the significant difference between overexpressed LdMeta1 GFP and Ld GFP, Graphpad prism version 6 was used for stastical analysis. An unpaired t-test (twotailed) was applied to compare the rate and quantum of parasites. P value was taken <0.001 was taken as extremely significant, 0.001 to 0.01 as very significant, 0.01 to0.05 as significant and >0.05 as non significant.

CHAPTER IV

RESULTS

This research work on '**Role of meta1 gene in infectivity of *Leishmania donovani* to the Balb/c mice macrophages**' have shown very promising results that *Leishmania* parasite with over expression of Meta1 gene are very much efficient in penetrating to the macrophage cells. This kind of work has been done for the first time so far we are aware. The results obtained are illustrated under following subheadings.

4.1. *L donovani* parasite counts

After revival from cryopreserve of both the parasites (LdMeta1 and LdGFP), 1 X 10^5/mL parasite counts kept in 5 ml HOMEM culture medium for 7 days found proliferated to approximately 1X10^7 parasites. The parasites were further processed for DNA extraction.

4.2 Isolation of Genomic DNA (gDNA)

Measurement of DNA for its purification and concentration by nano drop analyzer ND-1000 spectrophotometry in the extraction of 10 million parasites (1X10^7) lysed in lysis buffer found the DNA in purified form 304ng/ul. The DNA band in agarose gel under electrophoresis along with 1kb ladder and the band observed of the molecular weight 35 mega bp confirmed the pure LdGFP and LdMeta1 (Fig. 4.1)

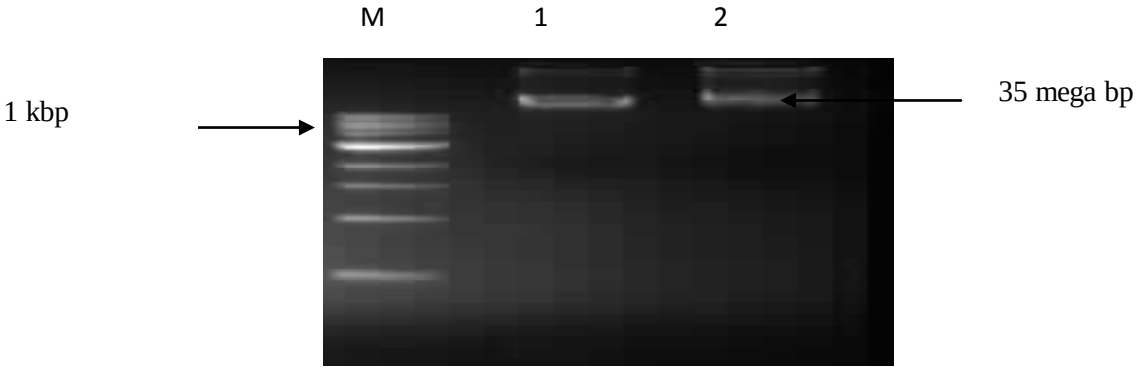

Fig 4.1 gDNA extraction of *Leishmania donovani*byLysis buffer method (M-marker 1kbp.Lane 1,35 mega bpgDNA of Ld GFP1 parasite; and lane 2, gDNA of Meta1 GFP parasite with 35mbp,)

4.3 PCR amplification of virulent *Leishmania donovani* with meta1 and *Ld* GFPgene using specific primers in separate parasite cultures

Leishmania donovani parasites with *Ld*GFP and *Ld*Meta 1 gene expression were confirmed by molecular analysis i.e. Polymerase Chain Reaction to identify specific amplicon or to amplify the given strains (Fig. 4.2 b) by using different combination specific primers (Meta forward/reverse and DRG forward/reverse) (Fig 4.2 a).

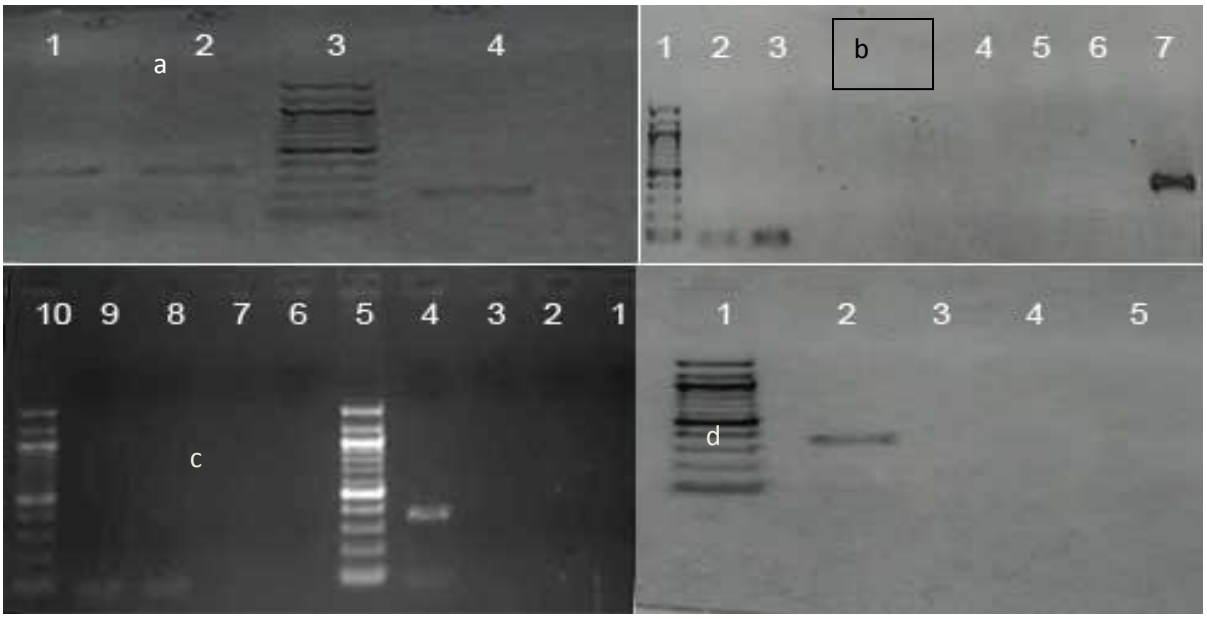

Fig: 4.2 PCR amplification from gDNA of Leishmania donovani Ldmeta1 and LdGFP with different primers combination (a.; b, c & d) (a). Amplicon of Ld GFP and Meta1; From left to right, lane 1 & 2 –LdGFP, Lane3- Marker, Lane4-Meta1 GFP;,(b). Gel picture DRG primer and meta primer; Lane1-marker, Lane2-no reverse primer, Lane3-no forward primer, Lane 4-no template, Lane 5 no forward primer, Lane 6–no reverser, Lane 7-meta1 amplicon, (c)-Amplicon with Meta1 primer; From right to left, Lane1-no reverse primer, Lane2-no forward, lane 3-no templatelane lane 4-Meta1,lane. Lane 5-marker, Lane6-DRG primer with meta primer in no forward, Lane7-no reverse lane8-no template, full reaction with DRG + meta1 primer,(d). Lane1-marker, Lane2-Meta1Lane 3-no template, Lane4-no forwad, Lane5- no template with Meta1 forward and DRG reverse in separate reaction.

The amplicon of *Leishmania donovani* with over expressed meta1 gene of nearly 200bp was amplified in agarose gel electrophoresis by using Meta forward and reverse primer (Fig 4.2 a, b). The *Ld*GFP parasites showed amplification of 162bp due to the DRG primer while using

combination of Meta and DRG primers. It was confirmed by this experiment that the above strains used for this study was *Leishmania donovani* with over expression of meta1 gene and *Ld*GFP.

4.4 stationary phase parasites to raise infection

L donovani promastigote parasites once revived from cryopreserved vial kept at the count of $1X10^5$ in culture flask showed multiplication of their number in log phase till 5^{th} day ($1X10^7$ -$1X10^8$). Then onwards, plateau growth was observed during its 6^{th}, 7^{th} and 8^{th} day of incubation which proved that the parasites are in stationary phase. Those stationary phase of promastigotes were applied for macrophage infection because all the virulent associated genes are up-regulated and metacyclogenesis is carried out during this stage of parasites increasing their infectivity.

4.5 J774 cell line growth and its doubling time

J774 cell line, the host cell culture at the count of 0.5×10^6 were found proliferated into 1×10^7 after 24 hour of incubation into the CO_2 incubator at 5% CO_2 and 37^0C temperature. The result confirmed that 24 hour time period is the doubling time for the J774 host cells.

4.6 Parasite–Host cell ratio determination

Different types of parameters were tested to see the maximum of infection such as the optimum ratio of cell and parasites. The ratio of parasites and host cell (J774 cell line) tested in 3 categories. The first case of 1:1 (Parasite:cell) and second of 1:5 ratio were not efficient to infect the host cells. Finally, we used ten times more parasites and found that enough parasites were easily got attached and internalized into the macrophages ($1X\ 10^7$). Hence this ratio was very suitable for this assay(tab.4.1).

Table 4.1. Ratio determination of J774 cell and *L donovani*parasites for better infection

S.N	parasites-cell ratio	Parasite count for infection	Internalized parasites
1	1:1	300	5
2.	1:5	300	10
3.	1:10	300	40

4.7 Macrophage infection assay

The cultured macrophage J774 cell line infected by stationary phase *L donovani* promastigote parasites studied using different parameters have exposed many interesting facts. However, the assumption behind this assay was that, when more parasites load gets internalized into the macrophages, the infective rate and quantum should be higher. Here, the over expressed meta1 and only LdGFP were compared to analyze their rate and quantum. This research has results of interaction between parasites and J774 cell line at the level of, exclussively external presence, attachment, internalizes and exclussively internal as mentioned below.

4.8 Stages of *L donovani* infection to macrophage cell

The *Leishmania donovani* parasites used at the ratio of 10:1 with macrophages were observed as attached, exclusively external**,** internalized and exclusively **in**ternal (Fig. 4.4). Based on the number of parasite count, score was given from +1 to +6 as mentioned in material and methods.

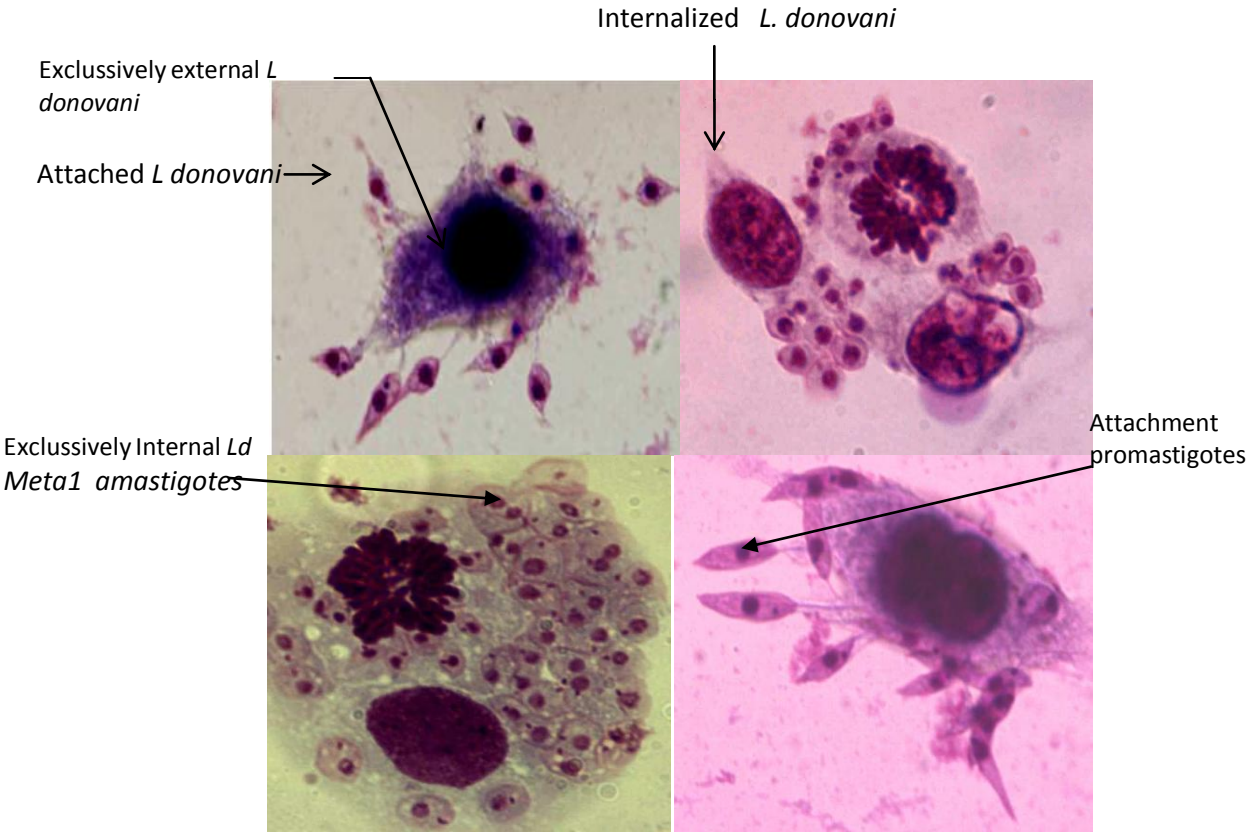

Fig: 4.3. Illustration of an example for the macrophage infected with *Leishmania* and the scoring a. exclussively external and attached, b. internalized *Ld* GFP and c. internalized Meta1 over expressed**.**

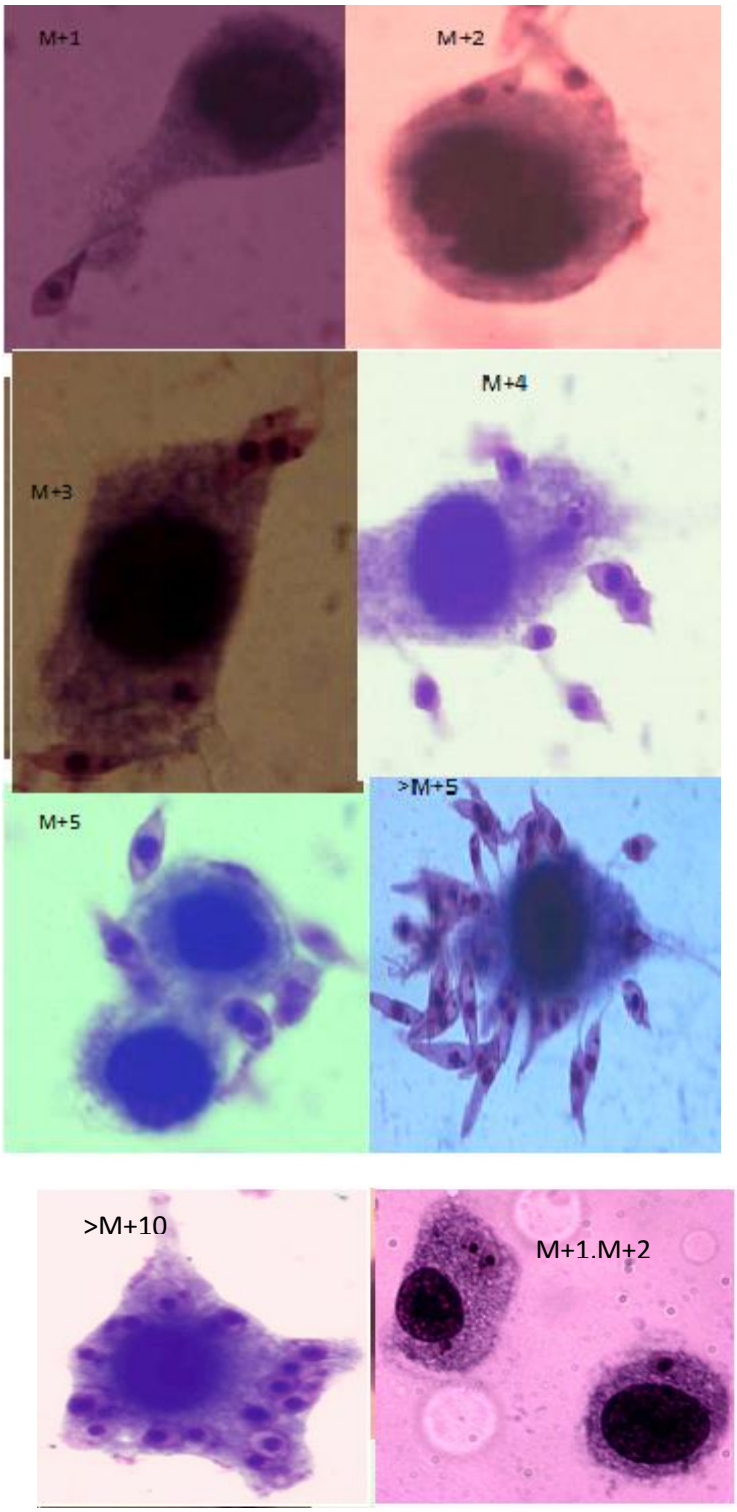

Fig: 4.4: Example of scoring of +1 to >5 in attachment category and >10 in internalized category in Meta and Ld GFP

The parasite load and quantum were analysed by Scoring of Macrophage infected by one parasites where the indication is M+1, similarly if two parasites were infected by host cell is

M+2 so ……>M+5(greater than five) and >M+10 parasites with macrophages were catagoried by phase contrast microscopy and were interpreted by statistical analysis.

Macrophages having one promastigotes and having greater than five promastigotes were calculated in attachment stage only however promastigotes having greater than ten were also analyzed in internalized category. Hence images (Fig 4.4) were the macrophages having scoring of +1 , >5 (or 5+) and >10 promastigotes.

4.8.1 Macrophage interacted by different catagories of L.*donovani* infection during host-parasite interaction.

Infection by *Leishmania* parasites in reference to the count of 300 macrophages per slide showed the parasites are in attached, internalized, only internal and only external phases. The infection seen to the macrophages by the test and control parasites at the studied phase are mentioned below.

4.8.1.1 Macrophages interacted by exclusively external *Leishmania*

After the raising infection by $1X10^7$ parasites, there were countable numbers of *Leishmania* outside the macrophages but none of them were able to venture the host macrophage cells. They were denoted as exclusively external parasites (Fig.4.5).

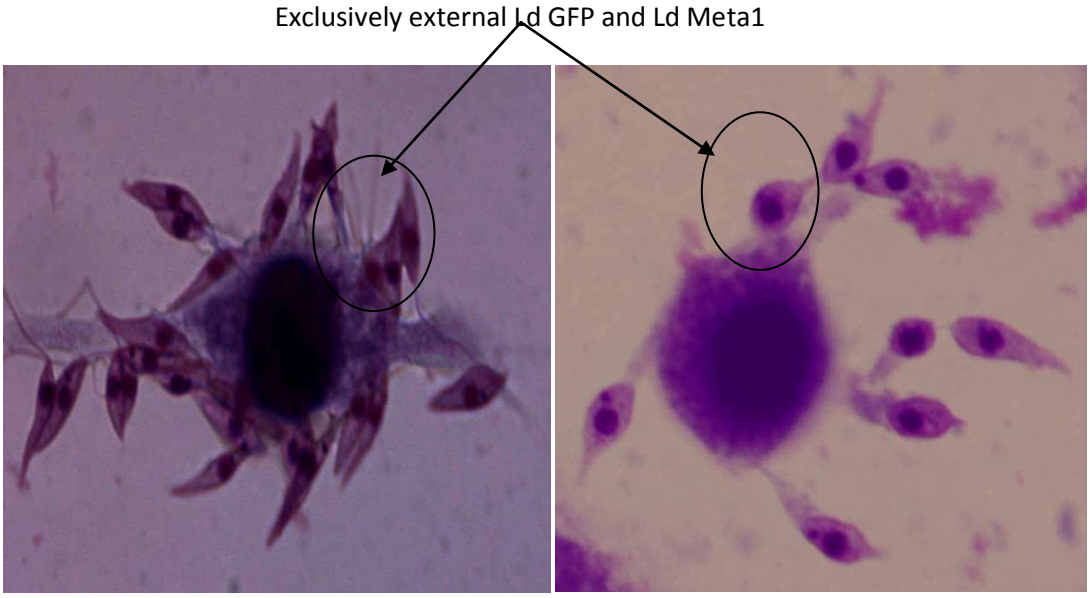

Fig: 4.5 exclusively external phase of Leishmania parasites, a.*Leishmannia* parasite infection in initial attachment phases. In *Ld* GFP b. only external in *Ld* Meta1 phase of GFP parasite

The exclusively external category reflected that mean number of 2012 *Ld* Meta1 Parasites out of 1×10^7 used for infections were found only external whereas in *Ld* GFP 3260 were found exclusively external to the macrophages,(fig:4.6) the exclusively external promastigotes were found significantly higher infected macrophages in the earlier time points in *Ld* GFP than control.

Table: 4.2.- Percentage of macrophages having only exclusively parasite at 4 time points -1hour, 4hour, 12hour, 24hour of Meta1 over expressing cell and *Ld* GFP.

	Exclusively external %		**p value**
Time points	**Ld Meta1**	**Ld GFP**	
1hour	**47 ± 15.7** (n=1271	**72± 7.4 n=1991**	<0.0001****
4hour	**22.55±20.5 n=658**	**22± 17.9 n=1110**	0.9
12hour	**0.4 ±0.35 n=80**	**0.5 ± 1.5 n=146**	0.8
24hour	**0.1 ±0.16 n=3**	**0.48± 4.5 n=13**	0.77

This study has shown that at earlier time points**;1** 1hr and 4 hr both the Ld GFP and Ld Meta1 showed significant difference (Pvalue-<0.0001****)(tab:4.2). However**,** at later time points at 12 h and 24 h, 0.4% vs 0.5**%** and 0.1% vs 0.48% only were exclusively external respectively. Hence there was subtle difference between two strains

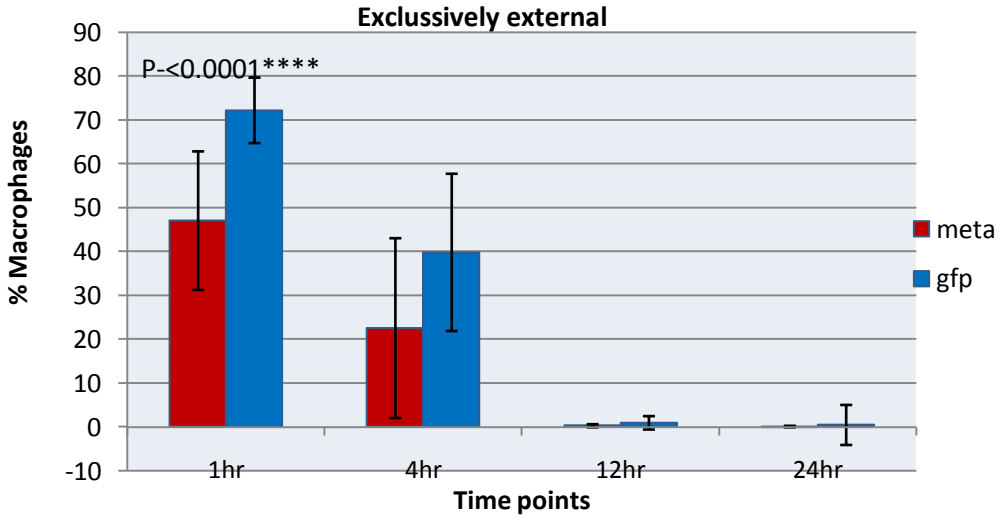

Fig 4.6: Bar graph showing comparison percent macrophages that have only external promastigotes 1hr, 4hr, 12hr, 24hr time points between *Ld* Meta and *Ld* GFP.

4.8.1.2 Macrophages interacted by attached *Leishmania*

The attachment category reflected that mean number (experiments was in triplicate) of 9,510 LdMeta1 parasites out of 1×10^7 used for infections were found attached to the macrophages, i.e. 93.0% of macrophages have been infected by LdMeta1 promastigotes (Fig. 4.8). The case to the LdGFP parasite was found a bit higher as 10,279 parasites out of 1×10^7used for infection were found attached, i.e. 95.0% of macrophages were infected at 1hr time point (tab. 4.3).The attachment category was analyzed as the indication of total infectivity. The 4 hr., 12 hr and 24 hr categories showed that 92.1 % vs 92.0%, 92.6.% vs 86.0% and 95.6% vs 89% were infected by LdGFP and LdMeta1 over expressed *Leishmania* parasite respectively. Hence gradually across the time points, the number of infected macrophage percent seemed to plateau till 12hr time points (Table 4.3,Fig 4.8). However, there was significant difference between two strains at 24hr time point (0.0001***). Therefore, both the LdMETA1 & LdGFP shared subtle differences in this category at earlier time points but at later time point LdMeta 1 showed significantly higher infective rate than Ld GFP. Further, to verify the changes in the internalization of promastigotes,internalization, exclussively internal & internal category were examined.

Table: 4.3 Percentages of Macrophages attached by *Leishmania* Parasite (Ld META1 over expressing cell and Ld GFP) at four different time points; 1hour, 4hour, 12hour and 24hour.

Time points	**Attachment %**		**p value**
	Ld Meta1	Ld GFP	
1hour	93.0 ±10.56 (n=.2362)	95.0 ± 2.05 (n=2438)	0.5
4hour	92.1±5.54 (n=2511)	92±11.9 (n=2279)	0.9
12hour	92.6 ±2.57 (n=2501)	86 ± 10.18 (n.=3094)	0.05
24hour	95.6 ± 2.90 (2136)	89 ±4.5 (2468)	<0.0001***

The number parasites raised to infect and the attached number of parasites are given in parenthesis.

Therefore this category contain atleast one promastigotes attached externally and taken randomly which do or do not contain internalized amastigotes so it has total infectivity and hence the percentages of promastigotes should increased as time increases. However in these results till 12 h time points the parasites remain constant but later increased to 95% in Meta1.

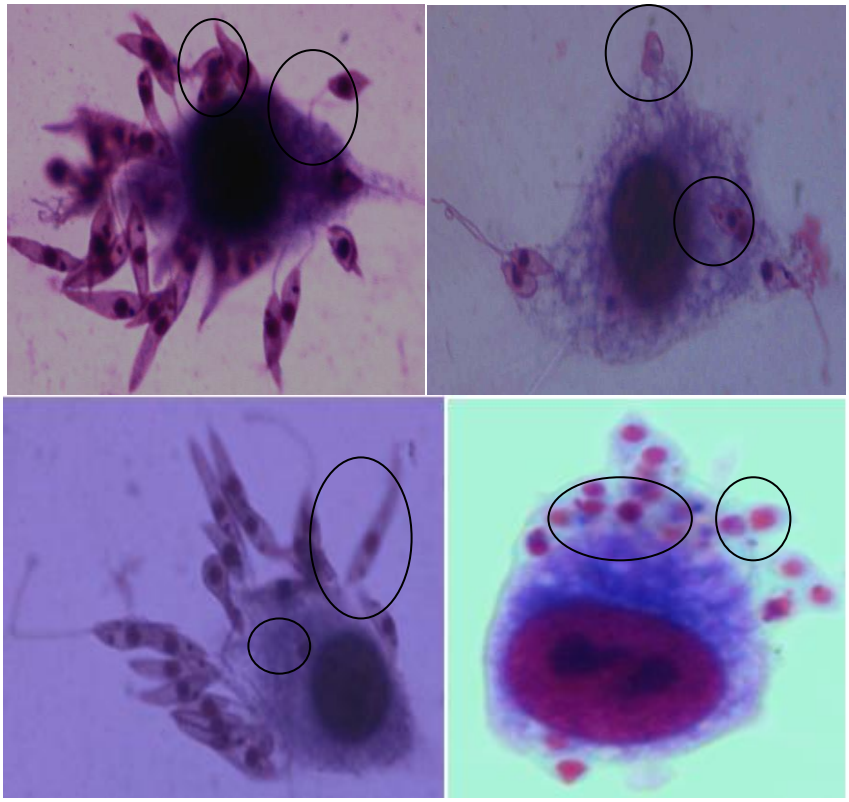

Fig: 4.7 Attached phases of *Leishmania* parasties (having at least one promastigotes external) a.*Leishmania* parasite infection in initial attachment phases. b. Attached Meta1 phase with one internal.c.more than five attached with one internal. d.more than ten internalized with three external promastigotes.

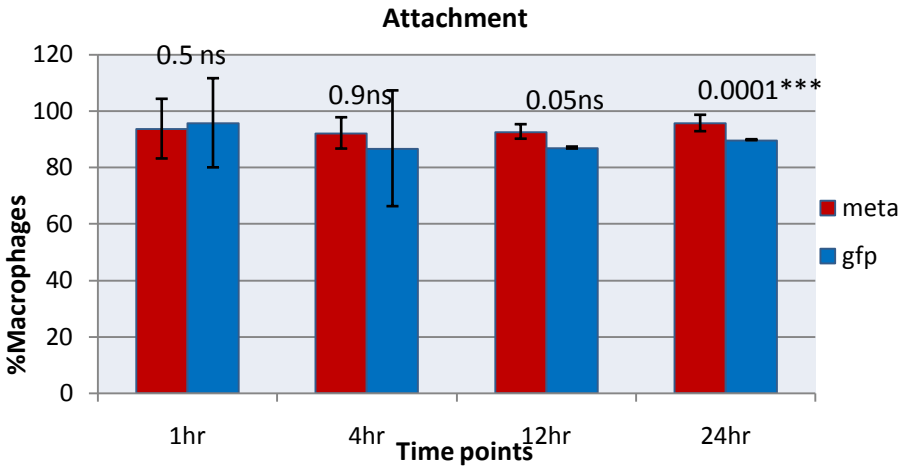

Fig: 4.8 Bar graph showing comparison percent macrophages that have atleast one attached promastigotes 1hr, 4hr, 12hr, 24hr time points between Ld Meta and Ld GFP.

4.8.1.3 Macrophages interacted by internalized *Leishmania*

The internalized category for *leishmania* parasite (Fig 4.9,10) included those macrophages that had at least one *leishmania* internalized with/without promastigote attached externally.

The internalisation category reflected that sum of Ld Meta1 parasites in all time points were6904 parasites internalized to the macrophages out of 1×10^7 used for infections, whereas in case of Ld GFP the sum of all parasites infected were 5218 (Table 4.4)**. T**he internalized promastigotes were found significantly higher in Ld Meta1 than Ld GFP.

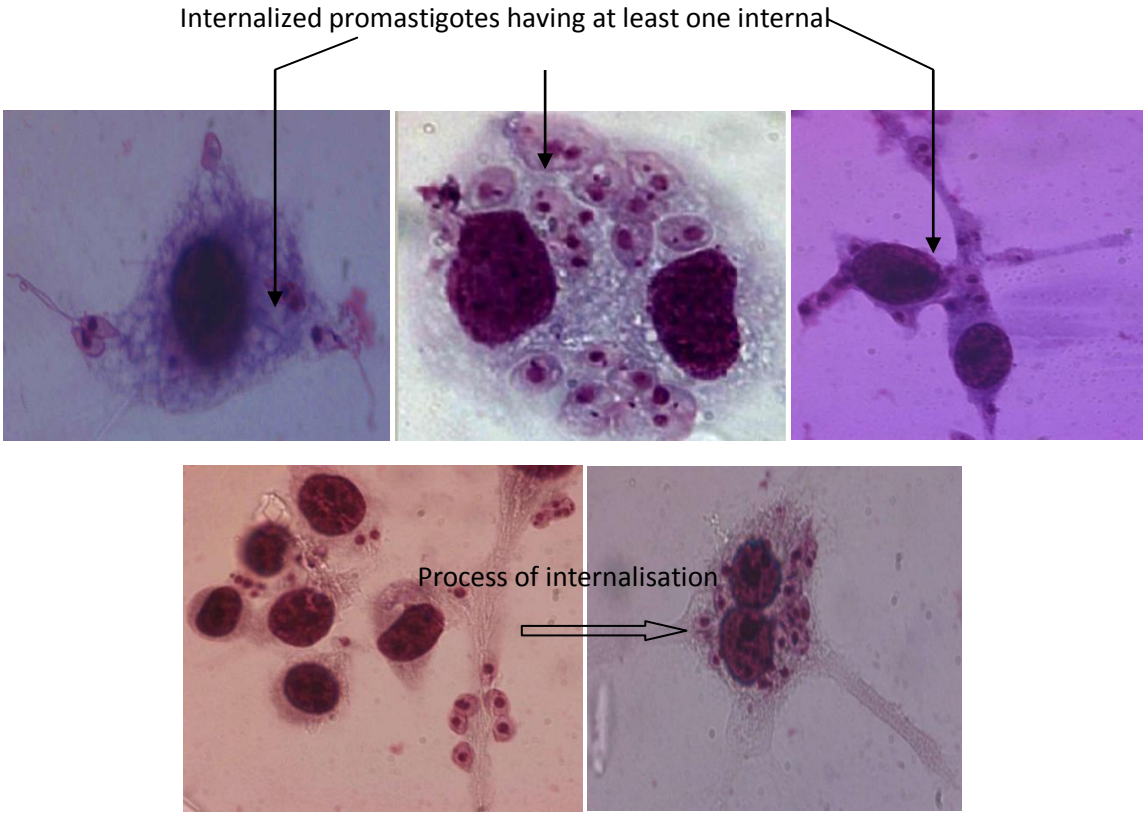

Fig: 4.9 Having atleast one internalised phase of Leishmania a) *Leishmannia* parasite infection in initial internalisation phases. b) internalized Meta1 phase of GFP parasite c) initial phase of internalization by pseudopodia. d) Process of int. by pseudopodia of Meta1 overexpressed

It has been observed that initially at 1hr time point 45% and 18% were infected by Meta1 and *Ld* GFP (Pvalue- 0.0005***) which means 45% of ld Meta1 parasites had internalized macrophage. At the time point of 4hr, the percentage of internalization hiked to 68% and 49% respectively in which the number of Ld Meta 1 parasites went up to **1845** from 1271 of

1 hr time point which is also significantly higher than control. Maintaining the trend, at 12 h time point, the internalized parasites increased to 92% vs 86% for Ld GFP Meta 1 and *Ld* GFP respectively. However, interestingly the *Ld* GFP got catch up the infectivity to 86% at this time point from 49% of 4 h and had no much difference with Ld Meta1 (p value,0.09).

Table: 4.4 Percentage of macrophages having internalized parasite at 4 time points -1hour, 4hour, 12hour, 24hour of *Ld*Meta1 over expressing cell and *Ld* GFP

	Internalized %		**p value**
Time points	**Ld Meta1**	**Ld GFP**	
1hour	45 ± 19.05 n=1271	18 ± 12.18 n=588	0.0005***
4hour	67.69 ± 17.9 N=1843	49 ± 12.37 n=1242	0.0070**
12hour	92 ±1.42 n=1202	86.96 ± 9.8 n=982	0.09ns
24hour	95.85 ± 3 N=2588	40± 19.3 n=2406	<0.0001****
Total (n)	6904	5218	

At the later time point of 24 hr, 96% and 40% macrophages were found infected by Ld Meta1 and Ld GFP respectively**.** Therefore, both the *Ld* META1 & *Ld* GFP in this category showed significant difference in general (Pvalue-<0.0001****).

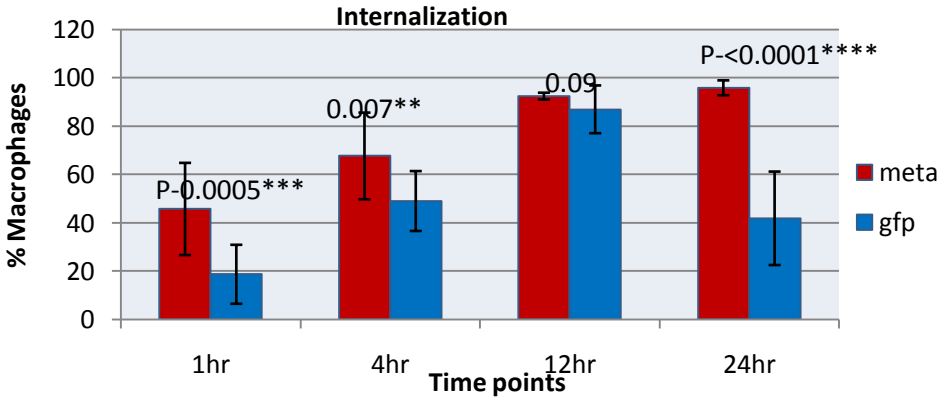

Fig: 4.10 Bar graph showing comparison percent macrophages that have atleast one internalized promastigote at 1hr, 4hr, 12hr, 24hr time points between *Ld* Meta and *Ld* GFP.

4.8.1.4 Macrophages interacted by exclusively internal *Leishmania*

The exclusively internal category reflected that mean number (experiments was in triplicate) of 51,95 *Ld* Meta1 parasites out of 1×10^7 used for infections were found exclusively internalised whereas it was 4133 in *Ld* GFP(Tab:4.5)

Table: 4.5.- Percentage of macrophages having only exclusively parasite at 4 time points - 1hour, 4hour, 12hour, 24hour of *Ld*Meta1 over expressing cell and *Ld* GFP

	Exclusively internal %		**p value**
Time points	***Ld* Meta1**	***Ld* GFP**	
1hour	5.7 ± 5.3 n=154	0.22 ±0.28 n=8	0.0017**
4hour	18.55±14.3 n=511	10.22± 8.4 n=319	0.09
12hour	77 ±10.83 n=2134	40.8± 7.2 N=1834	0.0001****
24hour	88± 8.08 n=2396	73 ± 9.2 N=1972	0.002**

So from this (Fig: 4.11,12) the exclusively internal promastigotes were found significantly higher in the overexpressed *Ld* Meta1 than *Ld* GFP.

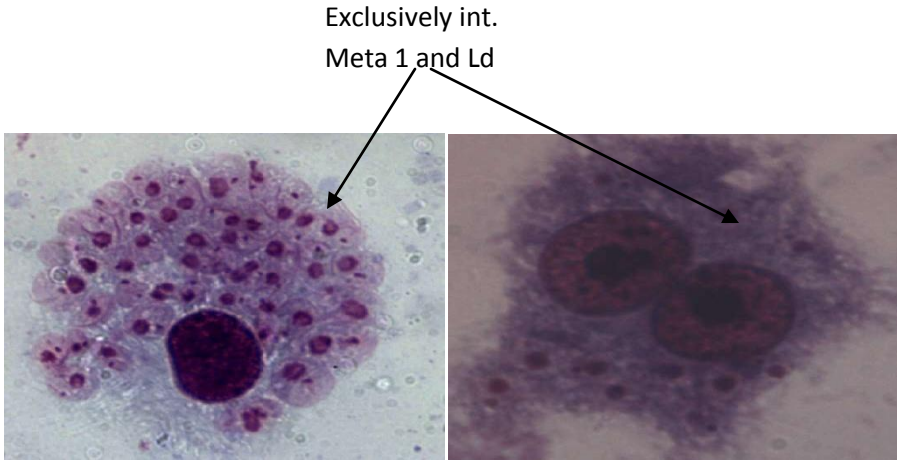

Fig: 4.11 Exclusvely internalised phase of Leishmania parasites a. *Leishmannia* parasite after infection and only internalized phases. b. only internalised Meta1 phase of parasites.

The exclusively internalized category includes those macrophages that have got only internal promastigotes without attached promastigotes. This, internalised category for Ld GFP showed that 18% of macrophages had only one promastigote internalized which is significantly less than the Ld META1 infected group(P-value-0.001**)(Table 4.5). Across the time points 18% vs 10% were only internalized, in Meta 1 and Ld GFP respectively, which showed subtle difference between two strains. At the later time points like in 12hr , 77% vs 40% were internalized which was significantly increased by 50% in case of Meta1(P value-0.0001****) than *Ld* GFP. Finally, at 24hr macrophages with 88% and 73% were internalized in *Ld*Meta1 and Ld GFP which showed that there was significant internalization in Meta1 than Ld GFP (Pvalue-0.002**).

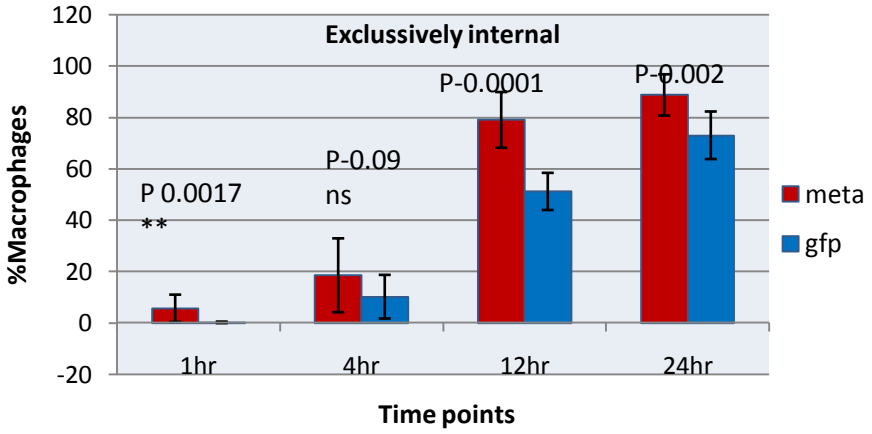

Fig:4.12 Bar graph showing a comparison of percent macrophages that have at least one promastigotes internalized without any of it attached externally at 1hr, 4hr, 12hr, 24hr time points between *Ld* Meta and *Ld* GFP.

4.8.2 Infectivity level of *Leishmania donovani* load to macrophages during host-parasite interaction.

The above first part of the research work studied the infection load of *Leishmania* parasite (count) to infected macrophage by counting total infectivity to 300 macrophages which proved that infection rate is higher in overexpressed Meta1. The work further carried to elucidate by looking at promastigote infected on macrophages in this section . The categorization was done by counting the number of parasites able to infect host macrophage cells in groups of less than five (M<5) and more than five (M>5) in different time points as mentioned above, i.e. 1 h, 4 h, 12 h and 24 h. In general, the distribution

pattern expressed that, greater than 5(M>5) group increased as time proceeds in. *Ld* Meta1 parasites showed more M>5 group than control parasite, *Ld* GFP (4.13).

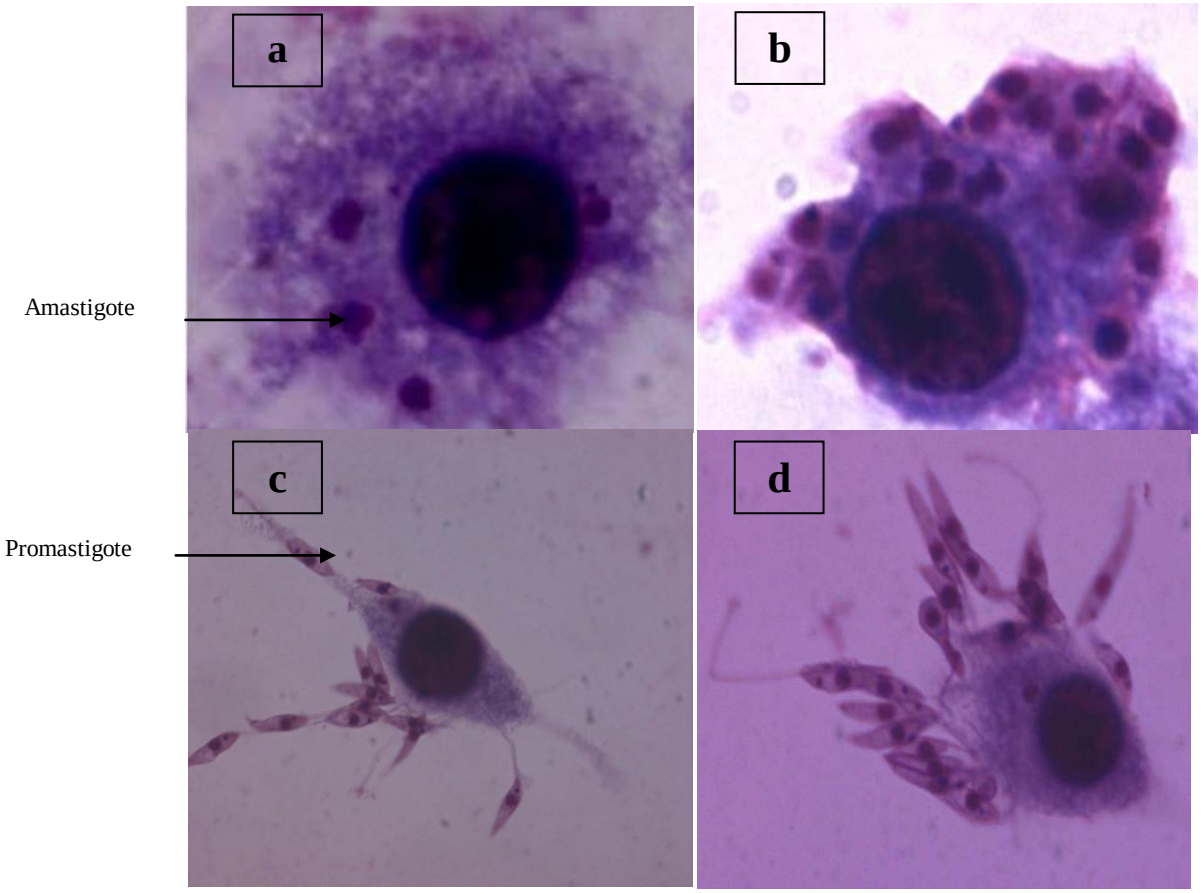

Fig:4.13 *Leishmania donovani* parasite infected to host macrophage cells by (a) < 5 intenalized (b) >5 internalized (c) <5 attached (d) >5 Attachment promastigotes

4.8.2.1 *L. donovani* parasites infective to macrophages exclussively external parasites.

Whereas for only external category of *Ld* GFP (Fig4.14, 15, tab: 4.6) at 1hr 34%vs59%and 9%vs12%,, having less than five and greater than five promastigotes were only attached and have not get internalized at 1h time point in Meta1 and *Ld* GFP respectively which is significantly higher promastigotes of less than five in *Ld* GFP than Meta1 over expressed (p value-<5-0.0001****) but in *Ld* Meta1 the no. of macrophages didn't differ significantly. And similar case was found at 4 hr time points where having less than five *Ld* GFP showed significantly higher than Meta 1(pvalue-0.005**)higher. However, at 12hr, both less than five and greater than five category showed significant increase in number than Meta 1.

(pvalue-<50.02 and >5-<0.0001****)Whereas in case of Meta 1 it lowered to 0.03% suggesting that Meta 1 were significantly better at internalization at earlier time point than Ld GFP and had higher parasite load than their counterparts.

Table:4.6 Percentage of macrophages having distribution of< 5 and >5*Leishmania* exclusively external at 4 time points -1hour, 4hour, 12hour, 24hour of LdMeta1 over expressing cell and Ld GFP.

	exclusively ext.%			Pvalue
Time points	**distribution**	**LdMeta1**	**Ld GFP**	
1hour	<5	34.296±3	59.8±14.5	<0.0001****
	>5	9.888±3.1	12.3±10.2	0.33ns
4hour	<5	14.38±11	27.3±10.1	0.006**
	>5	8.16±9.7	12.5±14.3	0.42ns
12hour	<5	0.26±0.27	0.89±1	0.02*
	>5	0.037±0.11	0.074±0.14	<0.0001****
24hour	<5	0.11±0.16	0.48±0.68	0.002**
	>5	0±0	1±0	0

Therefore , exclusively external parasite burdens were found significantly higher in virulent Ld parasites. which concluded that internalization is faster in Meta1.

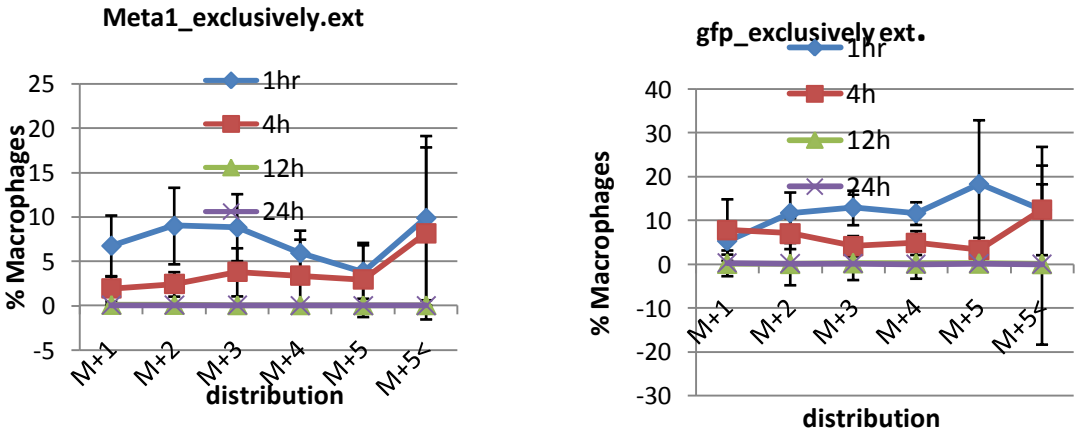

Fig: 4.14 Different percentages of parasites (Ld Meta1 and Ld GFP) able to attach exclusively.

This (fig: 4.14) is the indication of the distribution of meta1 and Ld gfp separately where in case of meta only ext. is incresed till M+5 and later it reached to ten so trend line is same in this case however in Ld GFP at one hour time point first it is at zero level and then it increased till M+5 but at four hour time point greater than five is increased to greater than ten. in both time point. Hence, in this category, *Ld* GFP shows significantly increased parasites load than Meta1 overexpressed.

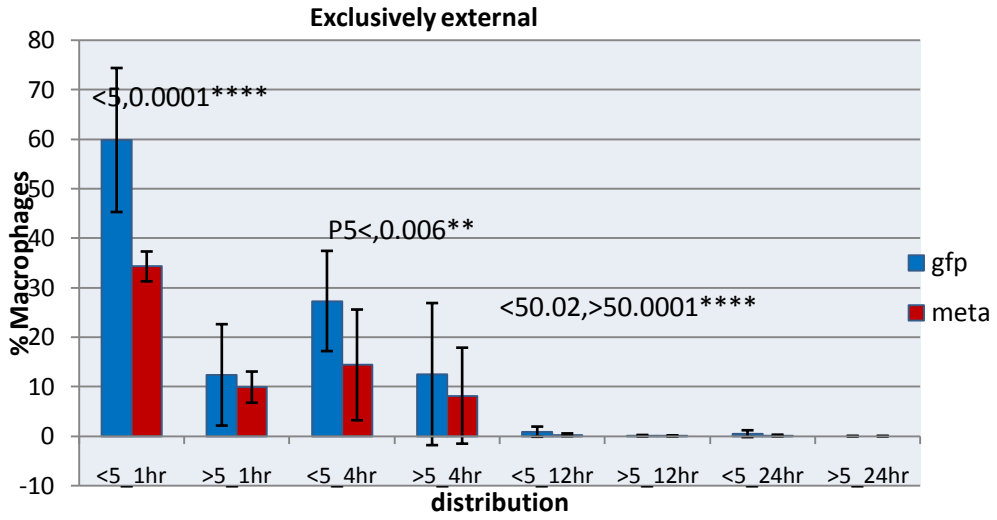

Fig: 4.15 Bar graph showing a comparison between percentages of *Ld*GFP&Meta1 over expressing cells in terms of percentage of Leishmania infecting macrophages cells having only external.

4.8.2.2 *L. donovani* parasite infective to macrophages as attached parasite

The distribution for attachment category (Fig 4.16,17, Table 4.7) expressed that, greater than 5(M>5) group increased significantly as time proceeds in *Ld* Meta1 parasites while the case did not remain true for the *Ld* GFP. There was significant increment in percentage of 5+ infections at 1 h, 4 h and 12 h but the infection percentage was insignificantly increased at 24 h time point by *Ld* GFP. Thirty seven percent of macrophages were found infected by more than five Ld Meta1 promastigotes while only27% was infected in the case of *Ld*GFP at 1hr time point. Similarly 53% has been infected by Meta1 and 34 % by *Ld* GFP at 4 hr & which is comparatively lower in *Ld* GFP. Similarly, there were high percentage of infection by *Ld* Meta1 at 12 h and 24 h time point in comparison to *Ld* GFP.

Table:4.7 Percentage of macrophages having distribution of >5 and <5 *Leishmania* attached, at 4 time points -1hour, 4hour, 12hour, 24hour of LdMETA1 over expressing cell and Ld GFP

	Attachment%			
Time points	Distribution	LdMeta1	Ld GFP	
1hour	<5	62.25±10.2	58.22±11.2	0.002**
	>5	37.48±6.1	27.66±11.3	<0.0001****
4hour	<5	38.722±8.7	52.27±17.4	0.017*
	>5	53.388±5.6	34.38±20.9	0.0042**
12hour	<5	11.629±7.9	15.81±5.2	0.12ns
	>5	81±7.6	71.11±11.1	0.0144*
24hour	<5	5.407±4.6	18.74±14.2	0.0072**
	>5	90.222±7.1	70.96±18.3	0.0016**

Across the time points, there was an increment from 81±7.6% in 12 h to 90.2% (±7.1) in 24 h time point which were infected by more than five *Ld* Meta1 promastigotes. Till 12 h time points there is subtle difference between two strains however, at 24hr time points the parasite load was significantly higher in Meta1 (P value-<0.0001****). In case of less than five category, the number of *Ld* Meta1 parasites gradually decreased in attachment state (62%, 38%, 11% and 5%) while for the *Ld* GFP there is decrease from 1 hr to 12 hr time points (58.2%, 52.3% and 15.8%) but a bit increment was found at 24 hr time (18.74%)

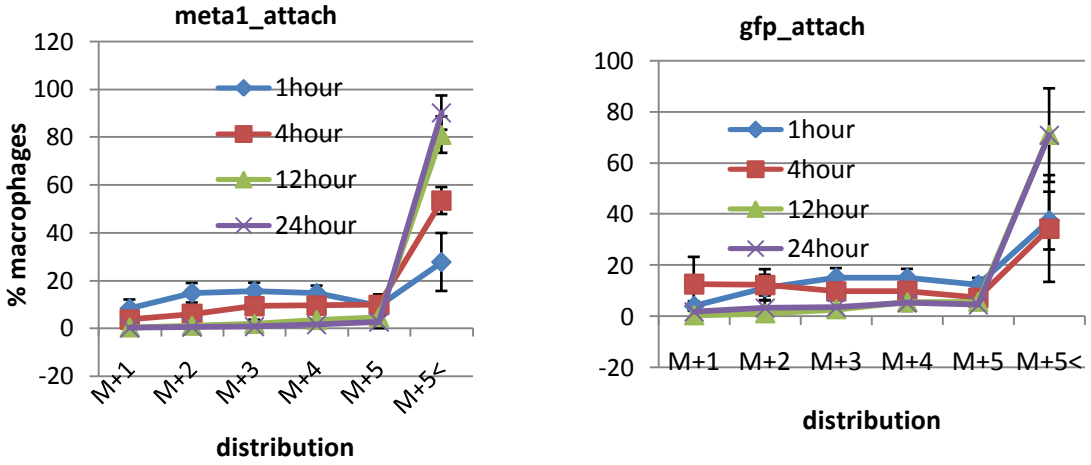

Fig: 4.16 Different numbers of parasites (*Ld* Meta1 and *Ld* GFP) able to infect macrophages.

This distribution graph showed that at 1h time points the infected macrophages were overlapped 4h in Meta1 and later it increased in Meta1 than *Ld* GFP at. 12h and 24 h hence, this data suggested that the rate at which attachment proceeds for *Ld* Meta1 promastigotes is slightly better as compared to *Ld* GFP.

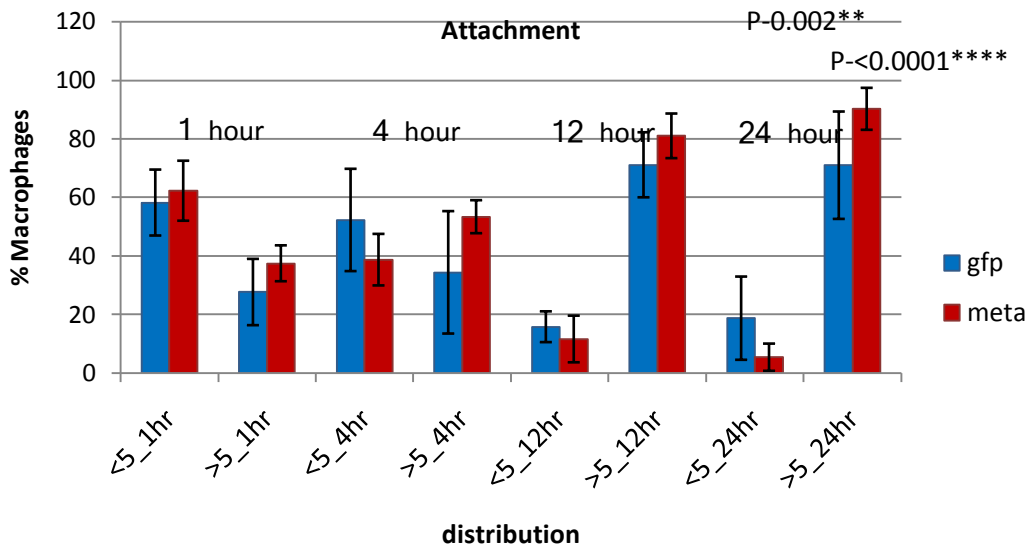

Fig: 4.17 Bar graph showing a comparison between percentages of *Ld*GFP&Meta1 over expressing cells in terms of percentage of *Leishmania* infecting macrophages cells having attached *Leishmani*a.

4.8.2.3 *L.donovani* parasite infective to macrophages as internalized parasite

For the internalized category of *Ld* META1,(Fig:4.18,19,tab:4.8) at 1hr time point 1.03%vs 0.62% of macrophages had internalized more than five promastigotes internalization by Ld Meta1 and *Ld* GFP promastigotes respectively and 44% vs 18% had less than five promastigotes got internalized in Meta1 and *Ld* GFP respectively hence at this time points parasite load in Meta 1 is significantly higher than *Ld* GFP (p value-0.0002***)so the later time points at 4hr 18.5 % vs 6.08 % in Meta1 and *Ld* GFP respectively and 49%vs42% in case of less than five promastigotes respectively hence Meta 1 showed significantly higher parasite load than *Ld* GFP(Pvalue-0.003** in >5 and 0.3 in <5) . At the later time points like 12hr macrophage having less than five and greater than five promastigotes were found ,12%Vs19%,and 79%vs,67% in Meta 1 and *Ld* GFP respectively and hence showed the parasite load is significantly higher in Meta1 than *Ld* GFP(Pvalue-<50.07,>5-0.0005***) similarly, at 24 thatpromastigotes5%vs19% and ,89%vs 69%having greater than five were significantly increased in over expressed Meta1 however, less than five as in case of *Ld* GFP.(Pvalue <5-0.002**and >5-0.001**) Which confirmed that parasites load is significantly higher in Meta1 with better internalization across the time points.

Table:4.8 Percentage of macrophages having distribution of <5 and >5*Leishmania* ,internalized, at 4 time points -1hour, 4hour, 12hour, 24hour of LdMETA1 over expressing cell and Ld GFP.

	Internalization %			PValue
Time points	distribution	LdMeta1	Ld GFP	
1hour	**<5**	44.74±18.6	18.14±11.9	0.0002**
	>5	1.037±0.9	0.629±0.58	
4hour	**<5**	49.14±16.6	42.97±11.5	0.03
	>5	18.55±13.8	6.08±3.73	0.003**
12hour	**<5**	12.96±8.7	19.18±6.8	0.07
	>5	79.44±8.3	67.778±6.1	0.0005***
24hour	**<5**	5.888±5.2	19.74±13.4	0.002
	>5	89.96±7.6	69.37±17.8	0.001**

The distribution graph showed that 4h time point the *Ld* GFP is increased initially but at later time points(12h,24h) Meta 1 showed increased parasite load than *Ld* GFP. Hence this indicated clearly that better infection or internalization in *Ld*Meta 1.

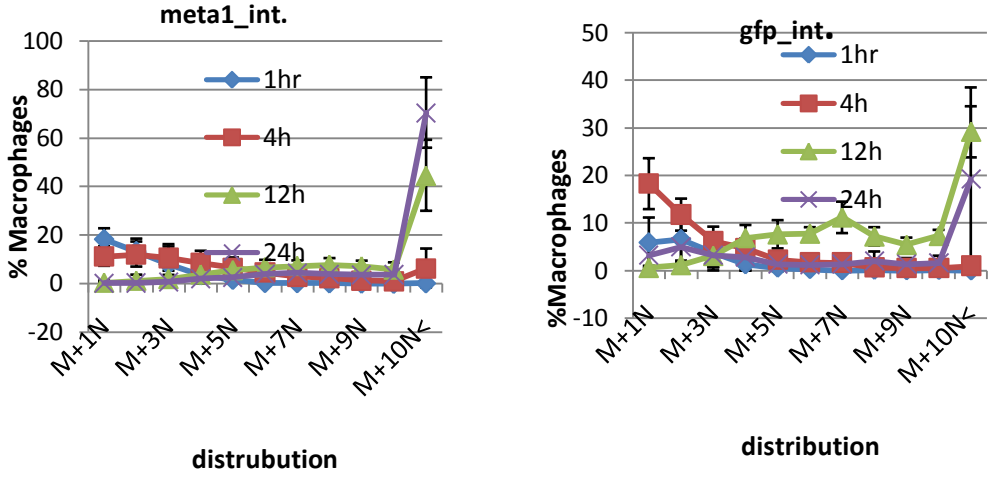

Fig 4.18 Different number of parasites (Ld Meta1 and Ld GFP) able to internalised macrophages

So, parasites burden was found higher in Over-expressed Meta1 than Ld GFP.

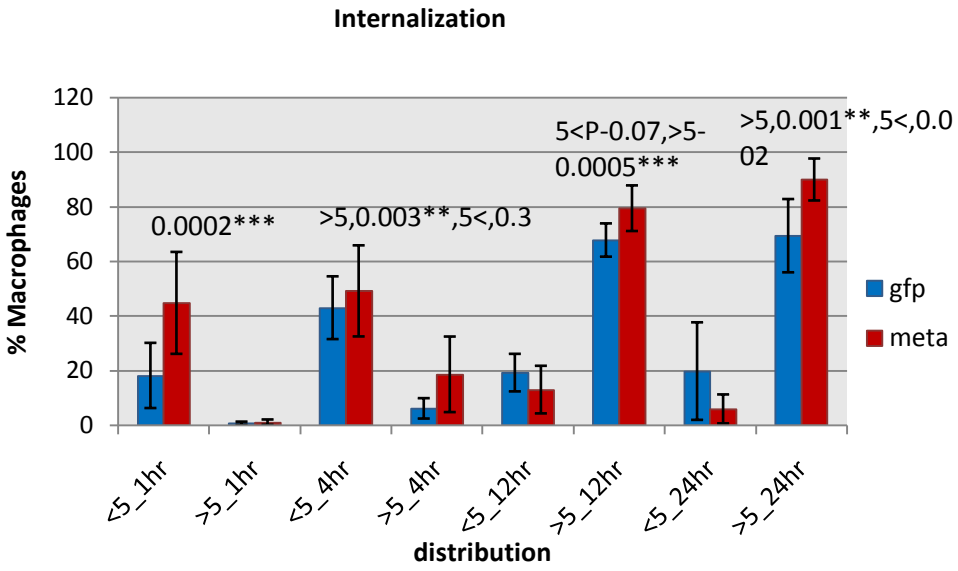

Fig: 4.19 Bar graph showing a comparison between percentages of LdGFP&META1 over expressing cells in terms of percentage of Leishmania infecting macrophages cells having internalized.

4.8.2.4 *L.donovani* parasite infective to macrophages as exclusively internal parasite.

On the contrary those promastigotes having nothing attached externally and all have got internalized found in this category. The exclusively internalized category for *Ld* GFP shows that having less than five were 5%vs0.2% 1.19% of *Ld* Meta1 and *Ld* GFP respectively which shows significantly higher parasite load in Meta1 than control(pvalue-0.001**). Similarly at 4hr time points having less than five and greater than five of 13%vs9%a and 5.4%vs1.19% were internalized macrophages respectively,which is significantly higher in Meta1 than Ld GFP(P value-0.001**) in case of greater than five category. Across the time points, at 12 hr 10%vs 13 % and 68%vs 37% were internalised less than and greater than promastigotes in Meta1 and Ld GFP respectively(Tab:4.9,Fig:20,21) where Ld GFP showed 69% less by Meta 1 of more than five group of promastigotesat 12hr which is highly significant than Ld GFP(Pvalue-<0.0001****) at later time points,4%vs16% and 83%vs56%were less than and greater than promastigotes of Meta1 and Ld GFP respectively at 24hr, which showed more than five promastigotes internalization in Ld Meta1 is highly significant than Ld GFP(Pvalue-,0.0001****).

Tab: 4.9 Percentages of infected J774 macrophages that had at least one *Leishmania*, exclusively internalised, and in META1 over expressing cells and Ld GFP

	Exclusively.int%			Pvalue
Time points	**distribution**	**LdMeta1**	**Ld GFP**	
1hour	**<5**	5.703±5.3	0.22±0.28	0.001**
	>5	0±0	1±0	0
4hour	**<5**	13.08±9.5	9.02±7.6	0.2
	>5	5.472±4.9	1.19±1.5	0.0017**
12hour	**<5**	10.96±7.0	13.37±3.8	0.1
	>5	68.07±14	37.8±4.2	<0.0001***
24hour	**<5**	4.925±4	16.62±12.6	0.003**
	>5	83.81±11	56.40±18.3	<0.0001***

Hence, from overall studies better internalization with higher parasite load was found significant in Meta 1 overexpressed(fig:4.19,20).

But the distribution was catagorised as greater than ten or less than ten in only internalized which showed that greater than ten increased in Meta 1 in both 12h and 24h time points than *Ld*GFP.

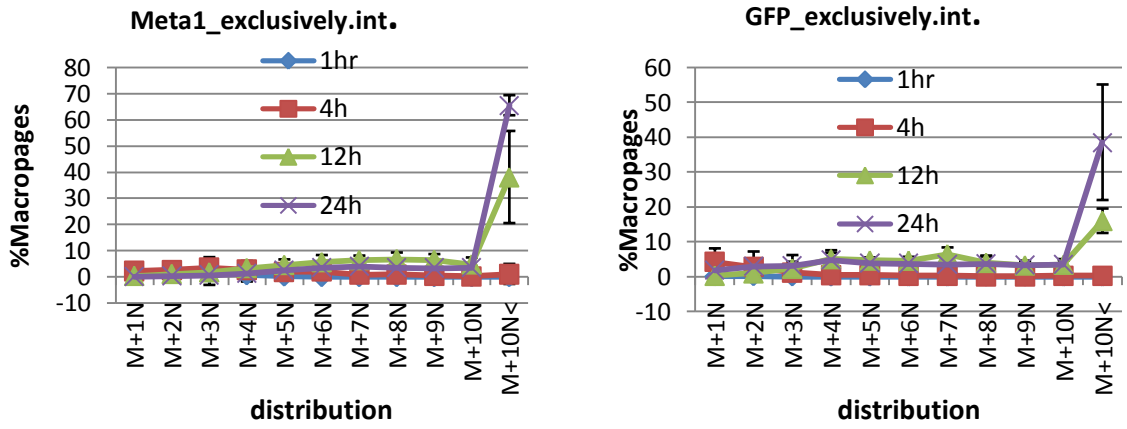

Fig:4.20 Different number of parasites (*Ld* Meta1 and *Ld* GFP) able to exclusively internalized.

Hence, only internal amastigotes were found significantly higher load in Meta1 than *Ld* GFP.

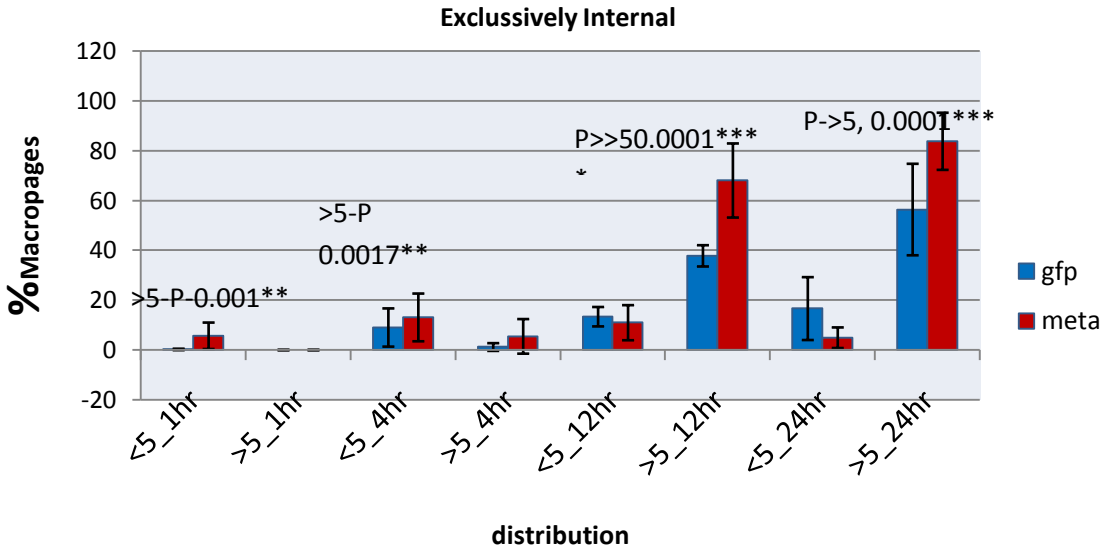

Fig: 4.21 Bar graph showing a comparison between percentages of *Ld*GFP&Meta1 over expressing cells in terms of percentage of *Leishmania* infecting macrophages cells having exclusively internal.

4.9 Kinetics of different strains of *leishmania* attached, internalised to the Macrophages at different time points.

To check infection rate and quantum, two group of parasites *Ld*meta1 (test) and *Ld*GFP control was used and found that the parasites in attached, internalized and multiply inside stages of the host cells as observed in the bright field of phase contrast microscopy. The principle of phase contrast microscopy is that it can penetrate the only stained cells so that giemsa stain helps to identify the stained parasites inside the host cells.

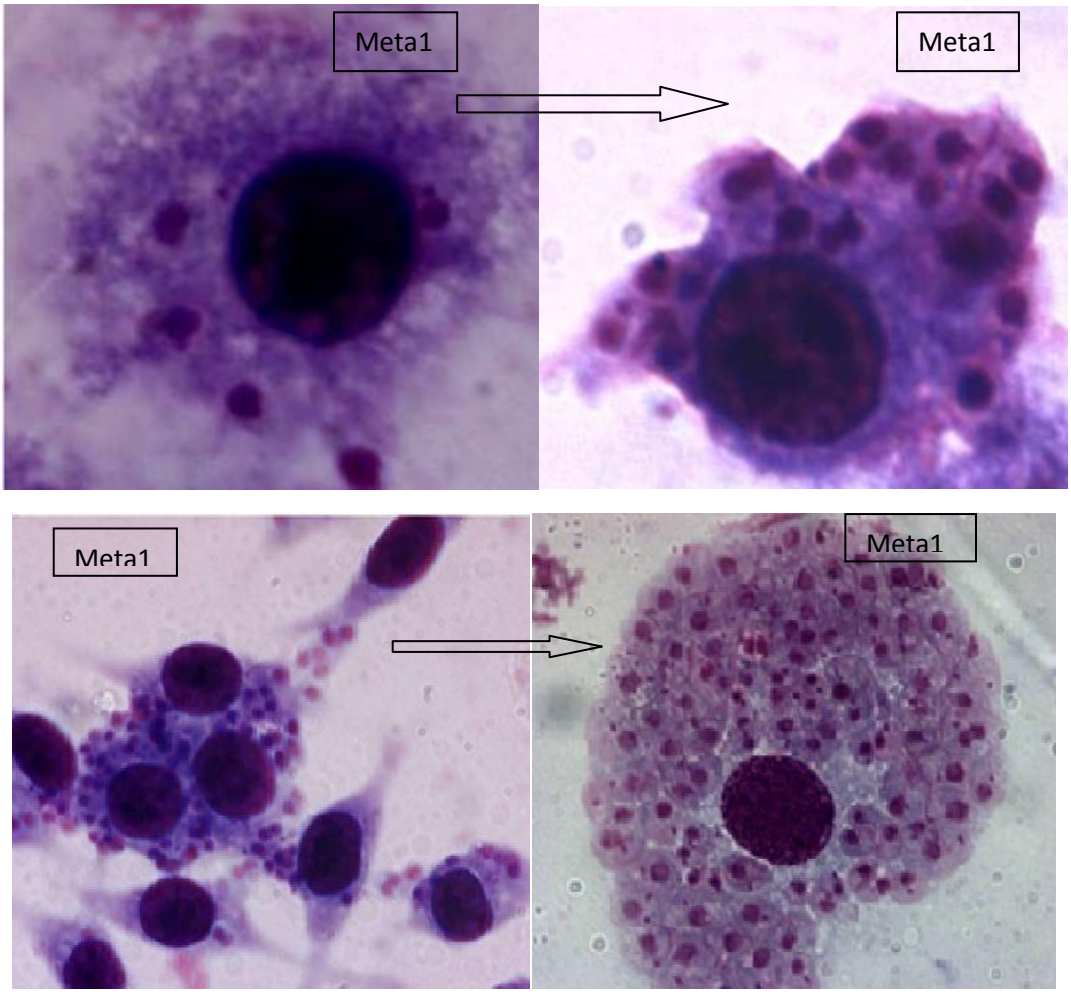

Fig: 4.22 Kinetics of macrophages infection by *Leishmania donovani* parasites with Meta1 overexpressed cells at a) 1 hour b) 4 hour c) 12 hour d) 24 hour time point, Axioplane 2 imaging- The giemsa stained slides were analyzed under bright field of phase contrast microscope images were captured and analysed in axioplan 2 microscope. J774 macrophage cell lines were infected with virulent *L. donovani* promastigotes at a ratio of 1:10 (cell: parasite). Infected cells were stained with 5% Giemsa stain at 1hr, 4hr, 12hr and 24 h., (a,b,c,d respectively).

It was found that, the number of parasites internalized increases from 1hour to 24 hour in case of over expressed Meta1 and nearly 100% promastigotes were internalized at 24 hour in Meta1. However, attachment parasites load were higher in case of *Ld* GFP so that, the internalization was still in the process as time increases. Hence morphologically, internalization is better in case of over expressed Meta 1 than control.

To confirm this experiment further, statistical data analysis was done above(Fig:4.,22,23)

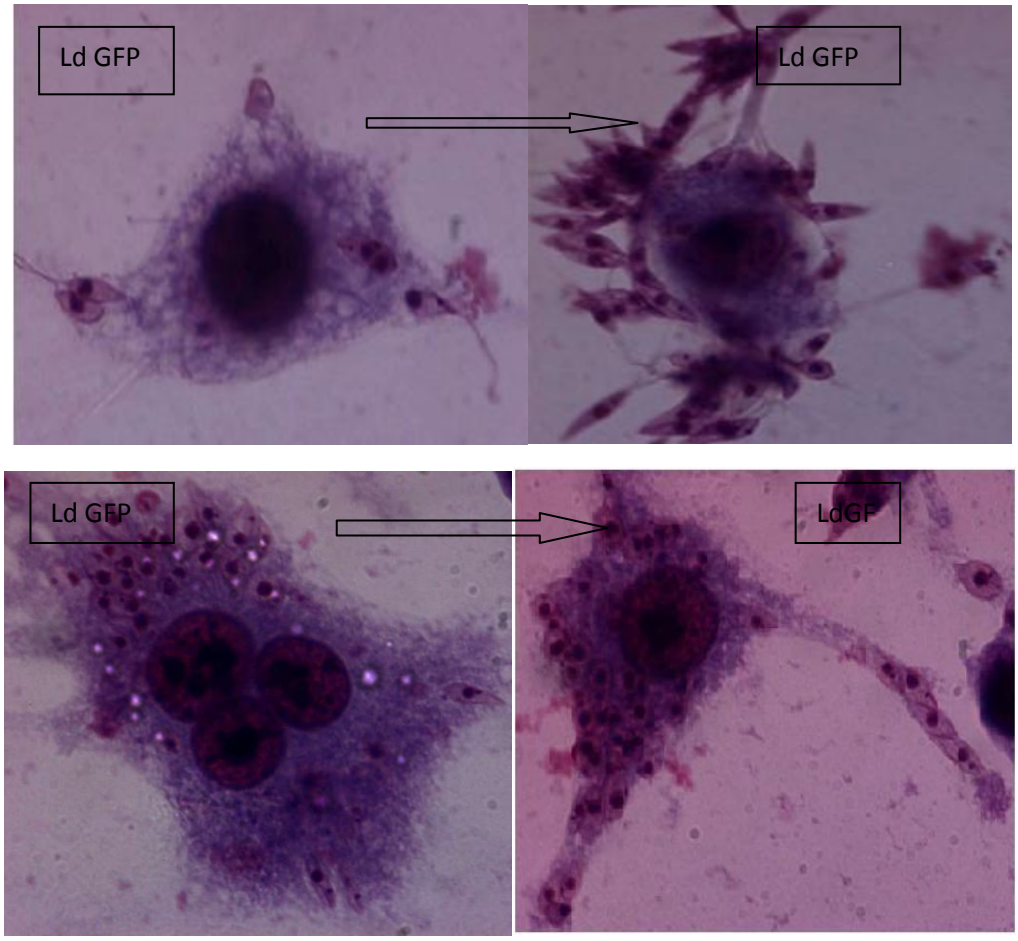

Fig: 4.23 Kinetics of macrophages infection by Leishmania donovani with GFP overexpressed cells at a) 1 hour b) 4 hour c) 12 hour d) 24 hour timepoint. Axioplane 2 imaging:The giemsa stained slides were analyzed under bright field of phase contrast microscope and images were captured and analysed in axioplan 2.Internalization of promastigotesin host cells. J774 macrophages cell lines were infected with virulent *L. donovani* promastigotes at a ratio of 1:10 (cell: parasite). Infected cells were stained with 5% Giemsa stain at 1hr, 4hr, 12hr and 12 h. (a,b,c,d respectively).

Thus, the number of internalized *Ld*Meta1 promastigotes per cell after infection was morphologically shown above fig:4:22 Hence, From above results we have shown that as time increases the number of parasite get internalized with better rate and higher quantum. Thus, the number of internalized LdGFP promastigotes per cell after infection was morphologically shown here (fig 4.23) Hence, From above results we have shown that the number of parasites is still in the process of internalization as time reaches to 24 hour so it takes time more than Meta 1 to get internalized promastigotes on macrophages.

4.10 Macrophages infection kinetics (1h, 4h, 12h and 24h) and their relation with *Ld* Meta1 and *Ld* GFP.

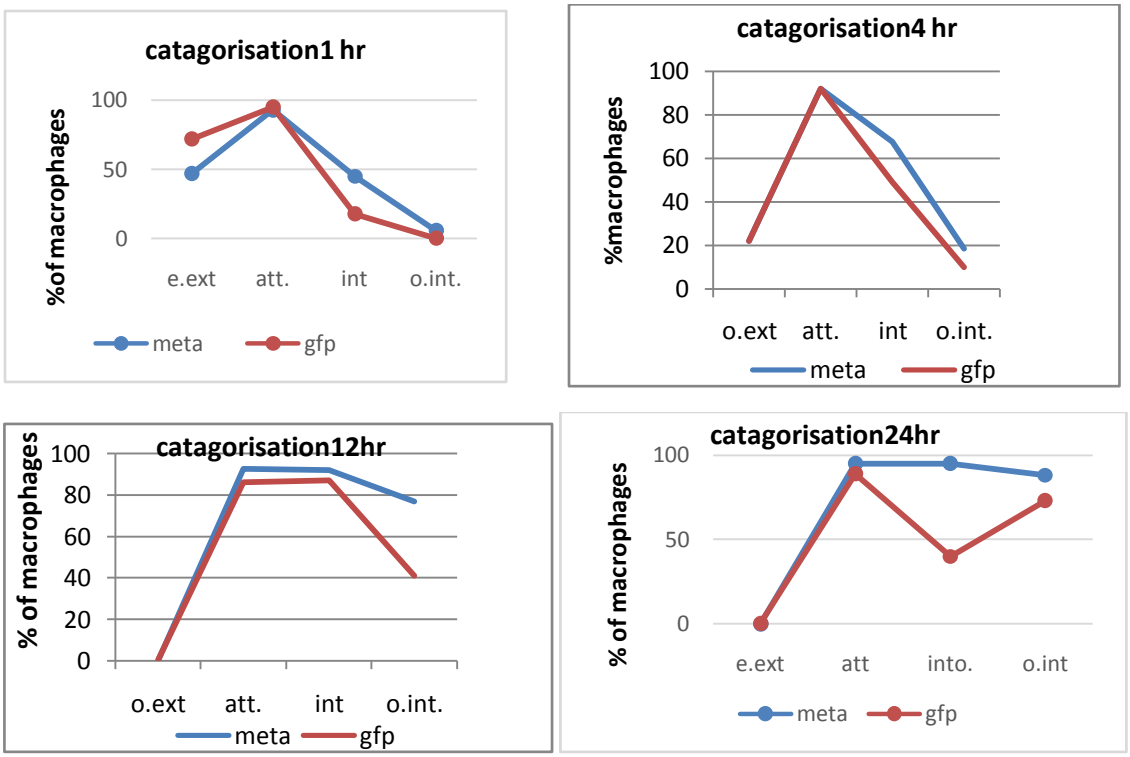

Fig:4.24 Total infectivity of macrophages having all catagorisation at 1h, 4h, 12h and 24h time points(0.ext-exclusively external, att- attachment, int-internalisation,o.int- exclusively internal)

Direct attachment is higher in than the exclusively external in both overexpressed Meta1 and GFP.There were no macrophages having condition of only external both in LdMeta1 and *Ld*GFP. The attachment cases were still huge 95% and 89% of Meta and GFP respectively. It is interesting that the internalizing rate is very high in *Ld* Meta1 in all time points except 1 hour while the rate is significantly decreased in *Ld* GFP at 4hour .similarly, at 12h and 24h internalization and exclusively internal infected Macrophages were extremely higher in LdMeta1 which shows the aggressive role of Meta 1 gene for penetration. Hence, the exclusively internal parasites, still the Meta have significantly high percentage of Meta1 in comparison to GFP.

CHAPTER V

DISCUSSION

In this study, virulent *Leishmania donovani* with over expressed Meta1 and virulent *Ld*GFP promastigotes (wild type) have been used to establish an *in vitro* model of infectivity by interaction of host J774 cells with parasites. The study investigated the interaction of Meta1 over expressed and only virulent *Ld* GFP with balb/c mice derived J774 host cells.The life cycle of parasite alternates between invertebrate (sand fly) and vertebrate (mammalian) hosts, and this obligate intracellular pathogen has major impact on both innate and adaptive immune responses by re-modulation of cytokines, chemokines and Toll-like receptor. So these modulations are associated with the recognition of *Leishmania spp.* by host cells, and they are critical in understanding of pathogen–host interactions (Kaplan et al.)

Previous, study demonstrated that whether Meta 1 expression correlates with the ability of the parasites to infect macrophages *in vitro*. They infected murine peritoneal macrophages with L. *(L.) amazonensis* at different growth stages. The infectivity was found to be remained low during the log and early stationary phases and increased significantly during the stationary phase of parasites. The study confirmed that the metacyclic promastigotes associated genes are up regulated during the stationary phase of the parasites(Mansueto et al., 2007). Present study showed that the infectivity is enhanced on host cells when *in vitro Leishmania donovani* infection assay was done by over expressed *Ld* Meta1 parasites using stationary phase of parasites.

The role of many molecules like GP63 , lipophosphoglycan , cysteine proteases etc. that are differentially expressed in the two developmental stages of the parasite have been studied for their involvement in adaptation of the parasite at 37^0C and by enhancing virulence of the parasite(Santos et al., 2013) . The infective stage amastigotes of cutaneous species, such as *L. mexicana* and *L. panamensis*, cannot survive well at 37°C *in vitro* as being better adapted to lower temperatures in skin (Descoteaux et al., 1992)In contrast, strains of *L. donovani* amastigotes that can be grown *in vitro* grows best at 37°C, indicating adaptation to growth at the higher temperatures of the internal organs. Since *Leishmania* is an intracellular organism, the clinical form of leishmaniasis depends on the behavior of the infected macrophage(Gupta et al., 2001). We therefore, addressed the question of how different species cause different pathologies by comparing difference in infection of *Ld* Meta1 in macrophages identically infected *in vitro* with *L. donovani,* which cause visceral leishmaniasis. So this study elucidated the adaptation of parasites at human body temperature till 24 hour and most of the parasites converted into the amastigotes in over expressed Meta1.

Meta1 (PF03724) is currently described as a small domain family of unknown function in *Leishmania* Meta1. A virulence-related function has been demonstrated by scientist for the Meta1 gene which, when over expressed, resulting into the hyper virulence in mice (Alexander and Russell, 1992). However the exact function of Meta1 protein is yet not known underlying mechanism remained to be explored.

Previous study also illustrated that, Meta1 protein expression varies between the two species(Uliana and Silvia, 1999) *Ld* Meta1 is expressed maximally in axenic amastigotes where as in *L. Major* Meta1 is down regulated in amastigotes and expressed maximally in metacyclic promastogotes(Ramos et al., 2004) . This suggests a principle role of Meta1 in visceralising the mammalian host rather than the insect vector species. Present study also explained the similar result with infection rate in mammalian host in response to Meta1 protein and has shown that infection rate was significantly higher in mammalian host. In case of amazonensis, the virulence capacity is increased when the Meta1 GFP protein is over expressed (Uliana and Silvia, 1999). On *Meta1* over expression in *L. amazonensis*, parasites were found to be more virulent than wild-type(Uliana and Silvia, 1999) hence above study also supported the present study because *Leishmania donovani* also causes visceral leishmaniasis and results showed that infection rate was significantly higher in over expressed Meta1 than wild type Ld GFP and hence better infectivity observed.

Previously the infection assay was done in *L. mexicana* virulent and attenuated which causes the cutaneous leishmaniasis and found that virulent parasites has significant increase in infection than attenuated at 48 hour time points only (Uliana et al., 1996,) where as present study illustrated infection assay on the visceral leishmaniasis and result showed that virulent over expressed Meta1 have high infection rate and quantum than without over expressed.

Hence present investigation also supported the above studies done by many scientists and role of Meta1 gene. However, in case of *Leishmania donovani* no infection assay study was reported till now on J774 macrophages cell line, hence to the best of our knowledge, this is the first study to investigate virulence mechanism of *Leishmania donovani* by infection assay, which causes viscera leishmaniasis in response to Meta 1 gene. Hence present study elucidated the principle role of Meta1 protein on host cells by infection assay mechanism and shown that there is significant increment of internalized *Leishmania* in case of over expressed parasites. Present study elucidated that there are significant host parasite relation in reference to the interactive time points as studied in 1hr, 4hr, 12hr and 24hr. There were huge numbers of parasites come to external site of host cells which remarkably decreased (47% to 0.1% in test and 72% to 0.5% in control parasites) as the interacting time increases from 1 hr to 24 hr. In similar condition, the attachment state of both parasites was found consistent at the range of 90%. Interestingly, the result demonstrated by the

internalized parasites were in gradually increment from 45% to 96% by *Ld*Meta1 while 18% to 87% by *Ld*GFP however, the latter had less (40%) number of infection in 24 hr time. Similar to the trend of internalized, the exclusively internal parasites increased in increasing time points (5.7% to 88% in test and 0.22% to 73% in control parasites).

It is assumed that when more promastigotes get internalized it will get converted into amastigotes and the infective rate should be faster. This study also have illustrated that, only external *Leishmania* that have not get internalized were significantly increased in *Ld* GFP than Meta1 so it is not necessary and sure that all attached promastigotes should get internalized after attachment which proved that better internalization is case of Meta1 over expressed. In present study better internalization means parasites had capacity to fight with the immune system of host cell and is susceptible to infection.

Similar to the trend of internalized, the exclusively internal parasites increased in increasing time points (5.7% to 88% in test and 0.22% to 73% in control parasites).

Regarding susceptibility of macrophages to less than 5 and more than 5 parasites, the number of infected macrophages were decreased(35% to 0.11% in test and 60% to 0.5% in control). The number of cells attached by parasite showed that *Ld*Meta1 and Ld*GFP* parasites decreased when the numbers were less than 5 (63% to 6% vs 59% to 19%) and increased when the numbers were more than 5 (38% to 91% vs 28% to 71%). Similarly the internalized *Ld*Meta1 parasite to the macrophages by less than 5 parasites were in decreased order but the infected macrophage attached by more than 5 were in increasing trend. The susceptibility of macrophages for exclusively internalized more that 5 parasites were remarkably in increasing order from 0% to 84% for LdMeta1 while it was 1% to 57% for LdGFP. Finally, it is concluded that external *Ld*Meta1 and *Ld*GFP parasites were more or equal for exclusively external and attached parasites while the internalized and exclusively internalized were higher to former in comparison to latter. The result concluded that infection rate was significantly better and the parasite load was significantly higher in *Ld* Meta1 as compared to control.

So based on these two interpretations the role of over expressed Meta 1 is identified in present studies. Hence from this research work, role of Meta 1 gene is explored, the parasite load and total infectivity of host cells were significantly higher in over expressed Meta1 than *Ld* GFP. But the present study is done only *in vitro* under controlled condition so obviously the mechanism of infection could be different *in vivo* and to be explored.

CHAPTER VI

SUMMARY

Kala-azar or visceral leishmaniasis is however, transmitted by sand fly, *Phlebotomus argentipes*, the proven vector, it is caused by obligate intra cellular protozoan parasite. The disease is characterized by diversity and complexity. The disease is taken as "neglected" in the absence of effective, affordable or ease of treatment. The concept of "neglected disease" has been derived from the requirements in the development of new drugs to combat from the infectious diseases which have been ignored by public and private sector. Millions people are suffered from this diseases. The visceral leishmaniasis has got the most severe form of leishmaniasis, which is the second largest parasitic killer in the world after malaria responsible for an estimated 500,000 infections each year worldwide. An estimated 500,000 new visceral leishmaniasis cases and 60,000 deaths from visceral leishmaniasis occur each year. Visceral leishmaniasis (VL) caused by *Leishmania donovani* is a fatal systemic disease if left untreated. Scientists are still not able to develop proper vaccine for prevention, and mechanism of how it interacts with host cell. It is still not understood. Therefore, trying to search the mechanism of how *Leishmania* interact with host cell line *in-vitro* using J774 cell line.

So previously scientist explored that, there is one amastigote associated genes Meta1 which is derived from metacyclogenesis where virulence associated genes are unregulated and it plays crucial role in virulence but the exact function is unknown. Naturally, the process of metacyclogenesis is carried out when parasite is more prone to infect the human body. At the time all promastigotes degrade the stomodeal vulve and releases from their thoracic mid gut to reach to the mouth. The ready for infection stage hence carry out metacyclogenesis which is very essential for infection. Meta 1 gene is up regulated during metacyclogenesis however, its exact function is not known, so the function of Meta 1 needs to be elucidated by interacting parasites with host cells in vitro.

Host pathogen interaction mechanism done by Macrophage Infection Assay(MIA) to identify the role of Meta1 gene during infection have not been documented till now. MIA is very reliable and specific technique to find the role of Meta1 gene by infection assay in cell culture. Meta1 protein is produced due to activation of metacyclic promastigotes specific Meta1 gene at the infective stage of *Leishmania* and enhances its infectivity to the host cell and easily adopt inside human body temperature. This gene's upregulation at this stage was initially found in *L. Major* but the gene is conserved in all species. Many of the investigation proved that Meta1 has role in infection on human body and it helps to visceralise the disease. Hence, crucial function is to support *Leishmania donovani* to cause kala-azar. The gene plays role in human body rather than in insect vector and helps to survive inside

human body temperature. The over expression of Meta1 GFP gene in *L. amazonensis* parasites were found to be more virulent than wild type *Leishmania* hence, over expressed Meta1 gene are very much efficient in penetrating to the macrophage cells .

In this study, virulent and over expressed *L. donovani* promastigotes have been used to establish an *in vitro* model of infectivity & its subsequent process of attachment, internalization, multiplicity, exit of *Leishmania* at stationary phase at temp 37^{o}C by host macrophage cells. The J774 macrophages were infected with the stationary phase of virulent and *L. donovani* promastigotes over expressed at a ratio of 1:10 (target cell: parasites). *L. donovani* promastigotes were considered in stationary phase at 7^{th} day when their number reached to 1×10^{7}. The macrophages were seeded on 3 slides at a density of $0.5x10^{6}$ cells per slide using fresh RPMI 1640 media. The next day, virulent & overexpressed Ld strains were infected with macrophage for their respective time points (1h, 4h, 12h,24h) & were incubated under 95% humidity at 37°C in a 5% $CO_{2.}$ Free parasites were removed with vigorous PBS washing. For giemsa stain, the slides were fixed with 100% methanol & then were stained for 10 min. The scoring of 300 macrophages was carried out in random in each slide belonging to different groups using optical microscope at 100X magnification. The infected macrophages were categorized into following groups, M+1,M+2…..>M+10 and statistical analysis was performed to see significant difference between two strains in Graph Pad Prism Data Analysis software by t-test.

Two ways of interpretation is elucidated *viz* in reference to infected macrophages and in reference to parasite infected. There was significant difference between two strains at 24hr time point (0.0001***). Both the *Ld* Meta1 & *Ld* GFP shared subtle differences in this category at earlier time points but at later time point Meta 1 showed significantly higher infective rate than *Ld* GFP. In reference to macrophage infected in internalized category both the *Ld* Meta1 & *Ld* GFP showed significant difference. In general, earlier and later time points (p value, 0.0005 in 1h,<0.0001 in 24h) the exclusively external category does not have internal parasites so infected parasites were found higher in *Ld* GFP in earlier time points(Pvalue,<0.0001***) while the exclusive internal category did not have external parasites. So it showed significant increase in infected cells in *Ld* Meta1 at early and later time points (p value-0.001** at 1h and 0.0001**** at 24 hr).

In reference to parasites infected, in all categories, greater than five promastigotes were significantly higher in case of Ld Meta1 over expressed than *Ld* GFP, however less than five were higher in *Ld*GFP. Hence, parasites load were higher in Meta1 to be claimed that, Meta1 plays crucial role to infect host cells though both strain used here were virulent but over expressed Meta1 showed higher quantum and better internalization in reference to its counterpart

The life cycle of parasite alternates between invertebrate (sand fly) and vertebrate (mammalian) hosts

Previous, study demonstrated that whether Meta 1 expression correlates with the ability of the parasites to infect macrophages in vitro. They infected murine peritoneal macrophages with L. *(L.) amazonensis* at different growth stages. The infectivity remained low during the log and early stationary phases and increased significantly during the stationary phase of parasites.studies also illustrated that, META1 GFP protein expression varies between the two species. Ld META1 is expressed maximally in axenic amastigotes where as in *L. Major* META1 is down regulated in amastigotes and expressed maximally in metacyclic promastigotes.This suggests a principle role in META1 in the mammalian host rather than the insect vector in visceralising species hence present study also explained the infection rate in mammalian host in response to meta1 protein and have shown that infection rate was significantly higher in mammalian host.

It is assumed that when more promastigotes get internalized it will get converted into amastigotes and the infective rate should be faster. . In present study better internalization means parasites had capacity to fight with the immune system of host cell and is susceptible to infection. so this study illustrated infection assay on the Visceral leishmaniasis and result showed that virulent overexpreesed meta1 have high infection rate and quantum than without overexpreesed. . Hence present study elucidated the principle role of Meta1 protein on host cells by infection assay mechanism and shown that there is significant increment of internalized *Leishmania* in case of over expressed parasites Along with this assay the interpretation was done by two ways first is in reference to macrophages infected where catagorisation of interpretation were done as only external, attachment, internalization, and only internal macrophages infected promastigotes and were counted where as in second case, in reference to infected promastigote and the catagorisation were same so from first interpretation found the the total infectivity of macrophages out of 300 counted and from second interpretation, found the parasites load or burden but previously this type of interpretation is not observed.Similarly, counting of infected macrophages and parasites infected were performed by counting greater than ten , greater than five and greater than five and less than five in both cases because the trend line were found same by counting these numbers so it enhanced the interpretation and analysis of results. So based on these two interpretations the role of overexpressed meta 1 is identified in present studies. Hence from this assay, role of Meta 1 gene is explored the parasite load and total infectivity of host cells were significantly higher in overexpressed meta1 than Ld GFP. But the present study is done only *in vitro* under controlled condition so obviously the mechanism of infection could be different *in vivo* and to be explored.

	Exclusively external %	
Time points	**Ld Meta1**	**Ld GFP**
1hour	**47 ± 15.7** (n=1271	**72± 7.4 n=1991**
4hour	**22.55±20.5 n=658**	**22± 17.9 n=1110**
12hour	**0.4 ±0.35 n=80**	**0.5 ± 1.5 n=146**
24hour	**0.2 ±0.16 n=3**	**0.48± 4.5 n=13**

	Attachment %	
	Ld Meta1	Ld GFP
1hour	93.0 ±10.56 (n=.2362)	95.0 ± 2.05 (n=2438)
4hour	92.1±5.54 (n=2511)	92±11.9 (n=2279)
12hour	92.6 ±2.57 (n=2501)	86 ± 10.18 (n.=3094)
24hour	95.6 ± 2.90 (	89 ±4.5

	Internalized %	
	Ld Meta1	**Ld GFP**
1hour	45 ± 19.05 n=1271	18 ± 12.18 n=588
4hour	67.69 ± 17.9 N=1843	49 ± 12.37 n=1242
12hour	92 ±1.42 n=1202	86.96 ± 9.8 n=982
24hour	95.85 ± 3 N=2588	40± 19.3 n=2406
Total (n)	6904	5218

	Exclusively internal %	
	Ld Meta1	**Ld GFP**
1hour	5.7 ± 5.3 n=154	0.22 ±0.28 n=8
4hour	18.55±14.3 n=511	10.22± 8.4 n=319
12hour	77 ±10.83 n=2134	40.8± 7.2 N=1834
24hour	88± 8.08 n=2396	73 ± 9.2 N=1972

Tab: 4.10 summary of percentages of infected Macrophages in all catagorisation in Meta1 and GFP.

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	exclusively ext.%		
	distribution	**LdMeta1**	**Ld GFP**
1hour	<5	34.296±3	59.8±14.5
	>5	9.888±3.1	12.3±10.2
4hour	<5	14.38±11	27.3±10.1
	>5	8.16±9.7	12.5±14.3
12hour	<5	0.26±0.27	0.89±1
	>5	0.037±0.11	0.074±0.14
24hour	<5	0.11±0.16	0.48±0.68
	>5	0±0	1±0

	Attachment%		
	Distribution	**LdMeta1**	**Ld GFP**
1hour	<5	62.25±10.2	58.22±11.2
	>5	37.48±6.1	27.66±11.3
4hour	<5	38.722±8.7	52.27±17.4
	>5	53.388±5.6	34.38±20.9
12hour	<5	11.629±7.9	15.81±5.2
	>5	81±7.6	71.11±11.1
24hour	<5	5.407±4.6	18.74±14.2
	>5	90.222±7.1	70.96±18.3

	Internalization %		
Time	**distribution**	**LdMeta1**	**Ld GFP**
1hour	**<5**	44.74±18.6	18.14±11.9
	>5	1.037±0.9	0.629±0.58
4hour	**<5**	49.14±16.6	42.97±11.5
	>5	18.55±13.8	6.08±3.73
12hour	**<5**	12.96±8.7	19.18±6.8
	>5	79.44±8.3	67.778±6.1
24hour	**<5**	5.888±5.2	19.74±13.4
	>5	89.96±7.6	69.37±17.8

	Exclusively.int%		
Time points	**distribution**	**LdMeta1**	**Ld GFP**
1hour	**<5**	5.703±5.3	0.22±0.28
	>5	0±0	1±0
4hour	**<5**	13.08±9.5	9.02±7.6
	>5	5.472±4.9	1.19±1.5
12hour	**<5**	10.96±7.0	13.37±3.8
	>5	68.07±14	37.8±4.2
24hour	**<5**	4.925±4	16.62±12.6
	>5	83.81±11	56.40±18.3

Tab: 4.11 summary of percentages of infected parasites in all catagorisation in Meta1 and GFP.

CHAPTER VII

CONCLUSION

The current study reflects that promastigotes of the intracellular protozoan parasite of *Ld* GFP and *Ld*Meta1 strain of *Leishmania donovani*in stationary phase at 37^0c attaches macrophages in a time span of 1 hour, and as the infection time is prolonged, the number of the macrophages having attached *Leishmania* increases too. Similarly internalization started from 1 hour time points in case of Meta1 and within 24 hour nearly 90% macrophages had got internalized completely. The number of macrophages with parasites attached and internalized were counted and expressed in percentage of macrophages having atleast one *Leishmania* attached and atleast one Leishmania internalized thus, a comparative analysis was carried out between *Ld*GFP and *Ld*Meta1.

- In terms of extent of the infectivity, *Ld* Meta1 over expressed cells internalized with significantly higher parasite load per macrophage, while GFP internalized at relatively lower quantum.
- In terms of rate ,*Ld*Meta1 internalized at a relatively better rate across the time points
- Where as in case of *Ld*GFP only external group has been increased from 1hr to 24 hr time point. Thus it could be concluded that infection rate was significantly better and the parasite load was significantly higher in *Ld* Meta1 as compared to control.
- In future we can conduct the assay by gene expression profiling at 12 hour and 24 hour time points to understand further the process of internalization, transformation into amastigotes and parasite load. For expression profiling standardized the methods and materials for Meta 1 and *Ld*GFP but due to the lack of time we were unable to show difference between two groups.

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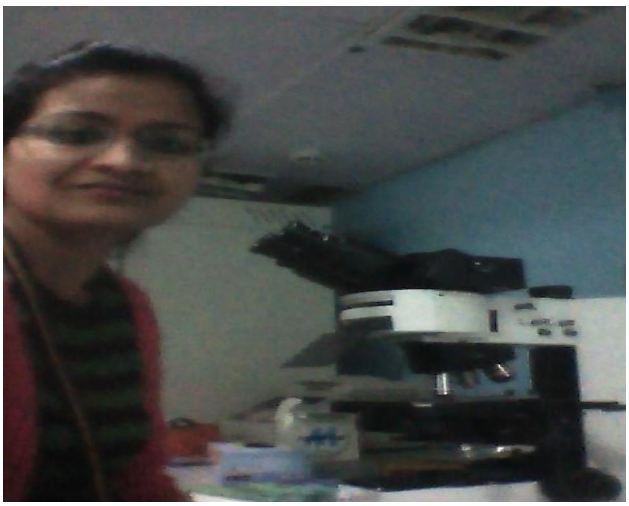


Thermo
Forma Class II, B2
Biological Safety Cabinet
MVE

Real time PCR(Absolute quantification of GP63 metalloprotenase) for standarisation(incomplete….)

Inc	Pos	Name	Type	CP	Concentration	Standard	Status
☑	A1	NTC	Negative Control	35.00	2.05E-4		>
☑	A2	NTC	Negative Control	35.00	2.05E-4		>
☑	A3	NTC	Negative Control	35.00	2.05E-4		>
☑	A5	0.15NG	Standard	25.14	1.56E-1	1.50E-1	
☑	A6	0.15NG	Standard	25.27	1.42E-1	1.50E-1	
☑	A7	0.15NG	Standard	25.14	1.55E-1	1.50E-1	
☑	A9	0.015NG	Standard	28.78	1.34E-2	1.50E-2	
☑	A10	0.015NG	Standard	28.71	1.41E-2	1.50E-2	
☑	A11	0.015NG	Standard	28.60	1.51E-2	1.50E-2	
☑	A13	0.0015NG	Standard	31.99	1.55E-3	1.50E-3	
☑	A14	0.0015NG	Standard	31.96	1.58E-3	1.50E-3	
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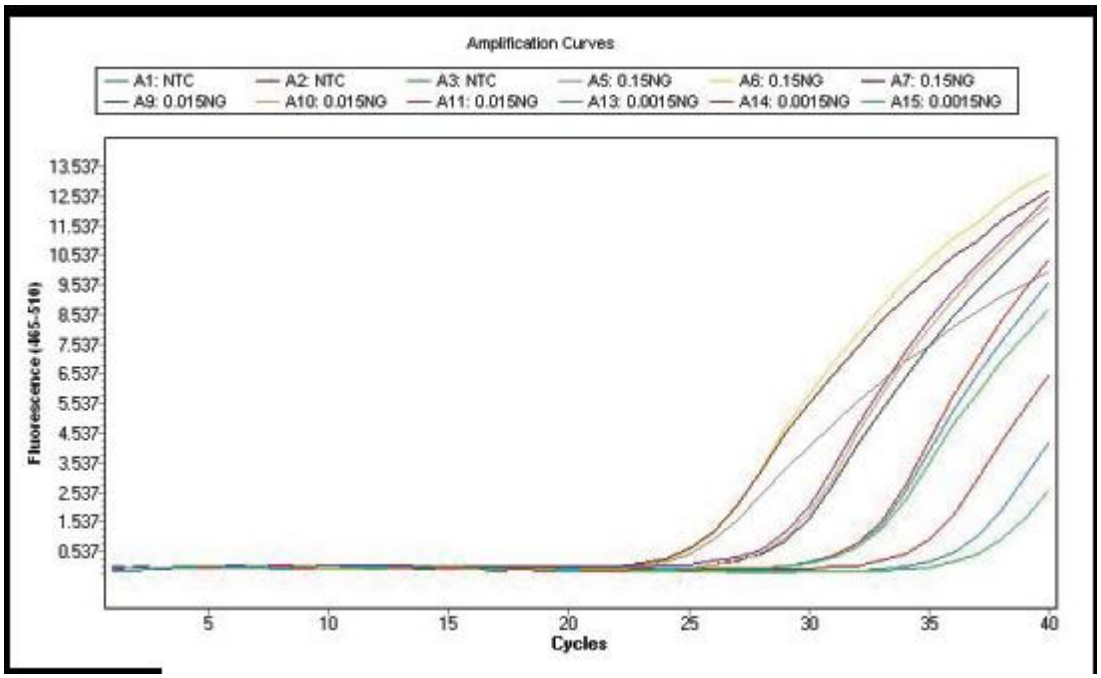

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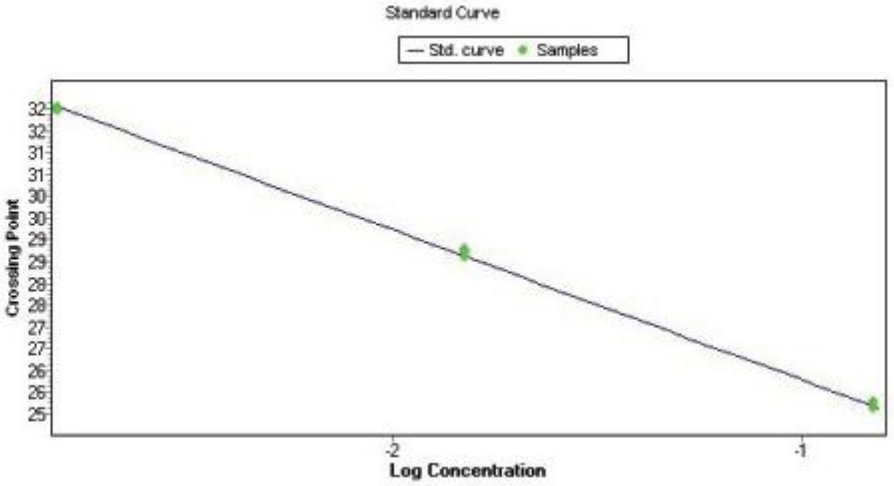

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