# Studies on drug susceptibility of Indian *Leishmania donovani* isolates to Paromomycin

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By

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**BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE**

PILANI RAJASTHAN

*CERTIFICATE*

This is to certify that the thesis entitled "**Studies on drug susceptibility of Indian *Leishmania donovani* isolates to Paromomycin"** and submitted by Vasundhra Bhandari ID No. 2010PHXF037P for award of Ph.D. Degree of the Institute, embodies original work done by her under my supervision.

Signature in full of the Supervisor ____________________________

Name in capital block letters ____________________________

Designation ____________________________

Date ____________________________

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## Abbreviations

| | |
|---|---|
| AmB | AmphotericinB |
| Ama | Amastigote |
| bp | Base pair |
| BLAST | Basic Local Alignment Search Tool |
| BMA | Bone marrow aspirates |
| $^oC$ | Degree Celsius |
| cDNA | Complementary DNA |
| CL | Cutaneous Leishmaniasis |
| ddNTP | Di-Deoxyribose nucleotide tri phosphate |
| DPH | Diphenyl hexatriene |
| DNA/RNA | Deoxyribose/Ribose nucleic acid |
| dNTP | Deoxyribose nucleotide triphosphate |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine tetra-acetic acid |
| FCS/FBS | Fetal Calf/Bovine Serum |
| Gms | Grams |
| HBSS | Hank's balanced salt solution |
| HRP | Horseradish peroxidase |
| HEPES | N-2-hydroxyethylpiperazine-N-2 ethane sulfonic acid |
| h | Hour |

| | |
|---|---|
| $H_2O_2$ | Hydrogen peroxide |
| HR | High Resistance zone to Antimony |
| HSP | Heat shock protein |
| IFA | Immunofluorescence assay |
| IFN-Ý | Intereferon gamma |
| iNOS | Inducible nitric oxide synthase |
| KA | Kala-azar |
| kb | Kilo base |
| kDa | Kilo Dalton |
| Ld | *Leishmania donovani* |
| Lm | *Leishmania major* |
| LPS | Lipopolysaccharide |
| LR | Low Resistance Zone to Antimony |
| M | Molarity |
| MIL | Miltefosine |
| mg/ ml | Milligram/ Milliliter |
| min/mins | Minute/Minutes |
| NADH | NAD/FAD dependent dehydrogenase: |
| $NaHCO_3$ | Sodium bicarbonate |
| N | Normality |
| ND | Not Determined |
| ng | Nanogram |

| | |
|---|---|
| NO | Nitric oxide |
| NOS | Nitric Oxide Synthase |
| NR | Non responder |
| PBS/ PBS-T | Phosphate Buffered Saline/ PBS with Tween 20 |
| PCR | Polymerase chain reaction |
| PKDL | Post Kala-azar Dermal Leishmaniasis |
| PMM | Paromomycin |
| PPG | Proteophosphoglycan |
| PP2A | Protein phosphatase 2A |
| Pro | Promastigotes |
| PSA | Parasite surface antigen |
| r | Correlation coefficient |
| rpm | Revolution per minute |
| RNase | Ribonuclease |
| RPMI | Roswell Park memorial Institute |
| RT-PCR | Reverse transcription- PCR |
| qRT-PCR | Quantitative real-time PCR |
| Sb | Antimony |
| SAG | Sodium Antimony Gluconate |
| SNAP | S-nitroso-N-acetyl-DL-penicillamine |
| SIN-1 | 3-morpholinosydnonimine |
| SSG | Sodium Stibo Gluconate |

| | |
|---|---|
| Sec/Secs | Second/Seconds |
| SD | Standard Deviation |
| SEM | Standard Error Mean |
| SJH | Safdarjung Hospital |
| TAE | Tris acetate EDTA |
| TAG | Tri Acyl glycerol |
| Tris | Tris (hydroxymethyl) amino acid |
| [T(SH)2]- | Trypanothione |
| (TrS)- | Trypanothione synthetase |
| U | Unit |
| UV | Ultra Violet |
| VL | Visceral Leishmaniasis |
| WT | Wild type |
| X g | Times gravity (centrifugal force) |
| X-gal | 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside |
| β-ME | Beta mercaptoethanol |
| μg/μl | Microgram/ Microliter |
| μM/mM | Micromolar/ Millimolar |
| % | Percentage |
| ~ | Approximately |

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In the nineteenth century, Cunningham, Borovsky, Leishman, Donovan, Wright, Lindenberg and Vianna each independently identified the parasite that causes leishmaniasis, to which Ronald Ross gave the generic name *Leishmania.* Leishmaniasis is a group of diseases with wide epidemiological and clinical diversity; the disease is endemic in large areas of the tropics, subtropics and the Mediterranean basin. The two major clinical forms of Leishmaniasis, cutaneous and visceral, are the result of infection by different species of the parasite. However, in addition to the infecting species, the clinical outcome of leishmaniasis also depends on the immune response of the host (Rivas *et al.* 2004). Environmental risk factors such as massive displacement of populations, urbanization, deforestation, new irrigation plans and individual risk factors such as HIV, malnutrition and genetic susceptibility make leishmaniasis an important public health problem (Desjeux 2004).

Visceral Leishmaniasis (VL) is a lethal, disseminated form of this infection caused by *L donovani/ L infantum* and stands second only to malaria as a parasitic cause of death. Prevalent in 70 countries, 90% of the VL cases occur in India, Bangladesh, Nepal, Brazil, Sudan and Ethiopia (Alvar *et al.* 2012). India alone shares almost 50 % of the world`s global VL burden. In India, states of Bihar, Uttar Pradesh and West Bengal are highly endemic foci of VL or kala azar

(KA) where periodic epidemics are common (Adhya *et al.* 2002). The governments of India, Nepal and Bangladesh have launched regional VL elimination programme in collaboration with world health organization.

Treatment options are quite limited and development of resistance towards the traditional antimony therapy has added to the problem. New treatments for VL have been introduced and others are on clinical trials. Amphotericin B (AmB), a polyene macrolide antifungal agent has shown to be highly effective in the treatment of VL. However, it has the disadvantage of being administered as slow intra venous infusions and causes potentially serious renal toxicity (Sundar and Rai 2005). Most promising treatments like liposomal Amphotericin-B (L-AmB) that have been found to be effective even at low doses in India (Sundar et al. 2002c) but are not affordable for most of the patients in endemic countries due to high cost. Moreover, unresponsive cases for L-AmB have also shown up in Sudan (Mueller *et al.* 2007). Miltefosine (MIL), an alkyl phospholipid, is the first oral drug for VL, registered in India in 2002. It has proved to be highly effective in treatment of VL in India both in adults (Sundar et al. 1998; Sundar 2001) and in children (Bhattacharya *et al.* 2004). However, its teratogenic potential severely hampers its general use in the clinic and a long terminal half life (150-200 hrs) increases the risk of development of resistant parasites in the field that could lead to rapid transmission of MIL-resistant population and put the lifespan of this important drug at risk. In India, where anthroponotic transmission of VL occurs, widespread use of MIL monotherapy might lead to the rapid emergence of clinical resistance (Sundar *et al.* 1998;

Bryceson 2001). Recent reports shows that after a decade of its use, MIL efficacy has drop down to 90% (Sundar *et al.* 2012).

Aminoglycoside paromomycin is recently registered as antileishmanial drug in India and has been found to be effective as monotherapy in comparison to SAG ( Sundar *et al*. 2007a ;Sinha *et al*. 2011) as well as in combination with other drugs (Sundar et al. 2011a). PMM is well tolerated and cheapest drug option therefore; it was designated as Orphan drug for the treatment of VL by FDA. Appropriate measures should be taken to ensure its long term effectiveness. There are a number of studies elucidating the PMM resistance mechanism in bacteria however, at present there are very limited studies on PMM resistance in *Leishmania*. Hence, there is an urgent need to understand the resistance mechanism operative in *Leishmania* and is crucial to monitor PMM sensitivity in the prevailing isolates.

One of the goals of the present study is to evaluate inherent susceptibility of the prevailing parasite population in India (exposed and unexposed to MIL) towards PMM. This will help to obtain a baseline data on the inherent susceptibility of parasites towards PMM before its introduction in the field. Additionally, MIL susceptibility in similar isolates will be performed to determine any cross tolerance to PMM.

There is a pressing requirement to attain pro- active knowledge about PMM resistance in clinical isolates of *L. donovani*. Towards this goal laboratory generated PMM resistant isolates will be used as a model to understand the

mechanism of resistance. Hence, the study aims to generate experimentally resistant PMM *L. donovani* isolates and utilize them to understand the mechanism of resistance which will lead to the identification of key intracellular targets and parasite defence mechanisms that may be exploited further.

## Introduction

Leishmaniasis is a complex of diseases caused by more than 20 species of the intracellular protozoan *Leishmania* (important species are mentioned in Table 1.1). The disease is widely spread and burden is high, with 350 million people considered at risk. There are an estimated 1.5–2 million new cases per year, up to 500,000 of which are visceral and 1,500,000 are muco-cutaneous/cutaneous. Whereas cutaneous leishmaniasis (CL) has a tendency to spontaneously self-heal with resulting scars, visceral leishmaniasis (VL) is fatal when left untreated, causing a global annual mortality estimated at 59 000. At present, Leishmaniasis is classified as an important neglected tropical disease which is highly prevalent in many poor and developing countries (Alvar *et al*. 2012). Transmission of the disease is via a bite of the sand fly, there are two groups of sand flies: genus *Phlebotomus* in undeveloped countries, and genus *Lutzomyia* in more developed countries.

The number of cases of Leishmaniasis is increasing, mainly because of manmade environmental changes that increase human exposure to the sandfly vector (Desjeux 2004). Leishmaniasis is spreading in several areas of the world as a result of epidemiological changes which sharply increase the overlapping of acquired immunodeficiency syndrome (AIDS) and VL. Continuing widespread migration from rural to urban areas, and ongoing fast urbanization worldwide are among the primary causes for increased exposure to the sandfly (Desjeux 2004).

Climate change and other environmental changes have the potential to expand the geographic range of the vectors and leishmaniasis transmission in future (http://www.cdc.gov/parasites/ leishmaniasis/epi.html). Increasing risk factors are making leishmaniasis a growing public health concern for many countries around the world.

**Table 1.1: List of main *Leishmania* species pathogenic to human**

| *Leishmania* species[1] | main disease | geographical distribution | reservoir | sand fly vector[2] |
|---|---|---|---|---|
| *L. (L.) donovani* | visceral (kala-azar) | Indian subcontinent, East Africa | human[3] | *P. argentipes, P. orientalis, P. martini* |
| *L. (L.) infantum* | visceral (infantile and HIV-co-infected) | Mediterranean basin, Central and West Asia | dog | *P. ariasi, P. perniciosus* |
| *L. (L.) infantum (syn. chagasi)* | visceral (infantile and HIV-co-infected) | Central and South America | dog, fox | *L. longipalpis* |
| *L. (L.) major* | cutaneous (oriental sore) | Central and West Asia, North Africa, Sahel of Africa, Central and West Africa | gerbil, rat | *P. papatasi, P. dubosqi, P. salehi* |
| *L. (L.) tropica* | cutaneous (oriental sore) | Central and West Asia, North Africa | hyrax, human | *P. sergenti* |
| *L. (L.) aethiopica* | cutaneous (diffuse) | Ethiopia, Kenya | hyrax | *P. longipes, P. pedifer* |
| *L. (L.) mexicana* | cutaneous (chiclero's ulcer) | Central America | forest rodents | *L. olmeca olmeca* |
| *L. (L.) amazonensis* | cutaneous | South America | forest rodents | *L. flaviscutellata* |
| *L. (V.) braziliensis* | cutaneous, mucocutaneous (espundia) | Central and South America | forest rodents, sloth, opossum | *L. wellcomei, L. complexus, L. carrerai* |
| *L. (V.) peruviana* | cutaneous (uta) | Peru | unknown reservoir (dog?), human | *L. peruensis, L. verrucarum* |
| *L. (V.) guyanensis* | cutaneous | South America | sloth, anteater | *L. umbratilis* |
| *L. (V.) panamensis* | cutaneous | Central America | sloth, humans | *L. trapidoi* |

**Types of Leishmaniasis**

In general, there are three major clinical manifestations of disease that are specific to the infecting *Leishmania* species:

(i) **Cutaneous leishmaniasis**: This is the most common form of Leishmaniasis, also known as 'Oriental sore' which first appears as a persistent insect bite. It has been estimated that 90% of CL cases occur in 7 countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria. In India, CL is endemic in Western Thar region of Rajasthan particularly in Bikaner region, where clinical manifestation varies from spontaneous healing lesions to chronic mutilating to rarely a chronic diffuse cutaneous lesion (Dogra *et al*. 1990) (Figure 1.1). The disease is spreading to other areas, a new foci of CL in Himachal Pradesh has been identified, where *L. donovani* and *L. tropica* are found to be the causative agents (Sharma *et al.* 2005). Simple skin lesions appear at the site of sandfly bite which self-heal within few months but leaves scars. The incubation period can last from few days to months. Gradually, the lesion enlarges, remaining red, but without noticeable heat or pain. Resolution of the lesion involves immigration of leucocytes, which isolate the infected area leading to necrosis of the infected tissues, and formation of a healing granuloma. Man is the definitive host whereas gerbils, cats, dogs, and rodent act as the natural reservoir of CL. Sandflies of genus *Phlebotomus* serve disease. CL is usually caused by *L. major, L. tropica, L. aethiopica*, in the old world and by *L. mexicana, L venezuelensis, L. amazonensis, L. braziliensis, L. panamensis, L. guyanensis* and *L. peruviana* in the new world.

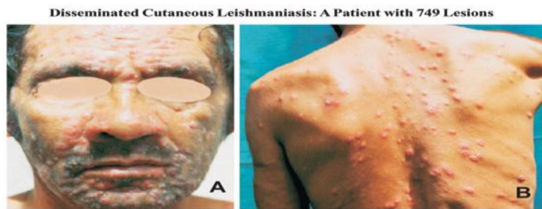



**Figure 1.1: Clinical signs of Cutaneous Leishmaniasis.** Patients with skin ulcers due to CL.

**(ii) Mucocutaneous leishmaniasis**: This form of disease, also known as espundia, causes extensive destruction of naso-oral and pharyngeal cavities with hideous disfiguring lesions, mutilation of the face (Figure 1.2) and great suffering for life. MCL is occasionally reported from Sudan and other Old World foci. Classical MCL is, however, restricted to *L. braziliensis* infections in which, following the apparently complete resolution of the initial oriental sore, sometimes many years later, metastatic lesions appear on the buccal or nasal mucosa. The reservoir hosts include rodents, opossums, anteaters, sloths and dogs etc. The causative agents of MCL in old world are *L. aethiopica* (rare), and in new world are *L. braziliensis, L. guyanensis, L. mexicana, L. amazonensis and L. panamensis* characterised by progressively destructive ulcerations of the mucosa that extend from the nose and mouth to the pharynx and larynx. Lesions are not self-healing and usually surface months or years after a first episode of cutaneous leishmaniasis.

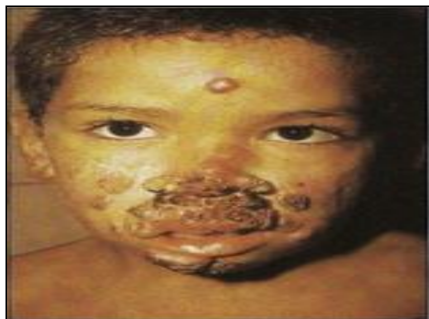


**Figure 1.2: Mucocutaneous Leishmaniasis:** patients with perforated nasal septum and mucosal tissue destruction.

### (iii) Diffuse cutaneous leishmaniasis (DCL)

This is a chronic, progressive, polyparasitic variant that develops in context of leishmanial-specific anergy and is manifested by disseminated non-ulcerative skin lesions, which can resemble lesions of lepromatous leprosy (Figure 1.3). Previously, DCL was restricted to Venezuela and Dominican Republic in the western hemisphere, and to Ethiopia and Kenya in Africa. Its main causative organisms are *L. aethiopica* and *L. mexicana* species complex. Recently a report has shown DCL, caused by *L. tropica* in India in HIV coinfection (Khandelwal *et al*. 2011).

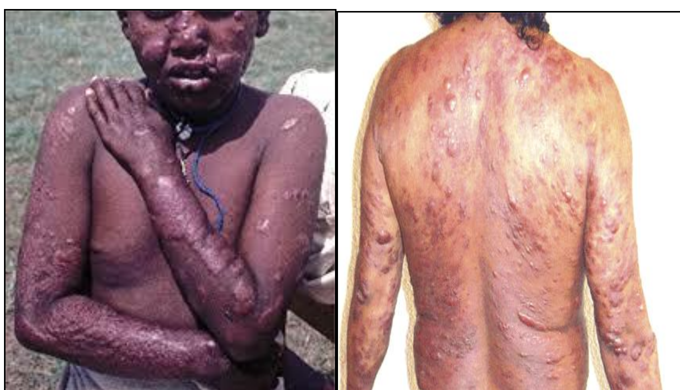


**Figure 1.3: Patients with clinical symptoms of Diffuse Cutaneous Leishmaniasis.**

**(iv) Visceral leishmaniasis** (VL) or kala-azar: VL is also known as Kala- Azar, Black Sickness, Black Fever, Burdwan fever, Dumdum fever or Sarkari Bimari etc. It is the most severe form of disease and if left untreated, is usually fatal. This poverty related disease is endemic in 70 countries and 200 million people at risk with an estimated 500,000 new infections annually concerning all age groups (Guerin *et al.* 2002) (Figure 1.4). More than 90% of the estimated VL cases occur in India, Bangladesh, Nepal, Sudan and Brazil with India alone sharing almost 50% of the world's total disease burden (Alvar *et al.* 2012). The parasite is

responsible for a spectrum of clinical syndromes, which can, in most extreme cases, move from an asymptomatic infection to a fatal form of VL. It is characterized by prolonged fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anemia, pancytopenia, and hypergammaglobulinemia and is complicated by secondary opportunistic infections affects mainly the bone marrow, spleen and liver of the patient and is fatal if left untreated (Figure 1.5). When successfully treated, patients still have a risk at developing post-kala-azar dermal leishmaniasis (PKDL).

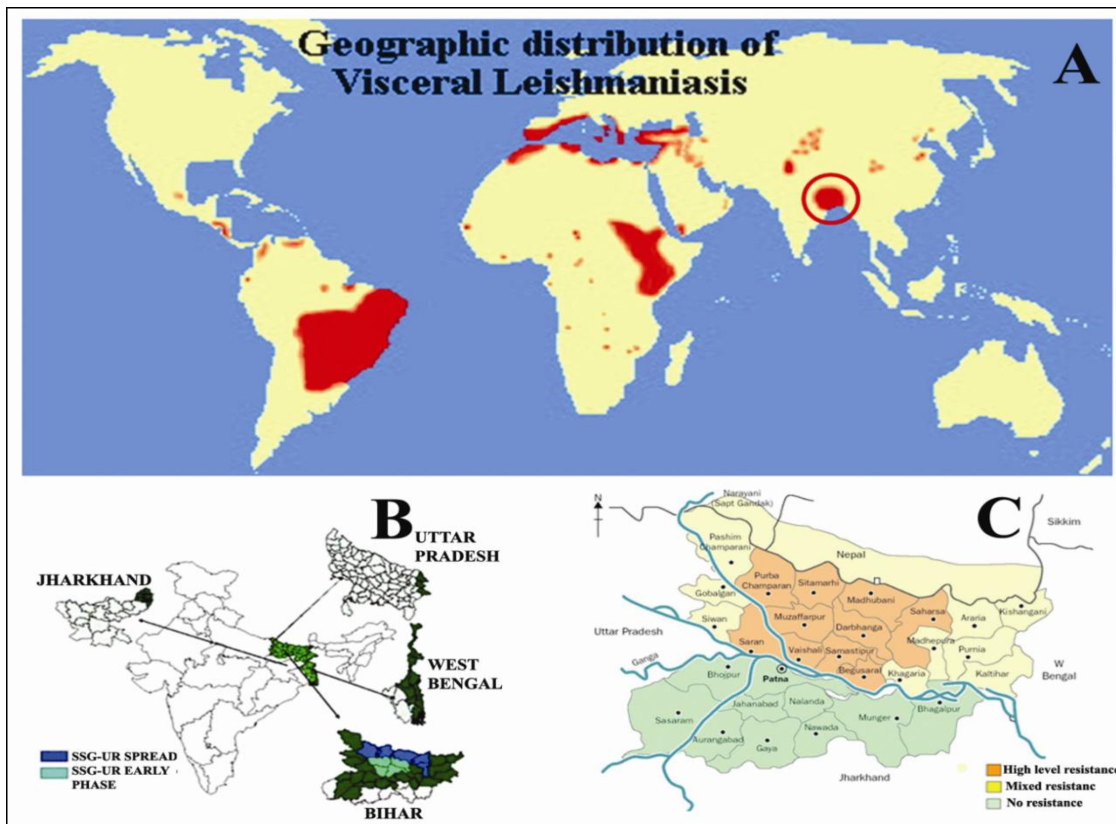



**Figure 1.4: Geographical distribution of visceral leishmaniasis (VL); (A)** Worldwide distribution of VL, **(B)** VL affected states of India and **(C)** VL affected districts of Bihar.

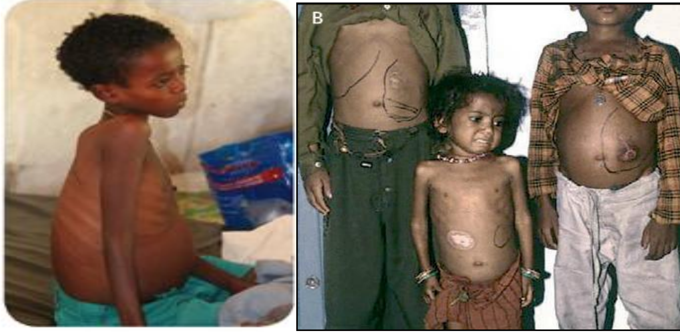



**Figure 1.5: Clinical symptoms of VL.** Hepato splenomegaly and wasting are the main features.

### (v) Post kala-azar dermal leishmaniasis (PKDL)

Post kala azar dermal leishmaniasis (PKDL) is a dermal sequel to VL. The disease is relatively common in the Indian subcontinent (India, Nepal, and Bangladesh), East Africa (Sudan, Ethiopia, and Kenya), and China, where the causative agent for VL is *L. donovani* ( Leng 1982; Guerin et al. 2002). PKDL was first described by Brahmchari in 1922 in cured VL patients with eruption and plaque in the skin, confirmed by demonstration of Leishman- Donovan bodies (LDB) in slit skin smear and termed as dermal leishmanoid. Later, the disease was renamed as PKDL since eruptions follow the visceral disease, commonly called as kala-azar. In India, it manifests in 5-15% of VL cases after months or several years of remission from infection, while in Sudan, it develops within weeks or months in 50-60% of cured VL cases, and most severe in children aged <3 years (Rees *et al.* 1984; Salotra and Singh 2006). The disease presents as erythema, hypopigmented macules and combination of papules, nodules and plaques, which first appear around the mouth, become denser and slowly spread over the entire body (Figure 1.6). In the absence of an animal host, cases of PKDL serve as a major reservoir for the *Leishmania* parasite and play an important part in KA transmission in India.

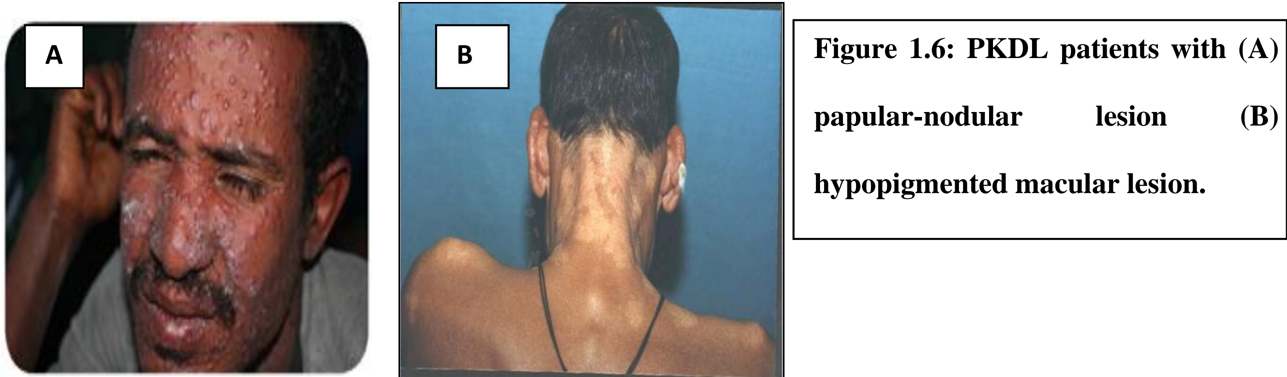



**Figure 1.6: PKDL patients with (A) papular-nodular lesion (B) hypopigmented macular lesion.**

**Transmission and life cycle**

The natural transmission of *Leishmania* occurs through the bite of sand flies (Figure 1.7). When a female sand fly bites an infected host (presumably at or after dusk), she will engorge amastigotes, a round immobile form of *Leishmania* that is only present in the host, and amastigote-containing cells together with the blood (McCall *et al.* 2013). Amastigotes will then transform to slender flagellated promastigotes in the abdominal midgut of the sand flies. Gut epithelial cells of the sand fly will secrete a chitinous matrix that will form a peritrophic membrane encircling the blood meal and the engorged parasites, but chitinases secreted by the promastigotes will cause it to break down sooner than normal to allow migration of parasites to the anterior part of the sand fly (Schlein *et al.* 1991). To avoid excretion with the rest of the digested blood meal, promastigotes attach themselves to the microvillar lining by their flagellum. Over a few days, they will migrate to the thoracic mid-gut and the stomodeal valve and will undergo a transformation from dividing non-infective promastigotes into non-dividing infective metacyclic promastigotes, a process called metacyclogenesis

(Da and Sacks 1987). In the anterior midgut, promastigotes will secrete a gel-like substance to create a plug that fills the anterior midgut and extends to the stomodeal valve into the foregut (Rogers *et al.* 2002). When the sand fly wants to feed, it will have to regurgitate first to overcome the obstruction by the plug, thereby expelling (metacyclic) promastigotes into the skin of the host (Bates 2007). In the host, metacyclic promastigotes will invade mononuclear phagocytes and transform into dividing amastigotes that are able to manipulate their host cell to allow long-term survival. When a host with an established infection is again bitten by a female sand fly, the amastigotes imbibed together with the blood meal will allow the life cycle to resume from the amastigote to promastigote transformation onwards. Sand flies (family Psychodidae, subfamily Phlebotominae) are dipteran insects of which over 700 species have been identified, but only ~ 10% of those are deemed to be possible vectors of leishmaniasis. For about 30 species there is convincing evidence of their vectorial capacity. In the Old World (*i.e.* Europe, Africa, the Middle East and Asia), *Leishmania* is transmitted by sand flies of the genus *Phlebotomus*. In the New World (the Americas), *Lutzomyia* sand flies are generally responsible for transmission of the parasite (Table 1.1).

Some sand flies can transmit several species of *Leishmania* (permissive vectors) while others can transmit only one specific species (specific vectors). Examples of permissive vectors are (i) *Lutzomia longipalpis*, the natural vector of *L. infantum (*syn. *chagasi)* that was also found susceptible for the full

development of *L. amazonensis* and *L. major* in the lab (Molyneux et al. 1975; Walters et al. 1993)  and (ii) *Phlebotomus argentipes*, the natural vector of *L. donovani* in the Indian subcontinent that can also support the development of *L. tropica* and *L. amazonensis* in the lab (Kamhawi *et al.* 2000). *Phlebotomus papatasi* and *Phlebotomus sergenti*, on the other hand, can only transmit *L. major* and *L. tropica* respectively (Kamhawi *et al.* 2000) and are therefore considered to be specific vectors. The main determinant in this *Leishmania* sand fly relationship is thought to be lipophosphoglycan (LPG). LPG is expressed at the surface of *Leishmania* promastigotes and protects them from digestive enzymes released into the blood-fed mid-gut and above all allows the parasites to attach to the mid-gut epithelium of the sand fly to avoid co-secretion with the digested blood meal (Sacks 2001). Specific vectors require *Leishmania* species with a complex LPG that is compatible with specific receptors on the endothelium of these sand flies' mid-gut. In permissive vectors, LPG seems to be less important. Here, the attachment of promastigotes to the mid-gut of sand flies seems to be mostly determined by lectin-like components on the promastigotes' surface that can bind conserved GalNac (an amino sugar derivative of galactose) on the microvillar border of the sand flies' mid-gut (Volf *et al.* 2008).

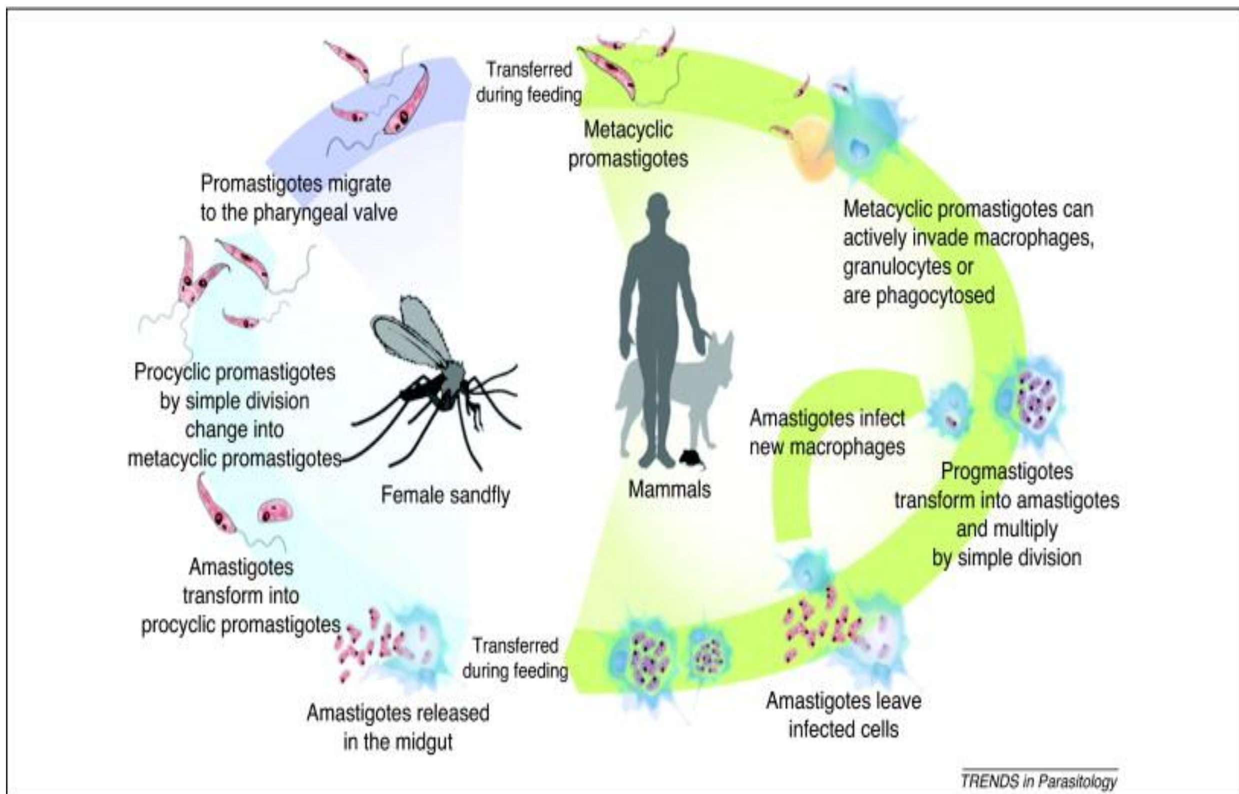



**Figure 1.7: Life cycle of *Leishmania donovani***

**Transmission patterns:**

There are 3 types of transmission considered for leishmaniasis:

(i) **Zoonotic transmission**: wild mammals are thought to be the only regular hosts for the parasite and humans are considered accidental hosts. For example in the Amazonian forest, rodents, sloths and opossums infected with *L. braziliensis* show no major symptoms. When humans (wood cutters, coca farmers, tourists) become infected, they risk developing mucocutaneous leishmaniasis.

(ii) **Anthropo-zoonotic transmission**: both humans and other mammals are regular hosts in the life cycle. For example *L. mexicana* circulates between rodents and humans causing cutaneous leishmaniasis in the latter. Another example is *L. infantum* in the Mediterranean basin which causes visceral leishmaniasis in children and immuno-compromised adults, while domesticated dogs are an important reservoir (who can also suffer from disease).

(iii) **Anthroponotic transmission**: humans are thought to be the only mammal host in the life cycle as considered in the case for *L. donovani* in the Indian subcontinent. However, caution should be taken here since some animals in Nepal have recently found to contain *Leishmania* infections as determined by PCR (Bhattarai *et al*. 2010; Khanal *et a*l. 2010). Whether or not these animals are also actively involved in the transmission cycle is not yet clear.

Important to note is that *Leishmania* can also be transmitted between hosts by the uptake of infected blood or cells through needles shared by intravenous drug users (Cruz *et al*. 2002) or blood transfusion (Desjeux 2004). However, these cases occur rarely.

**Survival of *Leishmania donovani***

*Leishmania* encounters hostile conditions such as (i) oxidative stress due to heme digestion in the blood meal (Vincent 1989) and midgut proteases in the

sand fly (Kamhawi 2006), (ii) complement lysis in the blood upon transmission (Dominguez *et al*. 2003) and (iii) reactive oxygen and nitrogen species (ROS and RNS) when phagocytised by cells of the host (Murray and Nathan 1999) during its life cycle. *Leishmania* successfully adapted to these different conditions allowing it to thrive in environments that would otherwise be exceptionally stressful. Their survival capacity relies mainly on (i) preventive host manipulating skills to suppress antileishmanial actions of the host and (ii) an active defence system against ROS and RNS through a unique protective redox metabolism (Vanaerschot *et al.* 2010). This section introduces some of the preventive and defensive measures *Leishmania* adopt to safeguard its survival.

**Preventive measures**

*Leishmania* already outwits its host at the initial stage of infection: it is known to enter the cell through specific receptors that allow 'silent' entry into the host cell, *i.e.* without alarming its antimicrobial defences (Brittingham and Mosser 1996; Mosser and Brittingham 1997; Dominguez and Torano 1999) . After being phagocytised, the parasite delays the maturation and acidification of the phagolysosome (Desjardins and Descoteaux 1997; Holm *et al*. 2001), allowing itself to transform into the amastigote stage from the promastigote stage which is better adapted to the host. Since amastigotes are hardly affected by the host cell, antigen presentation by this host cell is significantly impaired due to the lack of an adequate amount of digested peptides (Bogdan and Rollinghoff 1998). One of

the key factors of *Leishmania*'s manipulation of the host is that the parasite is able to activate central regulatory phosphatases of the host cell, thereby subverting crucial signalling pathways that normally lead to killing of an intracellular pathogen. As such, the major surface protein gp63 of *Leishmania* is able to enter the host cell's cytosol and activate SHP-1 and PTP1B (SH2-domain-containing phosphatase 1 and protein tyrosine phosphatase 1B respectively) (Gomez *et al.* 2009). Gp63 is also able to affect activated protein 1 (AP-1) and NF-κB, both important factors further downstream on the pathways that induce a microbicidal response of the host cell (Contreras *et al.* 2010). In addition, the activity of these factors is also negatively affected by a *Leishmania*-induced increased ceramide content of the macrophage (Ghosh *et al*. 2002). STAT1, another important transcription factor for the expression of IFN-γ induced genes such as iNOS and MHC II, was also shown to be directly inactivated by a proteasome-mediated mechanism induced by *Leishmania* (Forget *et al.* 2005; Shadab and Ali 2011). Given that all these and likely also many other host pathways are manipulated by *Leishmania*, it is no surprise that *Leishmania* infection has a great impact on the gene expression (Ettinger and Wilson 2008; Guerfali et al. 2008; Ghedira et al. 2011) and cytokine expression of the host cell: decreasing the expression of Th1- promoting cytokines such as IL-12 ( Weinheber et al. 1998; Cameron *et al.* 2004; Cheekatla *et al.* 2012) and increasing the expression of Th2- promoting cytokines, that are ineffective against *Leishmania*, such as IL-10 and TGF-β (Rodrigues, Jr. et al. 1998; Cabral et al. 2008). It is thus clear that *Leishmania* has a range of preventive measures

up its sleeve to avoid killing by the immune system of the host by circumventing both the innate and adaptive immune response. In the first stages of infection *L. donovani* inhibits dendritic cells to mount an adequate Th1-immune response by, among others, preventing them to produce IL-12 and avoiding activation of natural killer cells that can produce IFN-γ to stimulate the host cell to kill its invader. In addition, *Leishmania* also directly affects the IFN-γ signalling pathways of its own host cell to reduce the production of ROS and RNS.

**Defensive capacities of *Leishmania***

The mechanisms described above interfere with the host cell releasing its (antimicrobial and anti-leishmanial) effectors on the parasite. Nonetheless, the parasite is still exposed to toxic effectors such as ROS and RNS at particular stages of infection of the host cell. However, *Leishmania* also has a series of defence mechanisms ready to guarantee its survival. LPG on the surface of the promastigote and gp63, whether on the surface of promastigotes or excreted by amastigotes, can both protect the parasite from oxidative stress and/or hydrolytic enzymes imposed by the macrophage (Chan et al. 1989; Seay et al. 1996).

Although the main active defence system of *Leishmania* against extracellular and intracellular imposed ROS/RNS relies on its redox metabolism that is unique to Trypanosomatidae. This redox system consists of a cascade of enzymes with trypanothione as the main reducing agent (Figure 1.8). Trypanothione (T $[SH]_2$) consists of two glutathione (GSH) molecules linked by

spermidine and is unique for the Trypanosomatidae (Irigoin *et al.* 2008; Krauth-Siegel and Comini 2008; Van *et al.* 2011). When ROS and RNS are detoxified by members of this cascade (either trypanothione itself), $H_2O_2$ (Ariyanayagam and Fairlamb 2001), NO (Bocedi *et al.* 2010), tryparedoxin or tryparedoxin peroxidase (Flohe *et al.* 2002), $H_2O_2$ + NO (Iyer *et al.* 2008), the flavoenzyme trypanothione reductase (TR) will replenish the pool of reduced trypanothione ($T[SH]_2$) from oxidised trypanothione ($T[S]_2$) using NADPH as an electron donor. TR is therefore a central and very important enzyme for the intracellular survival of *Leishmania* (Dumas *et al.* 1997; Tovar *et al.* 1998a,1998b). Other thiols present in *Leishmania*, such as glutathione (GSH), cysteine and ovothiol, are also known to be able to participate in ROS detoxification, however, to a lesser extent in physiological conditions compared to $T[SH]_2$ and the redox cascade described above . The pool of these other thiols is kept reduced by the T $[SH]_2$/TR couple. Another important factor in the defence against oxidative stress, and especially superoxide ($O_2^-$), is superoxide dismutase (SOD) that transforms $O_2$ into $H_2O_2$ that can then be detoxified by other factors such as the cascade mentioned above. Several iron containing SODs have been identified in *Leishmania* (Dey and Datta 1994) and proved important for defence against $O_2^-$ and for intracellular survival (Ghosh *et al.* 2003). ROS and RNS can also be detoxified by thioredoxins such as those identified in *L. infantum* (syn. *chagasi*): (i) LcPxn1, mainly expressed in the amastigote stage and capable of detoxifying hydroperoxides, $H_2O_2$, HO. and $ONOO^-$, and (ii) LcPxn2 that is mainly expressed in promastigotes and apparently only able to detoxify $H_2O_2$ (Barr and Gedamu

2001,2003). After having detoxified ROS/RNS, these thioredoxins are most likely regenerated through spontaneous reduction by T $[SH]_2$ .

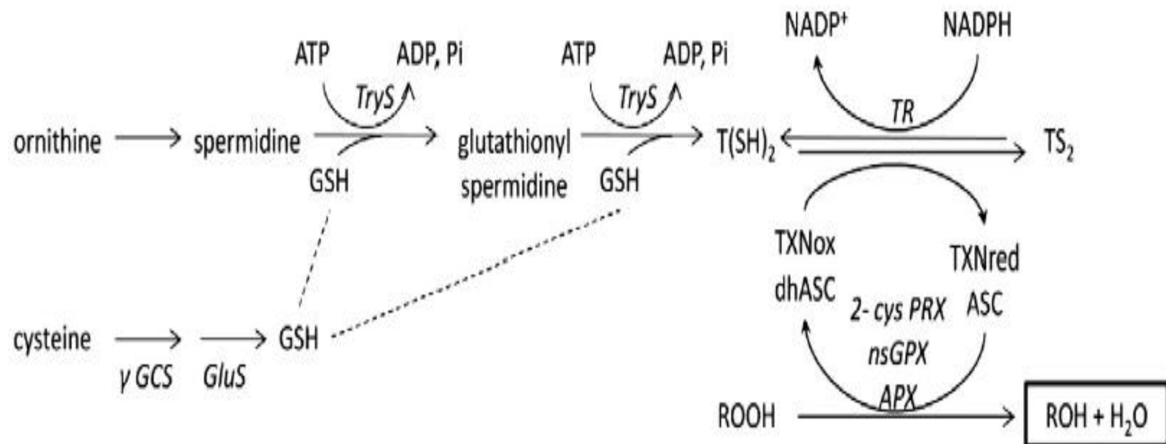



**Figure 1.8: Leishmania peroxidase cascade system for detoxification of hydroperoxides**. NADPH is the primary electron donor for reduction of $TS_2$ to T $(SH)_2$. The reducing enzyme and protein trypanothione reductase and tryparedoxin or ascorbate provide the necessary reducing equivalents to two-cysteine peroxiredoxin ( 2-cysPRX), non-selenium glutathione peroxidase (nsGPX) or ascorbate peroxidase (APX) for final reduction of hydroperoxides (ROOH). dhASC, dehydroascorbate; GluS, glutathione synthetase; Trys, trypanothione synthetase.

**Diagnosis of Visceral Leishmaniasis**

**Microscopic examination**

Demonstration of the amastigote form of the parasite by microscopic examination of tissue aspirates from spleen, bone marrow or lymph nodes is regarded as the gold standard most diagnosis for VL. The technique is advantageous as it is directly able to detect parasite load and is inexpensive to

perform. The specificity of this technique is high, although the sensitivity varies depending on the tissue used, being higher for spleen (93–99%) than for bone marrow (53–86%) or lymph node (53–65%) aspirates (Siddig et al. 1988; Zijlstra et al. 1992; Babiker et al. 2007). Results are dependent on technical expertise, hence well trained, competent microscopist and effective quality control (QC)/ quality assurance (QA) is needed for accurate diagnosis. Complications rarely occur but can be very severe up to death due to haemorrhages (Sundar and Rai 2002b). Therefore, there is a pressing need for less invasive diagnostic tools with a high sensitivity and specificity.

**Serological Methods of diagnosis**

Serological methods are based on identification of antibodies in the sera of VL patients. Recombinant antigens have considerably improved the sensitivity and specificity of immunological diagnosis over crude/total antigens. Among the recombinant antigens, a kinesin related protein, rK39, has been the most promising diagnostic antigen that has been tested widely. .

**(A) Agglutination tests**: Serum based Direct Agglutination Tests (DAT) or urine based Latex Agglutination Tests (LAT) is routinely employed for determining anti-leishmanial antibodies or antigens in VL patients. DAT is an easy to perform, widely applicable technique with high sensitivity (90-100%) and specificity (95-100%) and is in routine use in some regions (El *et al.* 1988; El *et al.* 2006; Jacquet *et al.* 2006). The test can be carried out using plasma, serum or even urine samples making it suitable for both field and laboratory

application (Chappuis *et al.* 2006; Sundar *et al.* 2007b). LAT such as (KAtex) assay detects stable, nonprotein *Leishmania* antigen in urine employing latex beads sensitized with antibodies raised against *L. donovani* antigen ( Sundar *et al.* 2005; Chappuis *et al.* 2006).

**(B) ELISA or Immunoblotting:** ELISA and Western blotting (WB) based on crude antigens and several antigenic molecules have been utilized for VL diagnosis. However, the diagnosis by these techniques is confined to referral labs due to the requirement of technical expertise. In the past, ELISA and WB have played a major role in identification of several antigens of diagnostic potential that could be translated to simpler diagnostic tools (Salotra *et al.* 1999). These detection tests are available for the diagnosis of VL but are not commonly used in field conditions since they have a low sensitivity and/or specificity are quite expensive, laborious and/or require specific technical equipment (Srivastava *et al.* 2011).

**(C) Immunochromatographic strip test:** Furthermore, the introductions of rk39 immuno-chromatographic strip test (ICT) based rapid diagnostic test has facilitated the field applicability of serological methods for control of VL. The test is easy to operate and even field workers with minimal training can perform it reliably. Apart from rK39, several other kinesin-related proteins such as K26, K9, KRP42, and KE16 have been tested for their serodiagnostic potential with variable success (Takagi et al. 2007; Mohapatra et al. 2010).

**Molecular test using PCR**

Molecular Approaches have become increasingly relevant due to high sensitivity (up to 100%), specificity and flexibility in choice of samples such as aspirates, blood, urine (Fisa *et al*. 2008) and buccal swaps (Vaish *et al.* 2011). Different DNA-targets have been utilized that have a high copy number in *Leishmania* (such as kinetoplast DNA, ribosomal DNA or mini-exon genes) or are very specific for *Leishmania species* (GP63, ITS1, HSP70, cysteine proteinases) can be amplified and detected for diagnosis and/or *Leishmania* species identification respectively. The ability of PCR assays to determine the species of *Leishmania* present in the clinical sample is an important advantage over the other techniques. DNA detection by PCR using blood and bone marrow are currently under use (Adhya *et al.* 1995; Osman *et al.* 1998; Salotra *et al. 2*001).

Quantitative PCR is a highly sensitive and specific tool used in referral labs for detection/assessment of parasite load in VL patients. Remarkable sensitivity and specificity and ability to quantify parasite load pre and post treatment indicated its robust potential in VL diagnosis and monitoring of the treatment (Mary et al. 2004; Wortmann et al. 2005; Verma et al. 2010). The method displays potential to provide threshold for distinguishing asymptomatics in endemic areas. However, it is important to note that PCR positivity alone is not a sign of active VL disease. PCR results should always be interpreted in the context of the patient's clinical symptoms or the lack thereof. However, the need for DNA extraction techniques, expensive reagents and detection methods limit the use of PCR for VL diagnosis to well-equipped clinics (Mondal *et al.* 2010).

Currently, improvement in VL diagnostics is required for successful decentralized (point of care) testing in field conditions and to detect VL-HIV co-infection. Techniques such as loop mediated isothermal amplification (LAMP) offer a reliable molecular diagnostic method for field application (Takagi et al. 2009; Adams et al. 2010). The diagnosis based on bioanalytics/biosensors promise as frontiers for point of care VL detection after adequate standardization. In addition to the VL confirmation, there is also a need to develop diagnostic markers which will also serve a test of cure after therapy, marker for asymptomatic infections and detect drug resistance.

**Treatment options in Visceral Leishmaniasis**

The absence of effective vaccines and vector control programs makes chemotherapy the single efficient way to fight leishmaniasis despite the considerable progress made in the study of the biochemistry, physiology and molecular biology of *Leishmania* parasites. Antimonials were the only VL treatment option in the Indian subcontinent 30 years ago, since economic returns on developing a new drug for leishmaniasis is so low that therapeutic switching represents the only realistic strategy. It refers to ''alternative drug use'' discoveries which differ from the original intent of the drug (Shakya *et al.* 2011). Amphotericin B, paromomycin, miltefosine and many other drugs are very successful examples of ''new drugs from old (Table 1.2, Figure 1.9)**.**

**Table 1.2: List of the most commonly used antileishmanial agent in Indian Subcontinent**

| **Drugs** | **Antimonials** | **Amphotericin B** | **Miltefosine** | **Paramomycin** |
|---|---|---|---|---|
| **Dosage & duration** | 20mg/kg/day for 28 days | 1mg/kg for 15 days on alternate days for 30 days | 50 mg or 100 mg for 28 days.<br>2.5mg/kg/day for 28 days in children | 15mg/kg/day for 21 days |
| **Administration** | Intramuscular | Intravenous | Oral | Intramuscular |
| **Cost (USD)** | 58 | 20 | 65-150 | 15 |
| **Toxicity** | Abdomen pain / nausea, cardiotoxicity | Infusion related chills, nephrotoxicity, cardiotoxicity | Gastrointestinal toxicity, nephrotoxicity, hepatotoxicity and possible teratogenicity | Nephrotoxicity, Ototoxicity (all relatively rare) |
| **Note** | >65% cases resistant in Bihar, India. Used as first line of treatment in other parts of world. | Used for treating antimony treatment failure cases in India | Now first line of treatment in Indian subcontinent | Cheapest drug with high efficacy in monotherapy & in combination therapy with MIL. |

**Pentavalent antimonials (SbV)**:

Sodium antimony gluconate (SAG) and meglumine antimoniate were the first pentavalent antimony agents to be reported active against kala azar and are still the mainstay of therapy for leishmaniasis in most of the world (Guerin *et al.* 2002). Antimonials have several disadvantages: patients have to be admitted to hospital for 3–4 weeks for parenteral therapy; toxic effects like nausea, abdominal pain, chemical pancreatitis and clinical pancreatitis in HIV co-infected patients may limit the drugs (Pintado and Lopez-Velez 2001). Long-term use at higher doses to combat resistance is restricted by cardiotoxicity (ST-segment inversion), QTc prolongation, and, possibly, fatal arrhythmia (Sundar *et al.* 2000). In the Indian subcontinent however, severe toxicity and an increasing treatment failure rate up to 65% in Bihar, India (Sundar 2001) urged the health authorities to abandon SAG as the first line treatment for VL.

(A) ***Mode of action:*** The mode of action of antimonials has not been clearly elucidated yet. Sb(V)] are considered as prodrugs that require *in vivo* reduction to the leishmanicidal trivalent form [Sb(III)]. The site (host macrophage, amastigote or both) and the mechanism of reduction remain unclear (Zhou *et al.* 2004). Previous work suggested that antimonials inhibit macromolecular biosynthesis in amastigotes, possibly due to the inhibition of glycolysis and fatty acid beta oxidation (Berman *et al.* 1985, 1987). Antimony induces efflux of the intracellular trypanothione, and also inhibits the enzyme trypanothione reductase (Wyllie *et al.* 2010). These two mechanisms compromise the thiol

redox potential of the cell and lead to the accumulation of reactive oxygen species (ROS). SbV binds to the ribose moiety and forms stable complexes with adenine nucleosides, which act as inhibitors of *Leishmania* purine transporters (Demicheli *et al*. 2002). Antimony also induces activation of certain important components of the intracellular signaling pathway in the host cells, which results in an early wave of ROS-dependent parasite killing and a stronger late wave of NO-dependent parasite killing (Mookerjee *et al.* 2006).

(B) ***Mechanism of resistance:*** Majority of the knowledge on antimony resistance in *Leishmania spp* has been derived from laboratory mutants developed by adapting to increasing drug concentration. Among the suggested mechanisms' of antimony resistance are gene amplification and the parasite's inability to convert SbV to SbIII. It is established that the trivalent antimony Sb(III) is the active form of the drug which is generated by reduction of Sb(V) by thiols either by the parasites or the macrophages, or both (Guimond *et al.* 2003; Croft *et al.* 2006;). A recent reports antimony resistant parasite specific glycans that induce IL-10 production from macrophages, which, in turn, up-regulate host MDR1 and contribute to resistance (Mukherjee *et al*. 2013). Earlier studies based on transcriptomic profiling and proteome mapping revealed the modulation of several genes and proteins in antimony resistant *Leishmania* parasites such as increased expression of multidrug resistance protein A (MRPA), thiol biosynthetic enzymes, HSP70 protein, ABC transporter, HSP83, proteophosphoglycans, a leucine rich repeat (LRR) superfamily protein, histone

H2A gene and the SbIII/thiol conjugate sequestering pump, and decreased expression of Aquaporin-1 (AQP1) (Brochu *et al.* 2004; Ouellette *et al.* 2004; Croft *et al.* 2006; Ashutosh *et al.* 2007; Mittal *et al.* 2007; Samant *et al.* 2007; Genest *et al.* 2008; Kumar *et al.* 2010; Singh *et al.* 2010)**.** Overall information on antimony resistance till date indicates that several mechanisms may coexist in the same cell and that different mechanisms may operate in field isolates compared with laboratory generated resistant parasites (Croft *et al.* 2006; Ashutosh et al. 2007).

**Amphotericin B (AmB)**

When increasing the drug dose and duration of SAG-treatment was no longer an option due to the high toxicity and the SAG-treatment failure rates reached at alarming proportions in the Indian subcontinent, AmB proved to be an effective alternative. It has an efficacy of >95% in all regions and primary unresponsiveness or relapses of AmB treated patients are uncommon (Mondal *et al.* 2010). AmB also cured SAG-unresponsive patients and the development of PKDL reduced significantly when using AmB treatment (Thakur et al. 1996a,1996b,1997,2008). However, its toxicity, frequently occurring side effects (infusion-related fever and chills, nephrotoxicity and hypokalemia) and the need to hospitalise the patient during the treatment course (around 4 weeks) are thus major drawbacks for using conventional AmB treatment.

**(A) Mode of Action of AmB**

AmB interacts with fungal membrane sterols and preferentially with ergosterol. Like fungi, *Leishmania* also have ergostane-based sterols as their major membrane sterol (Thakur *et al.* 1996b) and this likely explains the main efficacy of AmB against *Leishmania*. Several studies confirmed that AmB causes the formation of pores in the membrane that alters the permeability to ions responsible for cell death (Thakur *et al.* 1999,2008). In VL, liposomal amphotericin B is more than 95% effective and generally well tolerated; however, its high cost makes it unaffordable in endemic countries (Sundar *et al*. 2010). AmB is mainly used against VL in AIDS patients and post-dermal kala-azar although no cure can be obtained definitely (Matlashewski *et al.* 2011).

**(B) Mechanism of resistance**

AmB is widely used in the treatment of mycoses and fungal resistance has been only rarely reported (Bhattacharya *et al.* 2007). To date, no cases of AmB resistance in *Leishmania* infections have been reported in the field although resistant clones of *L donovani* have been produced in laboratory through stepwise increased concentrations (Sundar *et al.* 1998). Successive relapses after AmB treatments could contribute to an increase in AmB-resistant *Leishmania* isolates. In the laboratory, the selection of AmB-resistant strains by *in vitro* drug pressure is a long time process suggesting that a long term drug pressure should occur in the field before the mechanisms of resistance take place in parasites. The AmB-resistant parasite lacks ergosterol, the main target of AmB, possibly explaining the poor affinity of AmB for the modified membranes

of AmB-resistant parasites (Sundar et al. 1998). In AmB-resistant *L. donovani*, the membranes lacks C-24-alkylated sterols suggesting an inactivation of the enzyme system responsible for this alkylation, the S-adenosyl-L-methionine: C-24-delta-sterol-methyltransferase (SCMT). A recent report on AmB resistant clinical isolate represents altered membrane composition, increased expression of ATP-binding cassette transporters, and an up-regulated thiol metabolic pathway role in conferring AmB resistance (Purkait *et al.* 2012).

**Miltefosine (MIL)**

Miltefosine (Hexadecylphosphocholine) is an alkylphospholipid that was originally developed as an anti-cancer drug. It is a first oral antileishmanial drug which proved to be highly effective against VL with cure rates of 94%, including cases unresponsive to antimony (Sundar *et al.* 2002b; Olliaro *et al.* 2005). It was therefore, proposed as the first line VL therapy and remains the mainstay in the Kala-azar elimination program, which aims to reduce the VL incidence to 0.0001% in the Indian subcontinent by the year 2015. Since 2002, MIL has been used successfully in India for treatment of VL (Berman *et al.* 2006, 2008; Croft et al. 2006), although its use is compromised by teratogenicity and gastrointestinal side effects such as anorexia, nausea, vomiting and diarrhea (Sundar *et al.* 2002b). The long half life and reports of relapses following MIL treatment in VL, raise fears that recent gains in VL control may be lost (Sundar *et al.* 2012) responsiveness to MIL has been reported for cutaneous cases due to *L.*

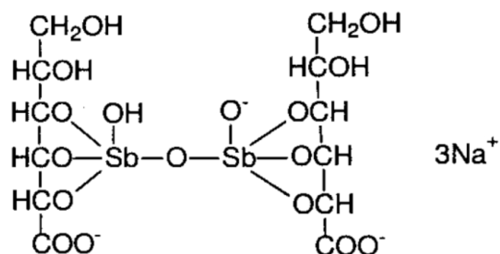
*braziliensis* and *L. (Viannia) guyanensis* in South America (Sanchez-Canete et al. 2009, Chrusciak-Talhari et al. 2011).

**(A) Mode of action**

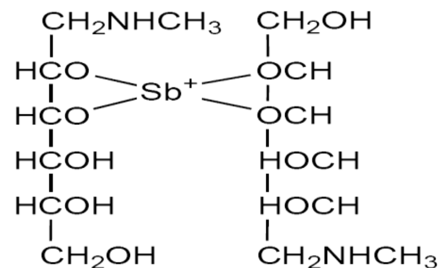
Although few data are available about the mechanism of action of miltefosine, we found recently that this drug kills the parasite by an apoptotic-like process in *L. donovani* promastigotes (Paris *et al.* 2004). These results were confirmed in amastigotes (Verma and Dey 2004). The apoptotic pathway in *Leishmania* is not well known but probably exhibits differences with those from Metazoans (Debrabant et al. 2003). Moreover, en effect on alkyl-lipid metabolism and phospholipid biosynthesis has been shown (Lux *et al.* 2000). However, the inhibitory effect observed on the enzymes involved in lipid metabolism is not compatible with physiological drug action.

(B) **Mechanism of resistance :**

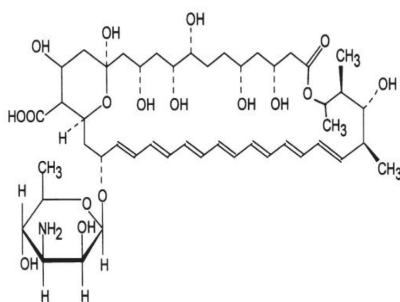
The mechanisms and related biological pathways that contribute to MIL resistance in the parasite are relatively poorly understood. Current evidence suggests that an impairment in drug uptake machinery involving amino-phospholipid translocase MIL transporter (LdMT) and an accessory protein, LdRos3 (CDC50/Lem3 family) in experimental MIL-resistant *Leishmania* lines was proposed to be the most likely mechanism of resistance (Perez-Victoria et al. 2003, 2006).



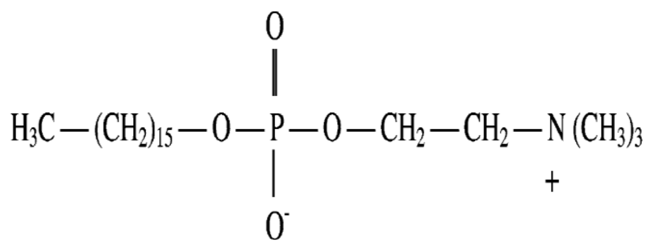

(A) Sodium antimony gluconate



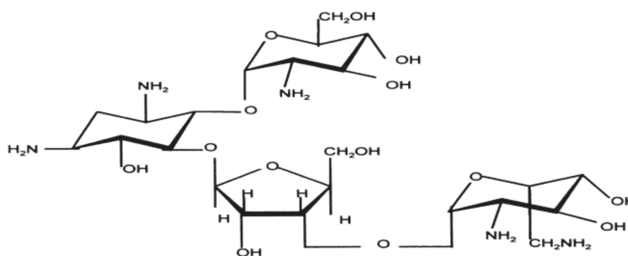

(B) Meglumine antimonite



(B) Amphotericin B




(D) Miltefosine



(E) Paromomycin Sulfate

**Figure 1.9: Chemical structures of different antileishmanial agents** (A, B): Different compounds of pentavalent antimonial drugs. (C) Amphotericin B (D) Miltefosine (E) Paromomycin sulfate.

**Paromomycin (PMM)**

Paromomycin is an antibiotic drug that was introduced for the treatment of visceral leishmaniasis in 2006 (Jha 2006; Mondal *et al.* 2010). Recently the phase IV trials results confirms its safety and efficacy as an antileishmanial agent (Sinha *et al.* 2011). PMM originated from the bacterial pathogen *Streptomyces rimosus* var. *paromomycinus* (Sundar and Chatterjee 2006). The spectrum of activity of paromomycin was found to encompass, like other aminoglycosides, most Gram-negative and many Gram-positive bacteria. Its activity includes *Mycobacterium tuberculosis* and non-tuberculous mycobacteria. Unusually, PMM is also effective against some protozoa and cestodes and it is the only aminoglycoside with clinically important antileishmanial activity. With regard to pharmaceutical information, PMM is an aminoglycoside antibiotic. It was first discovered during the 1950s, and it exists as paromomycin sulfate. It is a white and amorphous product. PMM is stable and water soluble. The chemical composition of paromomycin sulfate is *0*–2, 6-diamino-2, 6-dideoxy-β-L-idopyranosyl-(1→3)-*0*-β-D-ribofuranosyl-(1→5)-*0*-[2-amino-2-deoxy-α-D glucopyranosyl-(1→4)]-2-deoxystreptamine sulfate salt. Recently, advancements in pharmacology have allowed for the development of a newer modified version of PMM. The so-called "paromomycin-loaded albumin microsphere" has been in use for a few years. The preparation of paromomycin-loaded albumin microspheres (i.e., 5μm) helps to better target macrophages for treatment. This is useful for the treatment of leishmaniasis ( Khan and Kumar 2011;Khan *et al.* 2012a). Khan et al reported that "pharmacokinetic studies demonstrated nearly

80% reduction in $C_{(max)}$ of PMM when administered as paromomycin-loaded albumin microsphere, compared to other formulations at equivalent dose.” In addition, it has been observed that there are no symptoms of toxicity with the use of paromomycin-loaded albumin microspheres at the level that was reported to be toxic for classical paromomycin (Khan *et al.* 2012b). PMM is also being used in different formulations in various diseases (Table 1.3).

**Table 1.3: Formulations of paromomycin and their clinical usage**

| **Forms** | **Examples** |
|---|---|
| Oral | Amebiasis; cryptosporidiosis; tapeworm infestations |
| Ointment | Trichomoniasis; cutaneous Leishmaniasis |
| Inhalation | Cryptosporidiosis |
| Injection | Drug resistant tuberculosis; visceral leishmaniasis |

**(A) Mode of action:**

PMM could have ribosomes as a primary target the *Leishmania* (Maarouf *et al.* 1995). The mechanism of action of PMM has been well studied in *E. coli*. It is known to inhibit protein synthesis by interacting with the ribosomal subunits. It has also been shown to inhibit the anti-association activity of initiation factor 3 and promote association of the ribosomal subunits. It is known to bind to the major groove in the A-site of 16S rRNA in *E. coli* and induces misreading of mRNA (Eustice and Wilhelm 1984; Fourmy *et al.* 1996, 1998). It also appears to

have other effects in *Leishmania* including alterations in membrane fluidity and lipid metabolism and may also target key mitochondrial activities (Maarouf *et al*. 1997). The drug acts on RNA synthesis and modifies membrane polar lipids thereby affecting membrane fluidity and altering membrane permeability. Recent comparative proteomic analysis of the wild type and the PMM resistant *Leishmania* strains showed up-regulation of the ribosomal proteins, glycolytic enzymes and vesicular trafficking proteins in the PMM resistant parasite (Chawla *et al*. 2011).

(B) Mechanism of resistance

Studies on *in vitro* induced resistant strains showed a reduced uptake of the drug instead of mutations in the 30S or other ribosomal subunit as seen in PMM-resistant bacteria; however the exact mechanism of PMM resistance is as yet not entirely clear. The mechanism of action of PMM has been linked to the inhibition of cytochrome C reduction in *Candida krusei,* however there are only few studies in *Leishmania* elucidating the mechanism of PMM resistance. PMM resistance is readily induced in vitro (Maarouf *et al*. 1998). This PMM resistance was stable in the absence of continuing drug pressure and did not lead to loss of infectivity. El-On et al. produced *L. major* resistant to paromomycin (El-On *et al.* 1991), and Fong et al (Fong *et al.* 1994) in the USA produced *L. tropica* resistant to paromomycin. *Leishmania aethiopica* isolated from patients with diffuse CL after 60 days of PMM injections were three to five times less sensitive to the drug in vitro than those isolated before treatment (Teklemariam *et al*. 1994). In vivo

resistance in leishmaniasis has not yet been seen as the drug is not in clinical use for an extended period of time. The risk of secondary resistance is highest among VL patients co-infected with HIV, who have very high rates of relapse. If resistance mutations are stable, transmission from such patients would lead to primary resistance in others.

**Combination therapy**

Combination therapy is the policy to be developed in order to protect the novel antileishmanial agent from quickly becoming obsolete and preventing the development of resistance. This is especially important for the Indian subcontinent, where the emergence of resistance to antimonials significantly reduced the treatment options. A similar scenario with the current new drugs (MIL, AmB, and PMM) could be avoided or at least seriously delayed when they are administered in combination. Another major advantage is that the treatment regimen duration can be shortened compared to monotherapy without hampering treatment efficacy. This shorter regimen will not only increase treatment compliance, but also decrease toxicity, hospital costs and other indirect costs for patients. Ideally, the combination should include one fast acting drug to quickly reduce the parasite burden and another slower acting drug with another mode of action that can kill the remaining parasites (Croft *et al.*, 2006). A combination therapy needs to be evaluated for safety and optimized for either concomitant or sequential administration of component drugs.

A number of combinations therapies have been tried which includes sequential treatment with liposomal amphotericin B followed by MIL or PMM (as short as 7 days), as well as the concomitant administration of MIL and PMM (for 10 days) are very effective in India (>95%) (Table 1.4). Sodium antimony gluconate plus PMM for 17 days is more than 90% effective in East Africa. The shortened combination regimens are cost-effective in India. No combination has been tested so far in Brazil, Nepal and Bangladesh, although studies may be expected in the near future (Sundar *et al.* 2011).

**Table 1.4: Dosage and combinations of different regimens**

| **Drug Combinations** | **Dosage and duration** | **Cure Rate** |
|---|---|---|
| L-AmB + MIL | Single dose of 5mg/kg L-AmB+ MIL (7days, 50-100mg) | 97.5% |
| L-AMB + PMM | Single dose of 5 mg/kg L-AmB + 10 days 11 mg/kg intramuscular PMM | 97.5% |
| MIL + PMM | 50-100 mg/day MIL + 11 mg/kg/day PMM for 10 days | 98.7% |

The present investigation aims to evaluate the susceptibility of *Leishmania donovani* clinical isolates obtained from patients of visceral leishmaniasis (VL)/Post kala azar dermal leishmaniasis (PKDL) towards the antileishmanial drug, Paromomycin (PMM). Additionally, Miltefosine (MIL) sensitivity will also be determined in these isolates to evaluate the cross tolerance/ correlation if any towards PMM. The susceptibility of these isolates will be assessed with an *in vitro* intracellular amastigote assay. Further, PMM resistance will be induced experimentally in *L. donovani* isolates to study the mechanism of resistance and the fitness/ tolerance capacity of the PMM resistant parasites towards leishmanicidal host mechanisms.

## SPECIFIC OBJECTIVES

1. *Assessment of in vitro sensitivity towards Paromomycin and cross tolerance to Miltefosine in clinical isolates of L. donovani obtained from VL/PKDL patients.*
2. Generation and characterization of paromomycin resistant *L.donovani* parasites.
3. Studies on the mechanism of PMM resistance and parasite fitness utilizing experimentally resistant PMM lines.

***1. Assessment of in vitro sensitivity towards Paromomycin and cross tolerance to Miltefosine in clinical isolates of L. donovani obtained from VL/PKDL patients.***

We will evaluate the *in vitro* susceptibility of *L. donovani* clinical isolates from Indian VL and PKDL patients (exposed and unexposed to Miltefosine) towards PMM. The intrinsic sensitivity of these isolates towards PMM will reveal the current baseline sensitivity data in the prevailing parasite population and in miltefosine treated/relapse cases. Additionally, MIL susceptibility in these parasites will be performed to evaluate cross tolerance between PMM and MIL.

**2. Generation and characterization of paromomycin resistant *L.donovani* parasite.**

To investigate the mechanism of PMM resistance operative in *L. donovani,* two parasites line resistant to PMM will be generated experimentally by exposing the clinical isolates to increased drug concentrations. The lab generated PMM resistant parasites will be characterized based on the growth profile, altered PMM sensitivity, *in vitro* infectivity, cross resistance to other antileishmanial drugs. Further, PMM resistant parasites will be cloned to study the population homogeneity. The well characterized resistant parasite will be further employed as a tool for studying the mechanism of experimental PMM resistance as well as the parasite fitness against leishmanicidal mechanisms.

**3. Studies on the mechanism of PMM resistance and parasite fitness utilizing experimentally resistant PMM lines**

The mechanism of PMM resistance in the *L. donovani* is poorly elucidated. Several possible mechanisms of PMM resistance will be explored in *L. donovani,* including membrane fluidity, mutation in the small subunit ribosomal RNA gene, expression of ABC transporters and drug accumulation. Resistance might also be linked to tolerance to stresses imposed by the macrophage on the parasite as shown in other drug resistant isolates; therefore, we will also investigate the proficiency of the PMM-R parasite against macrophage killing mechanisms.

Hereby, this study aims to contribute to a better understanding of the mechanism of PMM resistance operative in *Leishmania* and shed light on impact of PMM-resistance on the biology of *L. donovani*.

## INTRODUCTION

In the absence of vaccines and the limited impact of vector control, chemotherapy is the key strategy for VL control in (Jha *et al.* 2005; Olliaro *et al*. 2005; Sundar and Chatterjee 2006). The situation is particularly grave in Bihar, India, where more than 60% of VL patients do not respond to traditional first line antimonial (sodium antimony gluconate) therapy. The use of Amphotericin B and its liposomal formulations, although highly effective even in antimony unresponsive patients, has limitations because of its renal toxicity, high costs and inconvenience due to slow I.V. based administration (Sundar and Rai 2002a, 2005). The first oral antileishmanial drug Miltefosine (MIL), an alkylphosphocholine, has proved to be highly effective against VL with cure rates of 94%, including cases unresponsive to antimony (Sundar et al. 2002b; Olliaro et al. 2005). Moreover, phase IV trial of MIL in India suggested doubling of the relapse and failure rate compared to phase III trials (Sundar and Murray 2005;Sundar and Rai 2005). Treatment failures (almost all relapses) were recently also observed in Nepal (Pandey *et al.* 2009). There has been a significant decrease in efficacy of the MIL from an initial cure rate of 97.5% to 90%. The long half life and reports of relapses following MIL treatment in VL, raise fears that recent gains in VL control may be lost (Sundar *et al*. 2012).

Paromomycin (PMM) is an aminoglycoside antibiotic exhibiting high efficacy towards VL (Sundar *et al.* 2007a). Data from Phase IV trials confirm the safety and efficacy of PMM to treat VL with advantages of shorter treatment course, higher safety profile and low manufacturing cost, making it an affordable

therapeutic option in Indian field conditions (Sinha *et al*. 2011). With a few chemotherapeutic options in hand, the primary concern is to safeguard the available treatment options against drug resistance by monitoring drug sensitivity and emergence of drug resistance in the field. In the present investigation, intrinsic sensitivity towards PMM was evaluated in the prevailing *L. donovani* population of hyper-endemic regions in order to obtain baseline data on sensitivity and in parasites obtained from MIL treated VL/PKDL patients (including post-treatment stage parasite isolates obtained either as residual parasites soon after completion of treatment or from cases that relapsed) to examine if there is an influence of MIL treatment background on PMM susceptibility. The PMM susceptibility was assessed with an *in vitro* intracellular amastigote assay as no correlation is observed at promastigote and amastigote stage (Kulshrestha *et al.* 2011). In addition, MIL susceptibility was also determined in VL/PKDL isolates to correlate it with treatment outcome and study cross tolerance to PMM.

## Materials and Methods

M199 medium, RPMI-1640 medium, penicillin and streptomycin were purchased from Sigma; L-glutamine was supplied by Invitrogen, USA. Fetal bovine serum (FBS) was purchased from Gibco, Grand Island, NY, USA, 16-well chamber slides from Nunc, Rochester, NY, USA, and Diff-Quik® stain solution obtained from Dade Behring, Newark, DE, USA.

## Preparation of drug stocks

PMM (Gland Pharma) stock solution of 20mM and MIL (batch 1149149) stock solution (20 mM) was prepared in water, filter sterilized and stored at −20°C until use. Drug stock solution was serially diluted freshly in RPMI media with 10 % FBS and penicillin (100 U/ml)/ streptomycin (100 µg/ml).

## Patients and parasites

Clinical isolates of *L. donovani* were prepared from splenic aspirates of VL patients reporting to KAMRC, Muzaffarpur, Bihar or from dermal lesions of PKDL patients reporting to Department of Dermatology, Safdarjung Hospital (SJH), New Delhi under the guidelines of the Ethical Committee of the respective Institute. All patients came from zones of high endemicity in Bihar, India. VL patients received MIL treatment for 28 days (50 mg capsule twice) while PKDL patients received MIL for 60 days (50 mg capsule, thrice daily). Splenic smears from all VL patients were examined microscopically at the pre and post treatment stages. Patients with negative or +1 smear grade were not treated further.

However, patients with grading >2 (Chulay and Bryceson 1983) were treated with amphotericin B deoxycholate.

Parasites isolated before onset of treatment were assigned XXX/0 codes. MIL treatment led to complete subsidence of VL symptoms, interpreted as clinical cure, although residual parasites could be cultured from splenic aspirates in a substantial number of patients at the end of 1 month of treatment. These post-treatment isolates were assigned with XXX/1 codes. All cases were followed up for one year. VL and PKDL cases that relapsed after an initial cure were treated with amphotericin B and cured. Parasites were isolated from each of the relapse cases at the time of reported relapse (after four, six and seven months of MIL treatment completion in VL and after 12, 18 and 32 months of MIL treatment in PKDL and were designated as XXX/month in which relapse occurred.

Parasites were routinely grown in Medium 199 with 10% heat-inactivated fetal bovine serum at 25°C.

**Drug susceptibility assay at intracellular amastigote stage**

The drug sensitivity of *L. donovani* parasites was assessed in intracellular amastigotes using J774 A.1 mouse macrophage adherent cell line. Briefly, J774A.1 cells (1 x $10^5$ cells/ml) were infected with stationary phase promastigotes at ratio of 10 : 1 (parasite : macrophage), plated into 16 well chambered tissue culture slides and incubated for 4 h at 37 °C in 5% $CO_2$. Excess, non-adhered promastigotes were removed by washing and macrophages incubated for 18-24 h. Infected cells were re-incubated for 48 h, with PMM (1, 5, 10, 20, 30 and

40μM) or MIL (1, 5, 10, 20 and 30μM). Macrophages were then examined for intracellular amastigotes after staining with Diff-Quik solutions. The number of *L. donovani* amastigotes was counted in 100 macrophages, at 100 x magnification. The survival rate of parasites relative to untreated macrophages was calculated and $IC_{50}$ were determined by sigmoid regression analysis. The assays were performed in triplicate and repeated at least twice.

## RESULTS

### Natural Sensitivity of *L. donovani* isolates to PMM

PMM susceptibility was determined at intracellular amastigote stage for 30 clinical isolates, including 11 pre-treatment isolates (prevailing isolates, six from VL and five from PKDL cases), thirteen post-treatment isolates (obtained at the end of MIL treatment from VL patients which depicted a clinical cure, although parasitology was still positive) and six relapse isolates (three VL and three PKDL). The clinical profile of patients and *in vitro* susceptibility of the isolates to PMM are summarized in Table 3.1.

The PMM $IC_{50}$ ranged from 3.41±0.29 to 10.70±1.12µM with mean $IC_{50}$= 7.05±2.24µM (Table 3.1). Furthermore, the PMM sensitivity was similar ($P>0.05$) in parasites non-exposed (mean$IC_{50}$=7.73±2.25µM) or exposed (mean$IC_{50}$ =6.79±2.25µM) to MIL (Figure 3.1).

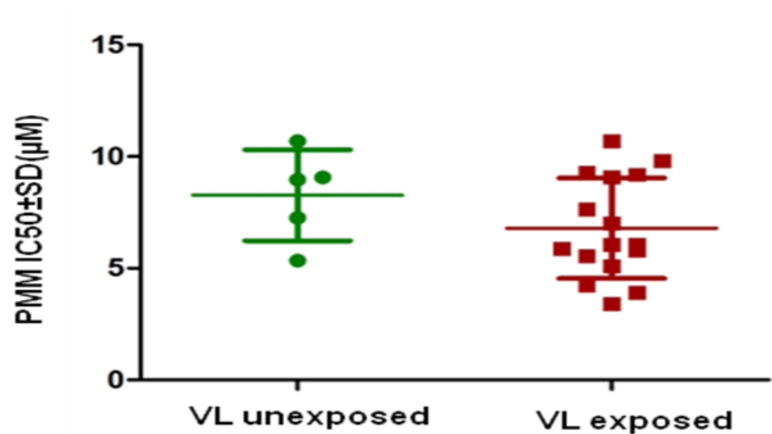



Figure 3.1: **Inherent PMM susceptibility profile of VL isolates exposed or non-exposed to MIL treatment**. Susceptibility of VL isolates at intracellular amastigote stage was determined by infection in murine macrophage cell line

J774A.1. Each individual value represents mean $IC_{50}$±SD from two separate assays.

The inherent PMM susceptibility of 8 PKDL isolates ranged from 4.92±0.34 to 8.62±1.82μM (Table 3.1). The PMM $IC_{50}$ of PKDL isolates was similar ($P>0.05$) in parasites non-exposed (n=5, mean $IC_{50}$ =6.12±1.40μM) or exposed (n=3, mean $IC_{50}$= 6.29±2.02μM) to MIL.

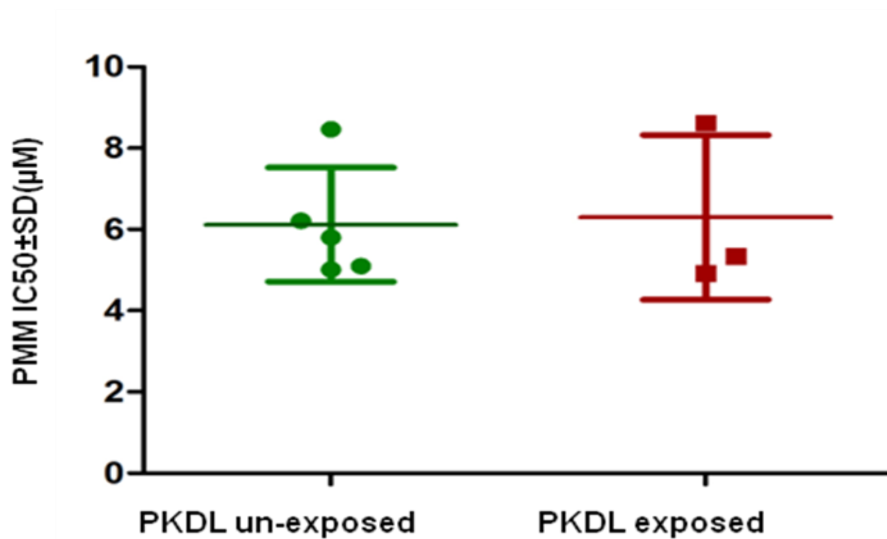



Figure 3.2: **PMM susceptibility profile of PKDL isolates exposed or non-exposed to MIL treatment**. Susceptibility of PKDL isolates at intracellular amastigote stage was determined by infection in murine macrophage cell line J774A.1. Each individual value represents mean $IC_{50}$±SD from two separate assays

The mean susceptibility of VL isolates (7.05±2.23μM, n=22) and PKDL isolates (6.18±1.51, n=8) was comparable ($P>0.05$).

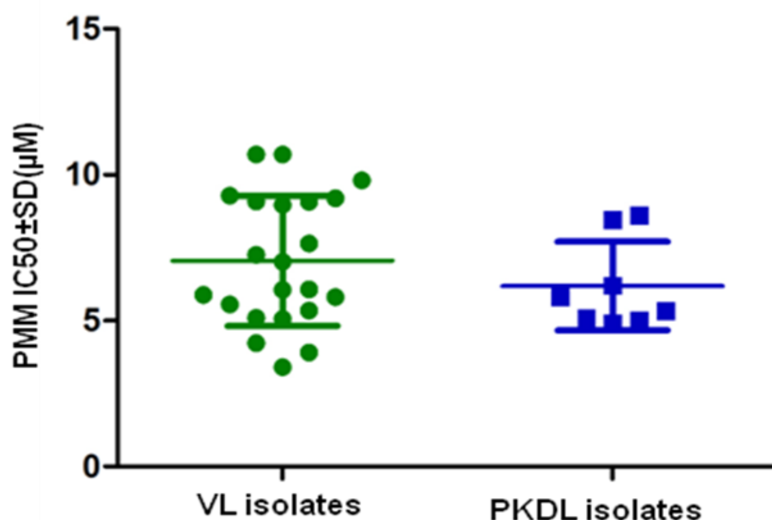



Figure 3.3: **Comparative *Paromomycin* susceptibility of all VL and PKDL isolates**. Sensitivity of VL and PKDL isolates at intracellular amastigote stage were determined by infection in murine macrophage cell line J774A.1. Each individual value represents mean $IC_{50}$±SD from two separate assays.

***In vitro* sensitivity to MIL**

The six VL pre-treatment isolates showed a sensitivity range of 0.95±0.06 to 2.91±0.24µM towards MIL with the mean $IC_{50}$±SD being 1.86±0.75µM (Figure 3.4). The post-treatment VL isolates had a mean $IC_{50}$ of 2.43±1.44µM (range 1.02±0.17 to 5.20±0.80µM) which was not significantly different in comparison with the pre-treatment group ($P>0.05$). The mean $IC_{50}$ of the three VL isolates from relapse cases (4.72±1.99µM) was significantly higher ($P<0.05$) than that of pre-treatment VL cases (1.86±0.75µM) (Figure 3.4).

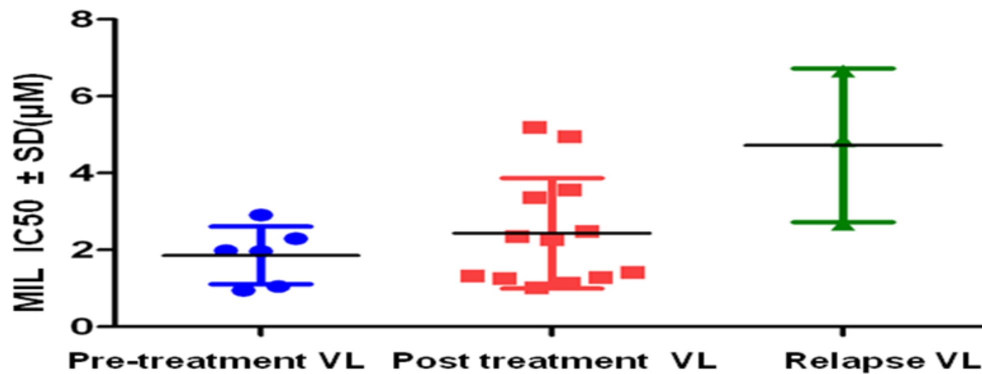



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Figure 3.4: ***In vitro* miltefosine susceptibility of VL isolates before and after Mil treatment including relapse isolates**. Sensitivity of VL isolates at intracellular amastigote stage was determined by infection in murine macrophage cell line J774A.1. Each individual value represents mean $IC_{50}$±SD from two separate assays.

The three PKDL relapse isolates showed a significantly higher ($P<0.05$), mean $IC_{50}$ of 16.13±2.64µM in comparison with the pre-treatment PKDL isolates (mean $IC_{50}$= 8.63±0.94µM) (Figure 3.5).

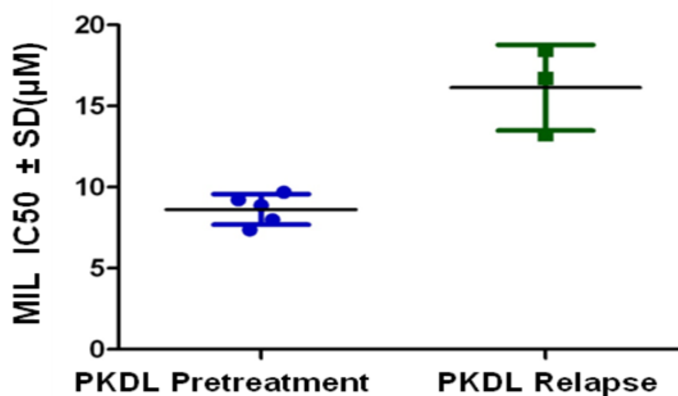


Figure 3.5: ***In vitro* miltefosine susceptibility of PKDL isolates before and after Mil treatment**. Sensitivity of PKDL isolates at intracellular amastigote stage

was determined by infection in murine macrophage cell line J774A.1. Each individual value represents mean $IC_{50}$±SD from two separate assays.

The mean susceptibility of all VL isolates (2.58±1.58µM, n=22) when compared with PKDL isolates (11.45±4.19µM, n=8) revealed that the latter were significantly (P<0.05) more tolerant to MIL (Figure 3.6).

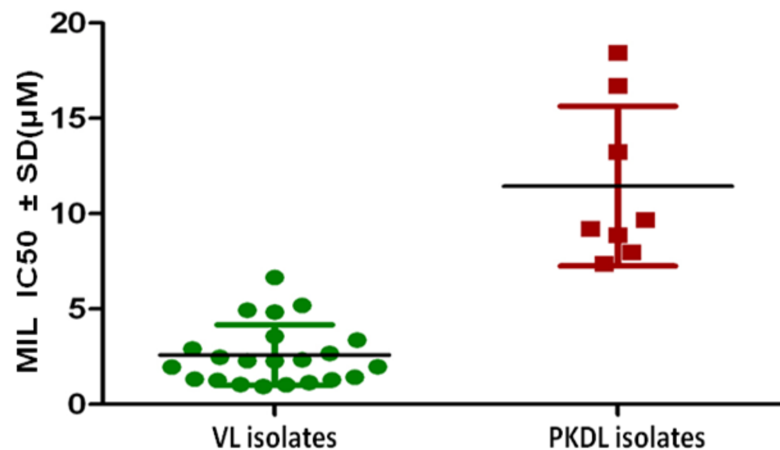


Figure 3.6: **Comparative miltefosine susceptibility of all VL and PKDL isolates**. Sensitivity of VL and PKDL isolates at intracellular amastigote stage were determined by infection in murine macrophage cell line J774A.1. Each individual value represents mean $IC_{50}$±SD from two separate assays.

There was no observed correlation and cross tolerance between MIL and PMM in VL (r=0.10, P>0.05) or PKDL isolates (r=-0.02, P>0.05).

**Table 3.1:** Drug susceptibility of Leishmania donovani clinical isolates following Miltefosine treatment in cases of Visceral Leishmaniasis and Post kala-azar dermal Leishmaniasis

| WHO code[1] | Patient age/sex | Clinical response to treatment | PMM IC50±SD (μM) | MIL $IC_{50}$±SD (μM) |
|---|---|---|---|---|
| MHOM/IN/2010/BHU782/0 | 18/M | Cure | 5.34+1.12 | 0.95±0.06 |
| MHOM/IN/2010/BHU869/0 | 10/F | Cure | 8.97±0.25 | 1.05±0.08 |
| MHOM/IN/2010/BHU994/0 | 60/M | Cure | 9.07±0.15 | 1.96±0.50 |
| MHOM/IN/2010/BHU828/0 | 10/M | Cure | 5.06±0.37 | 1.98±0.66 |
| MHOM/IN/2010/BHU902/0 | 7/M | Cure | 10.70±1.12 | 2.30±0.36 |
| MHOM/IN/2009/BHU815/0 | 12/F | Cure | 7.25±0.55 | 2.91±0.24 |
| MHOM/IN/2010/BHU796/1 | NA | Cure | 5.55±0.89 | 1.02±0.17 |
| MHOM/IN/2010/BHU1121/1 | 12/M | Cure | 6.05±0.65 | 1.14±0.11 |
| MHOM/IN/2010/BHU807/1 | 12/F | Cure | 9.08±0.65 | 1.26±0.15 |
| MHOM/IN/2010/BHU869/1 | 10/F | Cure | 3.91±0.03 | 1.32±0.26 |
| MHOM/IN/2010/BHU1042/1 | 6/M | Cure | 4.23±0.76 | 1.42±0.06 |
| MHOM/IN/2009/BHU815/1 | 12/F | Cure | 5.88±0.64 | 2.27±0.49 |
| MHOM/IN/2009/BHU741/1 | NA | Cure | 6.07±1.02 | 2.35±0.40 |
| MHOM/IN/2009/BHU800/1 | 35/M | Cure | 9.80±0.61 | 2.48±0.04 |
| MHOM/IN/2010/BHU902/1 | 7/M | Cure | 9.28±0.40 | 3.37±0.38 |
| MHOM/IN/2010/BHU1093/1 | 20/M | Cure | 3.41±0.29 | 3.57±0.35 |
| MHOM/IN/2009/BHU994/1 | 60/M | Cure | 5.10±0.65 | 3.72±0.52 |
| MHOM/IN/2010/BHU1080/1 | 12/M | Cure | 5.80±0.84 | 4.95±0.28 |
| MHOM/IN/2010/BHU814/1 | NA | Cure | 9.20±0.75 | 5.20±0.80 |
| MHOM/IN/2010/BHU1113/7 | 35/M | Relapse | 7.02±0.86 | 2.67±0.51 |
| MHOM/IN/2010/BHU872/6 | 18/M | Relapse | 10.70±1.02 | 4.84±0.39 |
| MHOM/IN/2009/BHU1062/4 | 7/F | Relapse | 7.64+0.76 | 6.66±0.62 |
| MHOM/IN/1998/NIPP44/0 | 18/M | Cure* | 5.01±0.38 | 7.37±0.07 |

| | | | | |
|---|---|---|---|---|
| MHOM/IN/2011/NIPP232/0 | 42/M | Cure | 8.46±0.77 | 7.99±0.15 |
| MHOM/IN/1998/NIPP48/0 | 15/M | Cure* | 5.80±0.85 | 8.88±0.18 |
| MHOM/IN/1998/NIPP49/0 | 23/M | Cure* | 5.10±0.37 | 9.23±0.22 |
| MHOM/IN/2001/NIPP93/0 | 25/M | Cure* | 6.21±0.37 | 9.69±0.52 |
| MHOM/IN/2010/NIPP195/12 | 21/M | Relapse | 8.62±1.82 | 13.26±0.89 |
| MHOM/IN/2010/NIPP214/18 | 35/M | Relapse | 4.92±0.34 | 16.70±0.65 |
| MHOM/IN/2011/NIPP214/32 | 36/M | Relapse | 5.34±0.75 | 18.45±0.79 |

[1]WHO code: country and year of isolation and the respective strain code, the number following the isolate ID indicates the number of months elapsed after start of MIL treatment. Parasites isolated from patients' prior start of MIL treatment were labeled as XXX/0 and one month following first treatment was labeled XXX/1. These patients cleared from VL symptoms after respective duration of MIL treatment and were interpreted as clinical cure, although residual parasites could be cultured from splenic aspirates (marked XXX/1). In the period of 1 year follow up, cases of relapse were observed in three VL patients that had shown an initial clinical cure, the isolates obtained were designated as XXX/month in which relapse occurred.

Cure*- Patients treated with S AG (1000mg intra muscularly), daily for four months.

## DISCUSSION

The present study reports the natural susceptibility of Indian *L. donovani* from VL/PKDL cases towards PMM and MIL. The study presents the scenario of drug susceptibility in the parasite population currently prevailing in the field (as yet unexposed to these drugs) and in isolates obtained from MIL post treatment cases (including relapses). At the end of MIL treatment all cases showed clinical cure although some of them were parasitologically positive and residual parasites could be cultured from splenic aspirates of such patients (Table 3.1). All VL/PKDL field isolates examined in the study were found susceptible to PMM suggesting its potential efficacy in VL and PKDL therapy. Comparative PMM susceptibility of VL and PKDL isolates revealed no significant difference in contrast to earlier trend of SAG susceptibility studies where PKDL isolates exhibits higher tolerance to SAG and acts as a reservoir of parasites (Singh *et al.* 2006). MIL therapy as treatment for VL is life saving and has pioneered the era of effective oral therapy for this potentially fatal disease. However, the long half life of MIL (150-200 h) poses the risk of development of resistance in natural population of parasites. It has been reported earlier that MIL resistant parasites can be easily generated *in vitro* (Seifert et al. 2003). The present study reveals for the first time, the intrinsic *in vitro* sensitivity of Indian *L. donovani* isolates from a set of VL and PKDL patients treated with MIL (including both responders and relapse cases). The sensitivity of the currently prevailing clinical isolates was

similar to the *L. donovani* parasites from the era of pre- MIL treatment reported earlier (Kumar *et al*. 2009).

The drug susceptibility of *L. donovani* parasites isolated at the end of therapy was comparable to that of pre-treatment isolates. On the contrary, parasites obtained from the cases that relapsed exhibited significantly reduced susceptibility to MIL, although the $IC_{50}$ values were below the expected serum threshold levels (Berman 2005), implicating the possible involvement of host factors in rendering tolerance to drug. Indeed, reports on VL relapse in HIV co-infected patients treated with MIL suggest that host immunity plays a role in the elimination of parasites from VL patients (Sindermann *et al*. 2004; Troya *et al*. 2008). Although we did not find any clinically resistant strains, the observation of strains with higher MIL tolerance (up to eight times compared to the sensitive ones) emphasizes the need for close monitoring of cases under MIL treatment. The study also investigated for the first time, the intrinsic susceptibility of PKDL isolates towards MIL. The *in vitro* susceptibility of PKDL isolates was significantly higher in pre-treatment isolates than in isolates originating from relapse patients, which were exposed to MIL for long duration (over two months). The other concern is significantly higher $IC_{50}$ of PKDL isolates (~ 4 fold) as compared to VL isolates, a trend similar to that reported earlier for SAG susceptibility in isolates from high endemic regions (Singh *et al*. 2006).

PMM did not exhibit any cross tolerance towards MIL in VL/PKDL isolates. PMM appears promising since (i) prevailing parasites (unexposed to drug) and isolates exposed to MIL were equally susceptible (ii) VL and PKDL isolates also

exhibited similar sensitivity. Regional policies concerning judicious use of the drug and monitoring the treatment outcome should be implemented and supervised by the health authorities in the endemic areas to minimize the risk of emergence of resistant strains.

**Introduction**

The decreased efficacy towards traditional treatment based on pentavalent antimonials (sodium antimony gluconate, SAG) in India, had appended the problem (Sundar *et al*. 2000). Amphotericin B is another highly effective antileishmanial agent but is expensive and associated with severe side effects and requires hospitalization (Sundar *et al.* 2010). High cost, teratogenic potential in women of child bearing age and reports of relapses following Miltefosine (MIL) treatment in VL, raise fears that recent gains in VL control may be lost (Bhandari *et al*. 2012).

Paromomycin (PMM) is an aminoglycoside antibiotic that has been used as an oral, topical, and parenteral drug for bacterial and parasitic infections. PMM was registered in 2006 for VL treatment in India and Phase III and IV trials further confirms the safety and efficacy to treat VL with a cure rate of approximately 94% (Sundar *et al.* 2007a; Sinha *et al.* 2011). It has shown to be effective in combination therapies with other drugs (Sundar *et al.* 2007a, 2011; Olliaro 2010), well-tolerated and currently the cheapest drug available ($15/patient) (Olliaro and Sundar 2009; Meheus *et al*. 2010) as compared to other chemotherapeutic options for VL treatment. It was shown that PMM is not hampered by antimony resistance (Kulshrestha *et al.* 2011), but appropriate measures should be taken to assure its long-term effectiveness. This issue needs to be proactively addressed in laboratory studies to help steer decisions on future treatment policies, diagnosis and epidemiological resistance monitoring. Therefore, it’s crucial to understand the mechanism of PMM resistance to safeguard the drug.

Studies towards understanding the mechanism of PMM resistance have been mostly confined to fungal and bacterial diseases. PMM resistance has been associated with various mechanisms such as drug uptake, mutations at the ribosomal binding sites or enzymatic inactivation of the drug in prokaryotes. There are limited studies/reports on PMM resistance in *Leishmania* which evince the involvement of mitochondrial membrane potential and decrease in the drug uptake (Maarouf *et al.* 1997,1998; Jhingran *et al.* 2009). Majority of the knowledge on antimony and other drugs resistance mechanism in *Leishmania spp* has been derived from laboratory mutants developed by adapting to increasing drug concentration (Seifert *et al,* 2003; Singh *et al* 2010). In the present study, PMM resistance was induced in two cloned field isolates with a defined antimony resistance background instead of laboratory strains used in previous studies (Maarouf *et al.* 1998). We further evaluated the phenotypic characteristics of experimental PMM resistant *L.donovani* based on its growth profile, altered susceptibility to PMM, susceptibility to other antileishmanial drugs, infectivity or virulence compared to wild type parasite.

The PMM resistant line which exhibits decreased susceptibility out of the two strains at amastigote stage was utilized to investigate the mechanism of experimental PMM resistance operative in *L. donovani.* We analyzed the changes in membrane fluidity, drug accumulation, the mRNA expression of ATP binding cassette transporter genes (MDR1& MRPA) and Protein phosphatase 2A (PP2A) and mutations in rRNA gene. Additionally, we investigated the tolerance of PMM-R parasite towards host leishmanicidal/defence mechanisms including

nitrosative, oxidative and complement mediated stresses. Finally, an understanding of resistance mechanism will shed light on how *Leishmania* parasites resist PMM pressure and the targets affected by it.

## Materials and methods

Medium 199, RPMI-1640, penicillin, streptomycin, resazurin, hydrogen peroxide, 3-morpholinosydnonimine (SIN-1), S-nitroso-N-acetyl-DL-penicillamine (SNAP-1), Diphenylhexatriene (DPH), Interferon-gamma (IFN-Ý) and Lipopolysaccharide (LPS) were purchased from Sigma; L-glutamine was supplied by Invitrogen, USA. Fetal bovine serum (FBS) was purchased from Gibco, Grand Island, NY, USA, 96 well plate from axygen, 16-well chamber slides from Nunc, Rochester, NY, USA, Trizol Reagent from Invitrogen, USA and Diff-Quik® stain solution obtained from Dade Behring, Newark, DE, USA.

## Preparation of drug stocks

PMM (Gland Pharma) stock solution of 20mM, MIL (Paladin) stock solution (20 mM) and SAG (Albert David) stock solution (1mg/ml) was prepared in water, filter sterilized and stored at −20 °C until use. Drug stock solution was serially diluted freshly in respective media with 10 % FBS and penicillin (100 U/ml)/ streptomycin (100 μg/ml).

## Parasites

Two SAG resistant *L. donovani* parasites, BHU573 and BHU568 were isolated from splenic aspirates of KA patients reporting to KAMRC, Muzaffarpur (hyper endemic region) Bihar, India, and the parasite were characterized as *L.donovani* by ITS-1 PCR RFLP (Kumar *et al.* 2007). The cloned line of both the isolates (BHU573 cl-3 & BHU568cl-1) were maintained in M199 medium supplemented with 25 mM HEPES, containing 10% fetal bovine serum at 25°C.

**Development of Paromomycin resistant lines**

Experimental *L. donovani* strain resistant to PMM was obtained by growing promastigotes *in vitro* with step-wise increase in the drug pressure (Seifert *et al,* 2003). WT parasites (5 X $10^5$ cells/ml) were initially exposed to 25μM PMM (M199 + 10 % FBS) and subsequently, in stepwise fashion, to 25, 40, 80 and 97 μM of PMM. At each step, parasites were cultured for at least for 5-8 passages to attain steady and optimal cell growth. A stepwise increase in drug concentration was undertaken only when the drug exposed cultures showed a growth rate equivalent to that of the wild-type clone cultures. Resistant clones at intermediate stages were also stored in liquid $N_2$ at various stages during their establishment. The growth profile of the PMM resistant parasite (referred as BHU573PMM-R & BHU568PMM-R) was compared with its corresponding wild type parasite (PMM-S). The PMM-R strains were passaged at intermediate stages of adaptation through macrophages to maintain infectivity.

**Cloning of PMM resistant parasites by limited dilution method**

Parasites were counted using hemocytometer and adjusted to a cell density of $1x10^6$ cells/ml and were further diluted in 20 ml medium to give $1x10^4$ cells/ml. 5µl and 30 µl from 10,000 cells/ml were added to 10 ml of complete M199 medium to get a working dilution of 50cell/10ml and 300cell/10ml. 100µl from each 10 ml cell suspension (50 and 300 cells) were aliquot into the 96 well plate. In each well 100µl of nutrient medium was added. The plates were left at 25ºC for 1-2 h before examining each well for single cells. Wells with single cells

were marked. The plates were then observed for growth of the clonal population after 10-15 days.

**Drug susceptibility of PMM-R parental and clonal population**

*In vitro* efficacy of parental resistant strains (BHU568PMM-R and BHU573PMM-R) and their clonal populations were evaluated towards PMM at both the stages of the parasite life cycle, i.e. the promastigote stage as in sand fly vector and intracellular amastigote stage as in mammalian host.

***Promastigote viability testing*** **using colorimetric Resazurin assay**

Resazurin dye (7-Hydroxy-3H-phenoxazin-3-one10-oxide) was employed for promastigote susceptibility testing. Resazurin is a redox indicator that converts to fluorescent and colorimetric, resorufin dye by the metabolically active cells. Log-phase promastigotes were harvested and counted in a counting chamber. Promastigotes were seeded into the wells at $10^5$promastigotes/well and exposed to the serially-diluted PMM from a range of 648μM to 0.63μM in 96-well plate. During incubation for 72h at 25°C, plates were wrapped in parafilm to avoid evaporation. After the stated incubation period, 50μl of Resazurin (0.0125% in PBS) is added to each well and the plates were further incubated for 24h. Fluorescence reading was obtained by using the filter combination 550-590nm. $IC_{50}$ were then calculated in comparison to untreated control using Origin 6.0 software.

**Drug Susceptibility assay for intracellular amastigote**

We studied the intracellular behavior of these parasites towards PMM using J774A.1 as described earlier (chapter3) and peritoneal macrophages (PEC) extracted from Balb/c mice for susceptibility assays. PECs were plated out at $2X10^5$ cells per 200µl per well in RPMI supplemented with 10% FBS in 16 well chamber slides incubated at $37^0C$, 5% $CO_2$. Twenty-four hours later, medium was gently removed and the macrophages attached to the slide were infected with late log promastigotes at a ratio of 10 parasites per macrophage in a volume of 200µl complete RPMI. After 24h of infection, non-internalised parasites were washed off and different dilutions of PMM (0, 5, 10, 20, 30, 40, 80, 100µM), MIL (0, 5, 10, 20, 30µM) and SAG (0, 10, 20, 40, 80, 100µg/ml) was applied in quadruplicate. After 48h of incubation, slides were fixed with methanol and stained with Diff-Quik solutions. The number of amastigote per cell was counted in 100 macrophages; the percent killing was calculated by sigmoidal regression analysis (Origin6.0).

***In vitro* infectivity**

The infectivity of PMM-resistant promastigotes was assessed using a murine macrophage J774A.1 cell line and peritoneal macrophages derived from Balb/c mice. The promastigote forms of WT and PMM-R parasites were used for macrophage infection from 6 day old cultures. The macrophages infected with

promastigotes were incubated for 48 or 72 hrs at 37°C at 5% $CO_2$ and slides were stained with Diff-Quik solution. The number of amastigote in100 macrophages/well was determined microscopically as described earlier.

**RNA isolation and real time PCR**

Total RNA was isolated from stationary phase promastigotes using Trizol Reagent following instructions recommended by the manufacturer and was reverse transcribed. All real time PCR reactions were performed in triplicate in 20µl volumes using SYBR Green for detection in an ABI Prism 7500 Sequence Detection System (Applied Biosystem). The analysis of gene expression was done using the $2^{-\Delta\Delta CT}$ method using Glyceraldehyde 3-phospho dehydrogenase (GAPDH) and Cystathione β-synthase (CBS) as endogenous controls (Decuypere *et al.* 2008; Kumar *et al.* 2012). The primers used in real time PCR are as follows**:** CBS (For "CGCCGATGTCAACTGGATG"& Rev "GCTCCTTCTTCAGCGTGTCG"), GAPDH (For "GAAGTACACGGTGGAGGCTG" & Rev "CGCTGATCACGACCTTCTTC"), PP2A (For: "GCGAAGGAGATTTTCACGAG" & Rev "CGCCCATGAAGACGTAGTTT"), MRPA (For "GCGCAGCCGTTTGTGCTTGTGG" & Rev "TTGCGTACGTCGCGATGGTGC") and MDR1 (For "CGAAGTGGGAAGTGAATGGT & Rev "CAGCATGTAGCCCATCAAGA").

**Measurement of fluorescent anisotropy by Diphenyhexatriene**

The fluidity of membranes was measured by Diphenylhexatriene (DPH), a fluorescence probe as described elsewhere (Mukhopadhyay *et al.* 2011). Briefly, 2mM stock solution of DPH in tetrahydrofuran was prepared and was stored at 4°C in the dark. The late log phase promastigotes were labeled using the 2 mM final concentration of DPH (2X$10^6$cells/ml) and incubated for 2h at 37°C. After incubation the cell suspension were washed thrice in PBS and then fixed with 2% paraformaldehyde for 15 minute. The cells were washed again and resuspended in 2ml of PBS. The DPH probe bound to the membrane of the cell was excited at 365 nm and emission was recorded at 430 nm in a spectrofluorimeter. The fluorescence anisotropy value (FA) was calculated using the equation: FA = $(I_{II} - I_{\backslash}) / (I_{II} + 2I_{\backslash})$, where $I_{II}$ and $I_{\backslash}$ are the fluorescent intensities oriented, respectively, parallel and perpendicular to the direction of polarization of the excited light.

**Full gene sequencing of rRNA**

DNA was isolated from late log phase promastigotes using Qiagen DNA isolation kit. Polymorphisms/Mutations in the *LinJ.27.rRNA6* gene were determined by direct sequencing of the amplicons resulting from the PCR using gene specific primers described in Table 4.1. Amplicons were passed through Qiagen PCR purification kit before sequencing. The sequencing reactions were carried out on Automated sequencing machine 3730 Version 3.0 (ABI PRISM) at

Sequencing Facility Department, Delhi University, South Campus, New Delhi. Sequences identified by DNA sequencing were aligned and polymorphism analysis was done by using Edit Sequence tool (DNASTAR).

**Table 4.1: Primers employed to sequence the full rRNA gene.**

| S. No | Primer sequences | Primer name |
|---|---|---|
| 1. | ATA TCG AGT AAC AAT TGG AGG | Full Gene F |
| 2. | TTG CTG TGT ATG TGG GAA AGG | Full Gene R |
| 3. | CTGTTGCTGTTAAAGGGTTC | LN_29_F |
| 4. | TGTTTCTTTGAATGTTCACG | LN_148_R |
| 5. | CTGTGACTAAAGAAGCGTGA | LN_220_F |
| 6. | ACCCTCCTTCATTCCTAGAG | LN_488_R |
| 7. | TTCAAGGATACCTTCCTCAA | LN_567_F |
| 8. | TTAAGTTTCACTCTTGCGAAC | LN_863_R |
| 9. | GGTTTAATTTGACTCAACACG | LN_903_F |
| 10. | AACATTGAGGAGCATCACA | LN_1222_R |
| 11. | AACGACTTTTGTCGAACCTA | LN_1265_F |
| 12. | GCAGGTTCACCTACAGCTAC | LN_1562_R |
| 13. | CCTTGTTACGACTTTTGCTT | LN_1543_R |

Foot note: Primers were designed for gene sequence analysis. The numeral indicated in the primer name represents position on the gene.

**Drug accumulation assay**

The uptake mechanism was studied as per the previously described (Maarouf *et al.* 1998) protocol with slight modifications. Briefly, log phase promastigotes were treated with 100µM of PMM for various time points (0-90 mins). The parasites were harvested and washed twice with PBS. Finally the pellet was re-suspended in PBS and digested by incubating in $HNO_3$ overnight at

room temperature. It was diluted with 1ml PBS and centrifuged at 12000 rpm, 10 mins and analyzed by LC-MS.

**In vitro promastigote stress tests**

The promastigote stress tests were performed as described elsewhere (Vanaerschot *et al.* 2010). Assays were performed in sterile 96-well microtiter plates using log-phase promastigotes ($10^5$/well), with each well containing different dilution of stress inducing agents, Hydrogen peroxide ($H_2O_2$, 9.76-10,000μM), 3-morpholinosydnonimine (SIN-1; 1.95-2000μM) and S-nitroso-N-acetyl-DL-penicillamine (SNAP-1; 0.98-1000μM). After 72h of incubation at 25ºC, 20μl resazurin (0.0125% ) in PBS was added to each well and the plates were incubated as before for a further 18- 24h except for SIN-1 where incubation period is 1h. Cell viability was measured fluorometrically (excitation λ, 550 nm; emission λ, 590 nm). The results are expressed as the percentage reduction in the parasite viability compared to that in untreated control wells, and the 50% inhibitory concentration ($IC_{50}$) was calculated by Origin 6.0. All experiments were repeated twice in quadruplicates.

**Amastigote stress tests**

For amastigote stress tests, primary peritoneal macrophages were extracted from Balb /c mice and plated out at $2X10^5$ cells per 200μl per well in RPMI supplemented with 10% FCS in 16 well chamber slides incubated at $37^0$C, 5% $CO_2$ . Twenty-four hours later, medium was gently removed and the

macrophages attached to the slide were infected with late log promastigotes at a ratio of 10 parasites per macrophage in a volume of 200µl complete RPMI. After 24h of infection, non-internalised parasites were washed off and different dilutions of SNAP (0, 25, 50, 100, 200, 400 and 800µM), was applied in quadruplicate. After 48h of incubation, slides were fixed with methanol and stained with Diff-Quik solutions. The numbers of amastigote per cell were counted in 100 macrophages; the percent killing was calculated by sigmoidal regression analysis (Origin6.0).

For determination of mean parasite burden, the plated peritoneal macrophages after 24h of infection was incubated with different dilutions of IFN-Ý/LPS (0.1U/ml:0.1ng/ml to 50U/ml:50ng/ml) as described elsewhere (Carter *et al.* 2005). After 48h of incubation, slides were fixed with methanol and stained with Diff-Quik solutions. The numbers of amastigote per cell were counted in 100 macrophages to calculate the mean % infectivity levels at each dosage.

**Complement mediated cell lysis (CML)**

Human serum was isolated from blood drawn from naive persons. A serial dilution of the human serum (50-0.78%) was prepared in 96 well plates. For complement assay, $10^6$ stationary phase promastigotes/ml in M199 + 20% FBS were incubated at $37^0$C for 60 min in 96 well plate containing graded concentrations of human serum. After incubation, 40µl of cold EDTA was added to each well. 24µl of resazurin was added in each well and plate was incubated

for 24h at 25ºC. Fluorescence was measured. The percent killing was calculated by sigmoidal regression analysis (Origin6.0) (Ouakad *et al.* 2011).

### Cytokine analysis

Peritoneal macrophages stimulated with IFN-Ý / LPS were infected with late log phase promastigotes, after 48h of infection the supernatants were collected and IL-10 levels were checked by sandwich ELISA (e-Biosciences) in accordance with manufacturer's instructions.

## Results

### Development of PMM resistant Parasite

*L. donovani* parasite (BHU573cl-3 and BHU568cl-1) was exposed stepwise to increasing PMM pressure up to 97μM and stable PMM resistance could be achieved in 32 weeks. PMM-R parasites exhibit similar morphology in comparison with PMM-S.

### Growth pattern of PMM-R parasites

The resistant parasites exhibited growth kinetics similar to the wild type parasite (Figure 4.1).

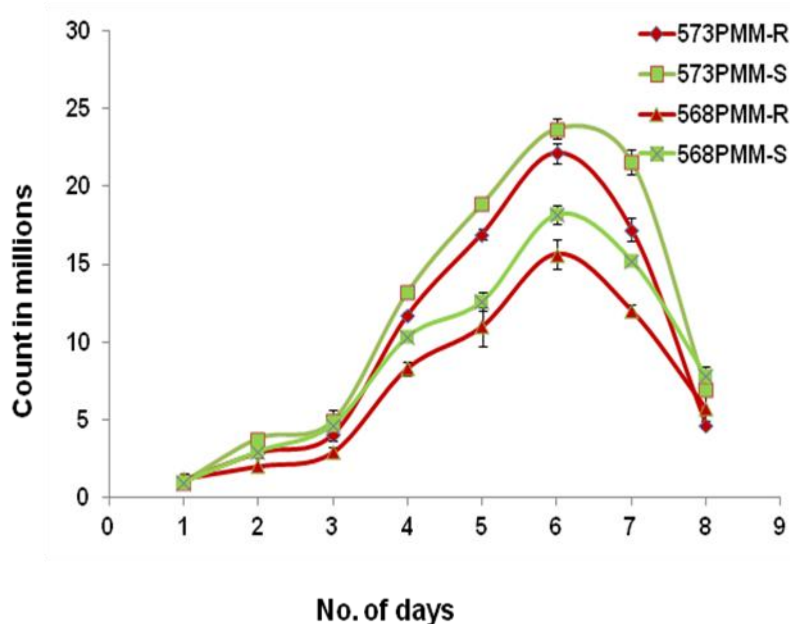


**Figure 4.1: Growth profile of cultured PMM resistant and wild type promastigotes in routine conditions for 8 days.** Wild type parasite (PMM-S) and parasite resistant up to 97μM PMM (PMM-R) were counted using hemocytometer

for eight days. Error bars indicate standard deviation of the mean (SD) based on 2 repeated experiments.

### *In vitro* infectivity studies of PMM-R parasites

The PMM-R infectivity in macrophages was assessed in comparison to the WT clone. This experiment mimics the initial stages of infection of the macrophage after transmission of *Leishmania* from the sandfly to the host. The infection levels of PMM-R parasites were similar at 48 and 72 h post infection and the percentage of infected macrophages at 72 h was comparable with 78.5 ± 1.4% for BHU573PMM-S and 83.3±2.82% for BHU573PMM-R parasites, likewise BHU568PMM-S (82.25±1.45%) and BHU568PMM-R (79.50±2.5%) also exhibited similar infectivity levels. The Diff-Quik stained PMM-R /PMM-S infected macrophages as observed at 100X magnification under oil immersion are depicted in Figure 4.2 A and B.

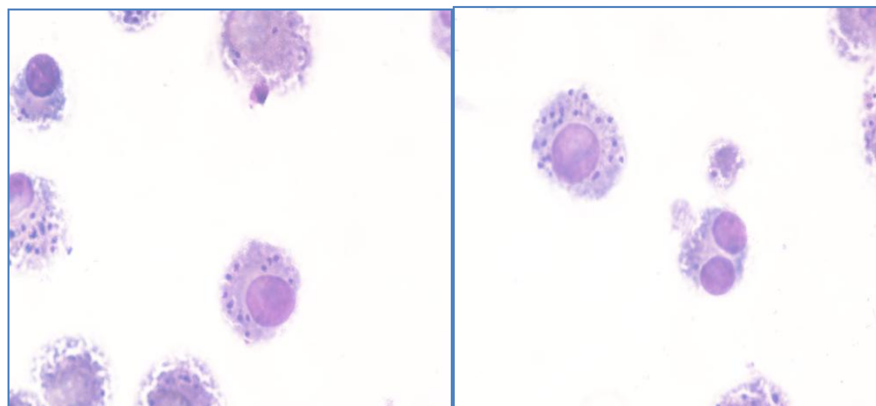

**Figure 4.2**: **Infectivity at 72h as determined by Diff-Quik staining**

(A) Wild type infected macrophages and (B) PMM-R infected macrophages

**Sensitivity profile of PMM-R strains at promastigote stage**

The PMM-R strains showed more than 9 fold increase in $IC_{50}$ value at promastigote level for both BHU573PMM-S & BHU568PMM-S. Mean$IC_{50}$ of BHU 573PMM-R strain was 334.2 ±25.13 μM which was 9.6 fold higher as compared to wild type BHU573PMM-S (Mean $IC_{50}$=34.6 ±1.08 μM). Similarly, mean $IC_{50}$ of BHU568PMM-R was 548.8 ± 93.54μM that was 11.6 fold higher as compared to WT parasite (mean $IC_{50}$=47.3 ± 14.1μM) (Table 4.2, Figure 4.3 ).

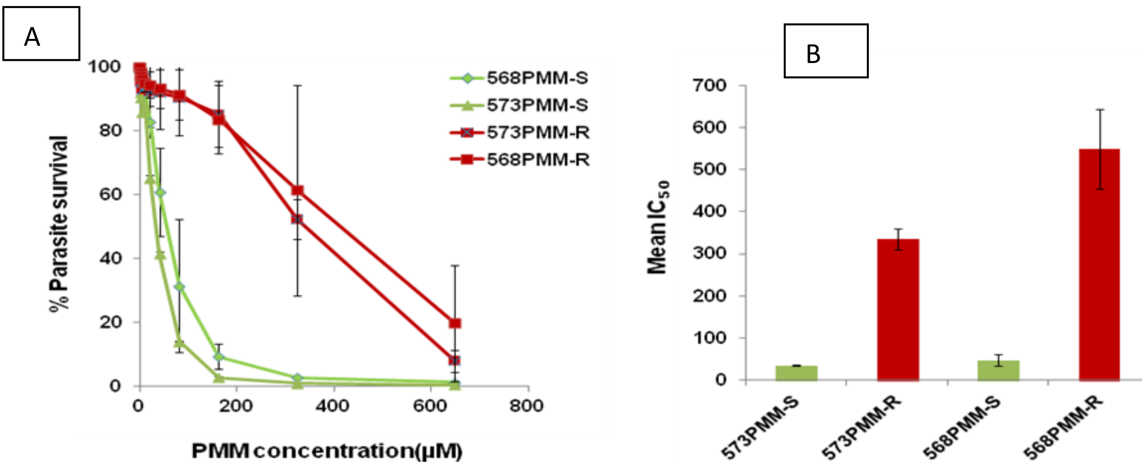


**Figure 4.3: Susceptibility of Paromomycin adapted parasite at promastigote stage.** (A) Dose response curve of the PMM-R parasite compared to sensitive parasite (PMM-S). (B)The mean $IC_{50}$ ±SD (μM) of the PMM-R parasite compared to control parasites.

**Comparative sensitivity profile of PMM-R strains at amastigote stage using peritoneum derived macrophages and J774A.1 cell line**

*In vitro* susceptibility at intracellular amastigote stage of BHU573PMM-R (mean $IC_{50}$=91.80±12.1) showed an increase of approximately 7 fold from WT

parasite (mean $IC_{50}$=13.09±0.48) using J774 cell line. Likewise, mean $IC_{50}$ of BHU568PMM-R strain was 83.06±10.7 approx. 6 fold higher as compared to WT (mean $IC_{50}$ = 14.20±0.61). The $IC_{50}$ was also determined in PECs which is considered a suitable model to test susceptibility. Similar fold difference was observed when susceptibility was determined at amastigote stage using PECs (Figure 4.4A & B, Table 4.2). Hence, resistance induced at promastigote stage was also evident at amastigote stage.

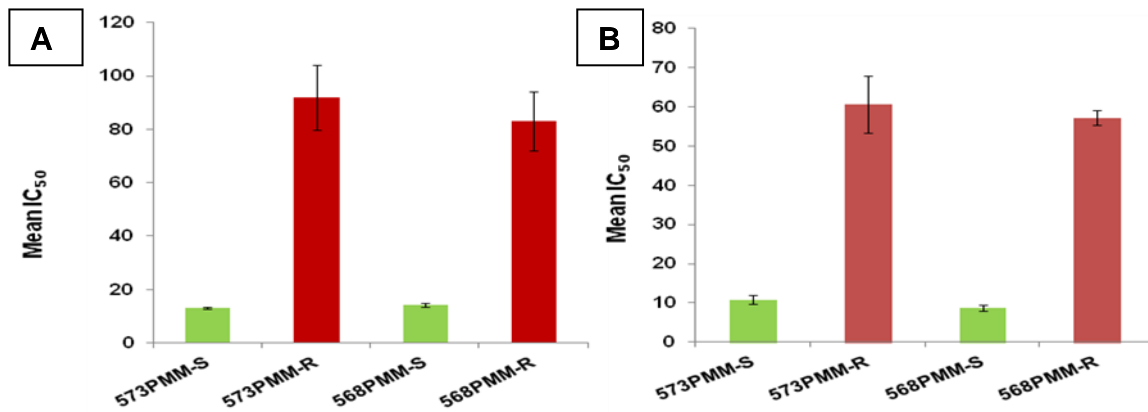


**Figure 4.4**: **Susceptibility of Paromomycin adapted parasite at amastigote stage.** The mean $IC_{50}$ ±SD (µM) of the PMM-R parasite compared to the WT at intracellular amastigote stage using (A) J774 A.1 mouse macrophage adherent cell line (B) Balb/c mice derived peritoneal macrophages.

**Table 4.2**: *In vitro* susceptibility of Paromomycin induced resistant parasites

| Parasite ID | $IC_{50}$Pro (μM±SD) | Fold increase | $IC_{50}$Ama (J774, μM±SD) | Fold increase | $IC_{50}$Ama (PECS μM±SD) | Fold increase |
|---|---|---|---|---|---|---|
| BHU568PMM-S | 47.3 ± 14.1 | **11.60** | 14.2 ± 0.61 | **5.85** | 8.82±0.77 | **6.49** |
| BHU 568PMM-R | 548.8 ± 93.54 | | 83.06±10.7 | | 57.21±1.82 | |
| BHU573PMM-S | 34.6 ±1.08 | **9.66** | 13.09±0.48 | **7.01** | 10.79±1.12 | **5.61** |
| BHU573 PMM-R | 334.2 ±25.13 | | 91.80±12.1 | | 57.21±1.82 | |

Foot note: Pro=promastigote, Ama=amastigote. $IC_{50}$ values represented are the mean of two independent experiments performed in quadruplicate for promastigote assay and in triplicates for amastigote assay.

**Sensitivity profile of PMM-R clonal population at promastigote stage**

The clonal population of PMM-R induced strains BHU573PMM-R showed an increase of 9-11 folds in $IC_{50}$ value at promastigote stage in comparison to the corresponding wild type 573PMM-S (Table 4.3). The susceptibility of PMM-R induced clonal population was identical. Similarly, the induced PMM-R clonal population of BHU568PMM-R isolates showed 8.5-11 fold decrease in susceptibility in comparison to wild type (Table 4.4).

**Sensitivity profile of PMM-R clonal population at amastigote stage**

BHU573PMM-R (mean $IC_{50}$=91.80±12.1) showed an increase of approximately 7 fold from WT parasite (mean $IC_{50}$=13.09±0.48). The clonal

population of BHU573PMM-R was homogenous and all clones showed an increase of approx. 6 fold in comparison to wild type (Table 4.3). Likewise, mean $IC_{50}$ of BHU568PMM-R strain was 83.06±10.7 approx. 6 fold higher as compared to WT (mean $IC_{50}$ = 14.20±0.61) and the clones also exhibited a similar sensitivity profile (Table 4.4).

Subsequent susceptibility profiling at both promastigote and amastigote stage revealed that the population is monoclonal and clones being resistant to PMM.

**Table 4.3**: Susceptibility profile of BHU573PMM-R clonal parasites

| S.No | Parasite ID | $IC_{50}$ PMM Pro(µM±SD) | $IC_{50}$ PMM Ama(µM±SD) |
|---|---|---|---|
| 1. | BHU 573 PMM-S | 34.6 ±1.08 | 13.09±0.48 |
| 2. | BHU573PMM-R | 334.2 ±25.13 | 91.80±12.1 |
| 3. | BHU573PMM-R Cl-1 | 375.5±10.45 | 83.8±1.69 |
| 4. | BHU573PMM-R Cl-2 | 414.0±28.90 | 89.05±1.67 |
| 5. | BHU573PMM-R Cl-3 | 391.4 ±12.10 | 85.45±2.90 |
| 6. | BHU573PMM-R Cl-4 | 346.9±10.10 | 88.35±0.91 |
| 7. | BHU573PMM-R Cl-5 | 367.0±18.90 | 83.72±0.96 |

Foot note: Pro= promastigote, Ama=amastigote. $IC_{50}$ values represented are the mean of two independent experiments performed in quadruplicate for promastigote assay and in triplicate for amastigote assay.

**Table 4.4**: Sensitivity profile of BHU568PMM-R clonal populations

| S.No | Parasite ID | $IC_{50}$ PMM Pro(µM±SD) | $IC_{50}$ PMM Ama(µM±SD) |
|---|---|---|---|
| 1. | BHU 568PMM-S | 47.3 ± 14.1 | 14.2 ± 0.61 |
| 2. | BHU568PMM-R | 548.8 ± 93.54 | 83.06±10.70 |
| 3. | BHU568PMM-R Cl-1 | 484.5±10.45 | 71.38±1.88 |
| 4. | BHU568PMM-R Cl-2 | 435.0±28.90 | 81.04±1.41 |
| 5. | BHU568PMM-R Cl-3 | 399.4 ±10.10 | 69.45±1.76 |

Foot note: Pro= promastigote, Ama=amastigote. $IC_{50}$ values represented are the mean of two independent experiments performed in quadruplicate for promastigote assay and in triplicate for amastigote assay.

**Cross-tolerance of PMM-R parasite towards other antileishmanial drugs.**

We evaluated the susceptibility of PMM resistant parasite PMM-R towards other antileishmanial drugs (SAG and MIL) at intracellular amastigote stage to determine if there are any changes in the susceptibility of the parasites towards these drugs compared to wild type parasite. The comparative sensitivity of the PMM-S and PMM-R line to other antileishmanial agents is reported in Table 4.5. Cross-resistance indices were determined by calculating the ratios of the $IC_{50}$ values of resistant lines to the WT. The susceptibility to various drug was

comparable and cross resistance index of parasites for various drug was close to one. Therefore, PMM-R does not show cross-tolerance towards other antileishmanial drugs (Table 4.5).

**Table 4.5: *In vitro* susceptibility of PMM adapted parasites towards other antileishmanial drugs**

| Isolate ID | SAG$IC_{50}$ (µg/ml±SD) | Cross Resistance Index | MIL $IC_{50}$ (µM±SD) | Cross resistance index |
|---|---|---|---|---|
| BHU573PMM-S | 26.13±0.74 | **1.10** | 6.73±0.66 | **0.91** |
| BHU573PMM-R | 29.01±1.28 | | 6.12±0.22 | |
| BHU568PMM-S | 25.13±0.34 | **0.94** | 3.70±0.28 | **1.19** |
| BHU568PMM-R | 23.56±0.45 | | 4.39±0.75 | |

Foot note: $IC_{50}$ values represented are the mean of two independent experiments performed in triplicate. Cross-resistance indices determined as ratios of $IC_{50}$ of resistant lines to the WT.

**Previously identified mutations of Paromomycin resistance**

The previous known mutations in rRNA gene which were suggested to be responsible for PMM resistance in *E. coli* was analyzed and additionally we sequenced the full gene to look for any other mutations. No previously known or any other mutations could be detected in PMM-R parasite as well as there was 100% similarity between PMM-S and PMM-R isolates (Figure 4.5).

```
PMM-S   TATTAATGCTGTTGCTGTTAAAGGGTTCGTAGTTGAACTGTGGGCTGTGCAGGTTTGTTC
PMM-R   TATTAATGCTGTTGCTGTTAAAGGGTTCGTAGTTGAACTGTGGGCTGTGCAGGTTTGTTC
        ************************************************************

PMM-S   CTGGTCGTCCCGTCCATGTCGGATTTGGTGACCCAGGCCCTTGCAGCCCGTGAACATTCA
PMM-R   CTGGTCGTCCCGTCCATGTCGGATTTGGTGACCCAGGCCCTTGCAGCCCGTGAACATTCA
        ************************************************************

PMM-S   AAGAAACAAGAAACACGGGAGTGGTTCCTTTCCTGATTTACGCATGTCATGCATGCCAGG
PMM-R   AAGAAACAAGAAACACGGGAGTGGTTCCTTTCCTGATTTACGCATGTCATGCATGCCAGG
        ************************************************************

PMM-S   GGGCGTCCGTGATTTTTTACTGTGACTAAAGAAGCGTGACTAAAGCAGTCATTTGACTTG
PMM-R   GGGCGTCCGTGATTTTTTACTGTGACTAAAGAAGCGTGACTAAAGCAGTCATTTGACTTG
        ************************************************************

PMM-S   AATTAGAAAGCATGGGATAACAAAGGAGCAGCCTCTAGGCTACCGTTTCGGCTTTTGTTG
PMM-R   AATTAGAAAGCATGGGATAACAAAGGAGCAGCCTCTAGGCTACCGTTTCGGCTTTTGTTG
        ************************************************************

PMM-S   GTTTTAAAGGTCTATTGGAGATTATGGAGCTGTGCGACAAGTGCTTTCCCATCGCAACCT
PMM-R   GTTTTAAAGGTCTATTGGAGATTATGGAGCTGTGCGACAAGTGCTTTCCCATCGCAACCT
        ************************************************************

PMM-S   CGGTTCGGTGTGTGGCGCCTTTGAGGGGTTTAGTGCGTCCGGTACGAGCTCCGGTTCGTC
PMM-R   CGGTTCGGTGTGTGGCGCCTTTGAGGGGTTTAGTGCGTCCGGTACGAGCTCCGGTTCGTC
        ************************************************************

PMM-S   CGGCCGTAACGCCTTTTCAACTCACGGCCTCTAGGAATGAAGGAGGGTAGTTCGGGGGAG
PMM-R   CGGCCGTAACGCCTTTTCAACTCACGGCCTCTAGGAATGAAGGAGGGTAGTTCGGGGGAG
        ************************************************************

PMM-S   AACGTACTGGGGCGTCAGAGGTGAAATTCTTAGACCGCACCAAGACGAACTACAGCGAAG
PMM-R   AACGTACTGGGGCGTCAGAGGTGAAATTCTTAGACCGCACCAAGACGAACTACAGCGAAG
        ************************************************************

PMM-S   GCATTCTTCAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGAGATCGAAGATGATTA
PMM-R   GCATTCTTCAAGGATACCTTCCTCAATCAAGAACCAAAGTGTGGAGATCGAAGATGATTA
        ************************************************************

PMM-S   GAGACCATTGTAGTCCACACTGCAAACGATGACACCCATGAATTGGGGATCTTATGGGCC
PMM-R   GAGACCATTGTAGTCCACACTGCAAACGATGACACCCATGAATTGGGGATCTTATGGGCC
        ************************************************************

PMM-S   GGCCTGCGGCAGGGTTTACCCTGTGTCAGCACCGCGCCCGCTTTTACCACCTTACGTATC
PMM-R   GGCCTGCGGCAGGGTTTACCCTGTGTCAGCACCGCGCCCGCTTTTACCACCTTACGTATC
        ************************************************************

PMM-S   TTTTCTATTCGGCCTTTACCGGCCACCCACGGGAATATCCTCAGCACGTTTTCTGTTTTT
PMM-R   TTTTCTATTCGGCCTTTACCGGCCACCCACGGGAATATCCTCAGCACGTTTTCTGTTTTT
        ************************************************************

PMM-S   TCACGCGAAAGCTTTGAGGTTACAGTCTCAGGGGGGGAGTACGTTCGCAAGAGTGAAACTT
PMM-R   TCACGCGAAAGCTTTGAGGTTACAGTCTCAGGGGGGGAGTACGTTCGCAAGAGTGAAACTT
        ************************************************************

PMM-S   AAAGAAATTGACGGAATGGCACCACAAGACGTGGAGCGTGCGGTTTAATTTGACTCAACA
PMM-R   AAAGAAATTGACGGAATGGCACCACAAGACGTGGAGCGTGCGGTTTAATTTGACTCAACA
        ************************************************************

PMM-S   CGGGGAACTTTACCAGATCCGGACAGGATGAGGATTGACAGATTGAGTGTTCTTTCTCGA
PMM-R   CGGGGAACTTTACCAGATCCGGACAGGATGAGGATTGACAGATTGAGTGTTCTTTCTCGA
        ************************************************************

PMM-S   TTCCCTGAATGGTGGTGCATGGCCGCTTTTGGTCGGTGGAGTGATTTGTTTGGTTGATTC
PMM-R   TTCCCTGAATGGTGGTGCATGGCCGCTTTTGGTCGGTGGAGTGATTTGTTTGGTTGATTC
        * **********************************************************

PMM-S   CGTCAACGGACGAGATCCAAGCTGCCCAGTAGAATTCAGAATTGCCCATAGGATAGCAAA
PMM-R   CGTCAACGGACGAGATCCAAGCTGCCCAGTAGAATTCAGAATTGCCCATAGGATAGCAAA
        ************************************************************

PMM-S   CTCATCGGCGGGTTTTACCCAACGGTGGGCCGCATTCGGTCGAATTCTTCTCTGCGGGAT
PMM-R   CTCATCGGCGGGTTTTACCCAACGGTGGGCCGCATTCGGTCGAATTCTTCTCTGCGGGAT
        ************************************************************

PMM-S   TCCTTTGTAATTGCACAAGGTGAAATTTTGGGCAACAGCAGGTCTGTGATGCTCCTCAAT
PMM-R   TCCTTTGTAATTGCACAAGGTGAAATTTTGGGCAACAGCAGGTCTGTGATGCTCCTCAAT
        ************************************************************

PMM-S   GTTCTGGGCGACACGCGCACTACAATGTCAGTGAGAACAAGAAAAACGACTTTTGTCGAA
PMM-R   GTTCTGGGCGACACGCGCACTACAATGTCAGTGAGAACAAGAAAAACGACTTTTGTCGAA
        ************************************************************

PMM-S   CCTACTTGATCAAAAGAGTGGGGAAACCCCGGAATCACATAGACTCACTTGGGACCGAGG
PMM-R   CCTACTTGATCAAAAGAGTGGGGAAACCCCGGAATCACATAGACTCACTTGGGACCGAGG
        ************************************************************

PMM-S   ATTGCAATTATTGGTCGCGCAACGAGGAATGTCTCGTAGGCGCAGCTCATCAAACTGTGC
PMM-R   ATTGCAATTATTGGTCGCGCAACGAGGAATGTCTCGTAGGCGCAGCTCATCAAACTGTGC
        ************************************************************

PMM-S   CGATTACGTCCCTGCCATTTGTACACACCGCCCGTCGTTGTTTCCGATGATGGTGCAATA
PMM-R   CGATTACGTCCCTGCCATTTGTACACACCGCCCGTCGTTGTTTCCGATGATGGTGCAATA
        ************************************************************

PMM-S   CAGGTGATCGGACAGGCGGTGTTTTATCCGCCCGAAAGTTCACCGATATTTCTTCAATAG
PMM-R   CAGGTGATCGGACAGGCGGTGTTTTATCCGCCCGAAAGTTCACCGATATTTCTTCAATAG
        ************************************************************

PMM-S   AGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTT
PMM-R   AGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTT
        **********************************************
```

**Figure 4.5: Multiple sequence alignment of LinJ.27.rRNA6 gene in PMM-R and PMM-S.**

**Increased membrane fluidity analysis**

The fluorescence anisotropy of PMM-R isolate was 2.5 times lower than WT indicating modifications within the isolate after induction of PMM resistance (Figure 4.6). As anisotropy is inversely proportional to membrane fluidity; the

membrane of PMM-R isolate is therefore more fluid as compared to PMM-S parasite.

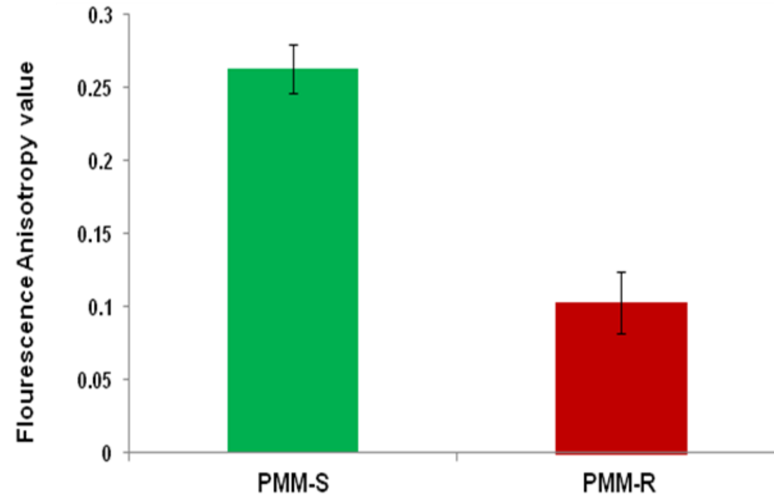


**Figure 4.6: Measurement of Fluorescence Anisotropy of the promastigotes of PMM-R strain**. The FA value was measured using DPH as probe. The fluorophore was excited at 365 nm, emission intensity was recorded at 435 nm for DPH using spectroflorimeter, and FA was calculated. Data represented to show the comparison of FA values between PMM-R and PMM-S isolates. Data represent the mean±SD of FA values from three independent experiments.

**Decreased drug accumulation**

The changes in the membrane of PMM-R isolate paved the way to further analyze the drug accumulation. Therefore, we compared the drug accumulation of PMM-R and PMM-S at different time points. There was an approx. 3 fold decrease of drug accumulation in PMM-R strain as compared to PMM-S parasite (Figure 4.7).

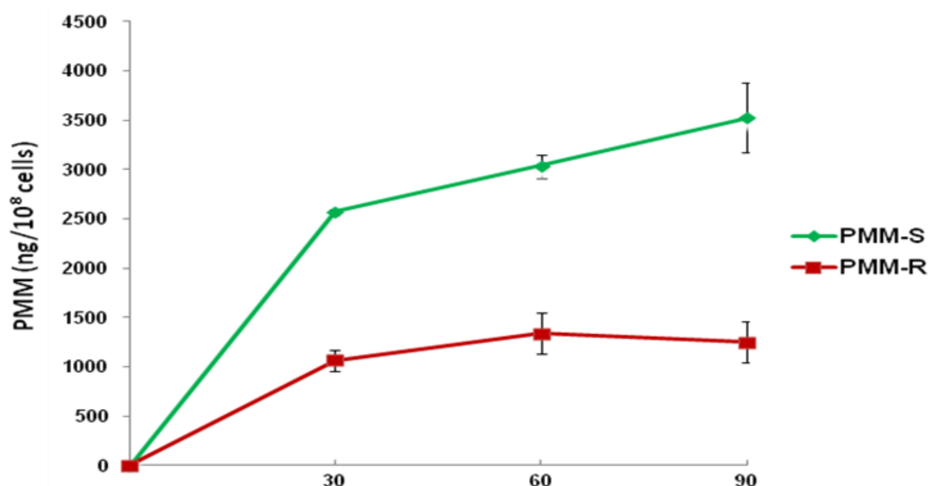


**Figure 4.7: Drug accumulation in PMM-S and PMM-R promastigotes.** PMM accumulation was analysed using LC-MS in PMM-R and PMM-S parasites after incubating with 100µM of PMM at different time points 0, 30, 60, 90 mins. Values given are mean ± SD of two different experiments.

### Expression analysis of genes involved in drug resistance

ABC gene family is well characterized and studied molecule in drug resistance of *Leishmania* here we had analyzed the expression of MDR1 and MRPA genes belonging to family of ABC transporter to understand their role in PMM resistance. MDR1 and MRPA were found to be up regulated by 6.83±3.01 and 11.47±0.22 fold respectively in PMM-R isolates. Protein phosphatase 2A was found to be 4.47±0.71 fold up in PMM-R parasite and has a suggestive role in phosphorylation of the transporter genes and thereby activating the expression of these transporters (Figure 4.8).

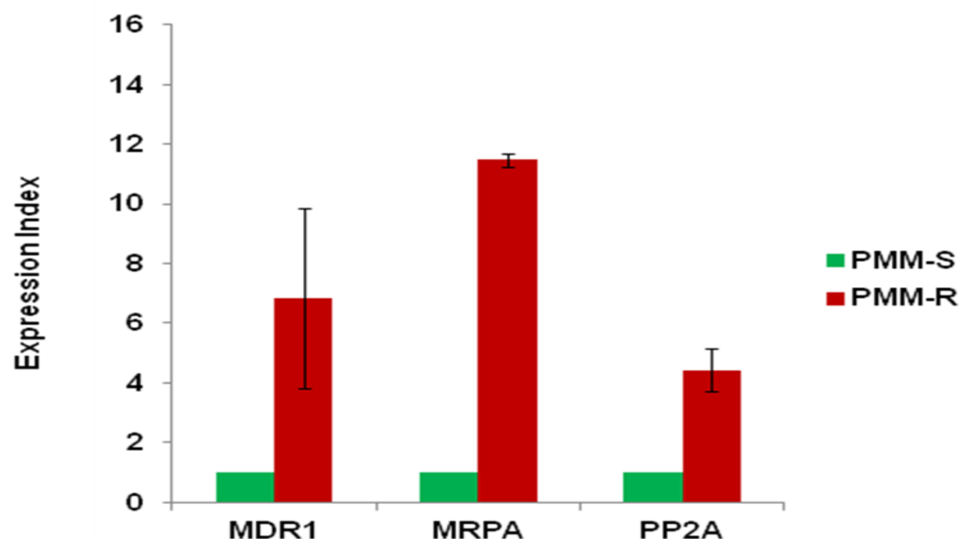


**Figure 4.8**: **Expression of MDR1, MRPA and PP2A in PMM-R isolate.**

Real-time PCR expression analysis of *L. donovani* ABC transporter (MDR1 & MRA) and protein phosphatase 2A gene (PP2A) was performed using GAPDH and CBS as internal control. Graph shows the expression index, defined as ratio of gene expression relative to that of PMM-S. Data represent the mean±SD of the results of three independent experiments.

***In vitro* promastigote stress test**

In this experiment, we compared the response of the PMM-R and PMM-S strain to various stresses that *Leishmania* encounters during their development in the sandfly gut and upon transmission to the host like nitrosative and oxidative stresses and complement lysis. The susceptibility of PMM-R and PMM-S strains towards $H_2O_2$ was similar with mean $IC_{50,}$ 176.03±12.27µM and 158.57±9.10µM respectively (Figure 4.9A). In contrast, PMM-R strain was more tolerant to SIN-1($NO+O_2$) donor and SNAP (NO donor) as compared to PMM-S isolate. The

mean $IC_{50}$ of PMM-R isolate was 646.14±36.57μM, 2 times more tolerant (P=0.045) to $NO+O_2$ stress and as compared to PMM-S strain with mean $IC_{50}$±SD= 328±4.24μM (Figure 4.9B). The PMM-R parasite was 9 fold more resistant (P=0.0048) to NO stress in comparison to PMM-S parasite with mean $IC_{50}$±SD of 476.58±1.06μM and 52.95±3.48μM respectively (Figure 5.5C).

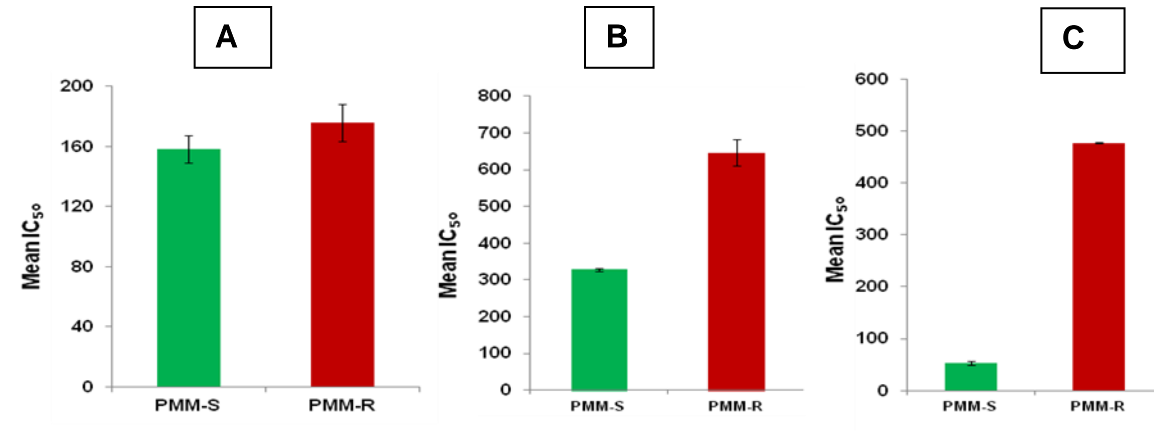


**Figure 4.9**: ***In vitro* susceptibility of PMM-R and PMM-S towards oxidative and nitrosative stresses at promastigote stage using resazurin assay.** A: Oxidative stress test, B: Nitrosative stress tests using SIN-1 and c) Nitrosative stress using SNAP. Each bar represents mean $IC_{50}$± SD of two independent experiments.

## ***In vitro* amastigote stress test**

The PMM-R isolate showed significantly low susceptibility (P=0.0028) towards nitrosative stress (NO) at amastigote stage as compared to PMM-S isolate with mean $IC_{50}$±SD, 88.12±5.17μM and 31.55±5.35μM respectively (Figure 4.10).

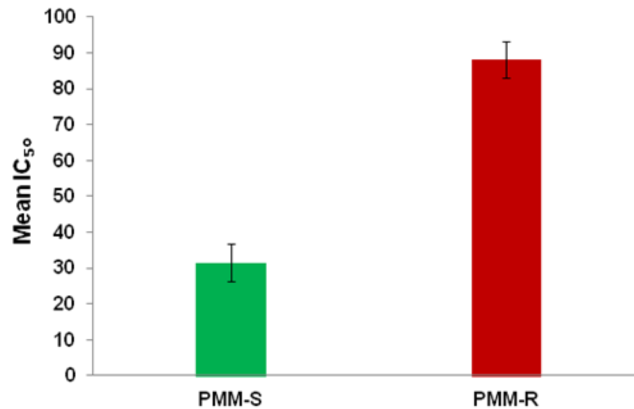


**Figure 4.10**: ***In vitro* susceptibility towards SNAP at amastigote stage**

Peritoneal macrophages infected with PMM-R and PMM-S strain were incubated for 48h with different dilutions of SNAP to determine the $IC_{50}$. Results are represented as mean $IC_{50}$±SD of two separate assays.

**PMM-R parasite more tolerant to complement mediated stress**

The PMM-R isolate showed a significantly high (P=0.005) tolerance to complement mediated lysis (mean $IC_{50}$=4.02±0.26%) when compared to PMM-S (mean $IC_{50}$=2.08±0.16%) indicating a higher survival capability as lysis by complement is one of the first immune mechanisms encountered by promastigotes upon inoculation by fly bite into the vertebrate host contributing to the fitness of PMM-R parasite (Figure 4.11).

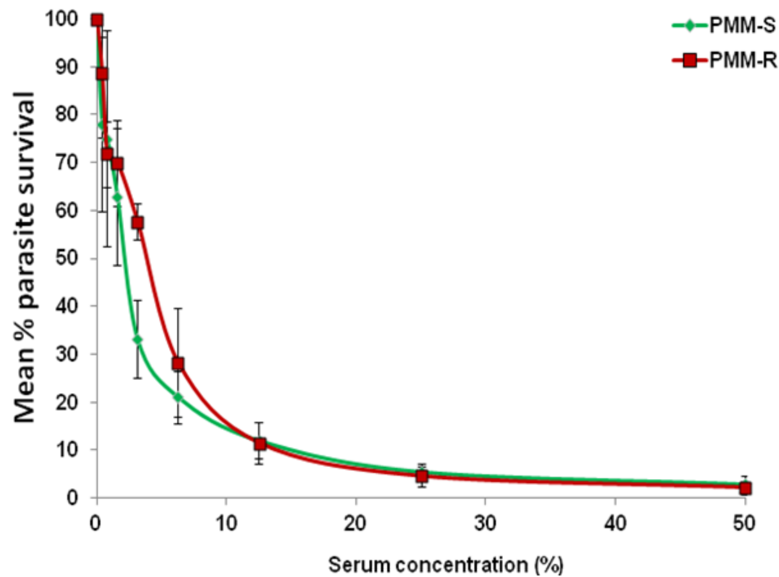


**Figure 4.11: Resistance to complement mediated lysis by PMM-R parasites**. Parasites were incubated with fresh human serum and percentage parasite survival was measured at different serum concentrations. The assay was performed thrice in triplicate. Values given are mean percentage survival ± SD.

**Increased IL-10 level in PMM-R infected macrophages**

We also assessed IL-10 level in supernatants of macrophages infected with PMM-R and PMM-S isolates. PMM-R isolate induces 1.8 times more IL-10 production in the host cells as compared to PMM-S parasite inhibiting immune response of the host and promoting parasite persistence or survival benefits to PMM-R strain as compared to PMM-S parasite (Figure 4.12).

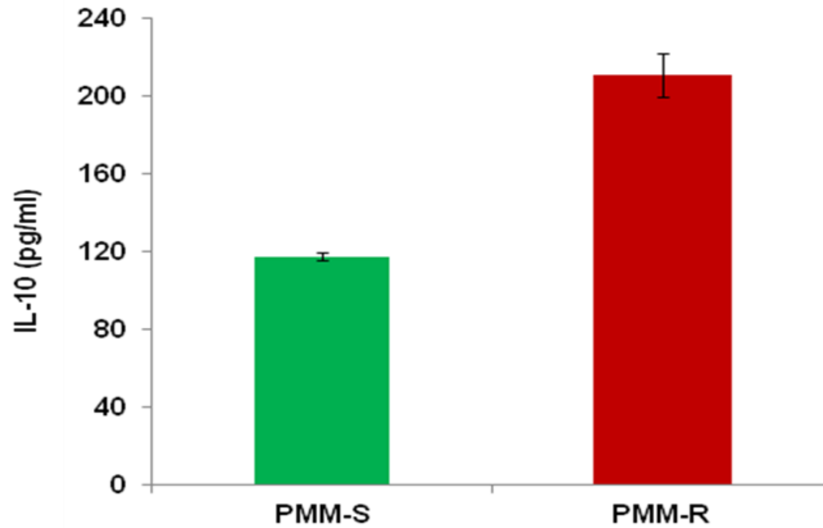


**Figure 4.12: Quantification of IL-10 levels.**

The IL-10 production in the supernatants of macrophages infected with PMM-S and PMM-R parasites was quantified by Elisa. The graph represents the mean ± SD of IL-10 produced in three independent experiments in triplicate

**Increased burden of PMM-R parasite in IFN-Ý/LPS stimulated macrophages**

Mean % infectivity of PMM-S and PMM-R isolates was determined in macrophages treated with different dilutions of IFN-Ý/ LPS (U/ml IFN γ: ng/ml LPS, 0.1, 1, 5, 10, 50). We observed relatively much decrease in parasite burden of PMM-S isolate as compared to PMM-R parasite at different concentrations of IFN-Ý/ LPS, the difference were significant ($P<0.05$) at concentration more than 5U/ml IFN: 5ng/ml LPS. This is indicative of resistance of PMM-R isolate to macrophage killing mechanisms (Figure 4.13).

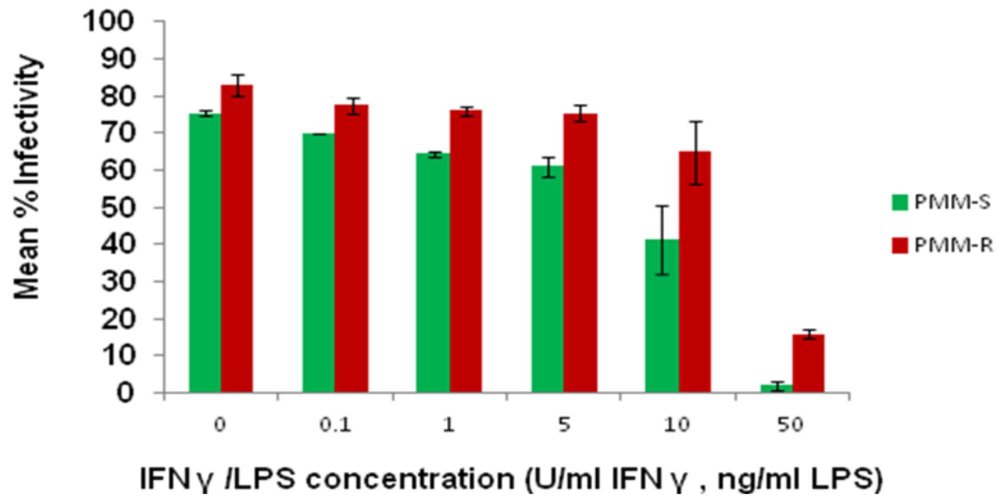


**Figure 4.13: Parasite burden of PMM-R and PMM-S in macrophages treated with different concentrations of IFN-Ý/LPS.**

The outcome of stimulus with IFN-Ý and LPS on parasite burdens of cells infected with a PMM-S or PMM-R strain was determined by infecting peritoneal macrophages with PMM-S and PMM-R promastigotes and then treating with different concentrations of IFN-Ý/ or left untreated (control). After 48h the percentage of cells infected for each treatment was determined. The graph represents mean% infectivity ±SD of two independent experiments in triplicate.

## Discussion

Paromomycin has already been extensively studied in clinical trials for its potential as monotherapy (Sundar *et al.* 2007a) or as combination therapy with other drugs (Olliaro et al. 2009; Meheus et al. 2010;). However, monotherapy holds a direct and enhanced risk for the development of drug-resistance, and even combination therapy is not devoid of risks particularly in foci where antimony resistance has already emerged. Hence, there is an immediate need to gain practical knowledge about PMM-resistance. Since PMM-resistant clinical isolates are not yet available, in the present study we induced PMM-resistance experimentally, considering drug selection pressure on the promastigote stage of antimony resistant parasites. PMM most likely may be used to treat patients unresponsive to SAG/ parasites belonging to hyper-endemic regions; therefore, two clinical isolates with established antimony-resistant background were used as parent strains for selection. Applying drug pressure to promastigotes in a stepwise manner resulted in resistance after 32 weeks producing levels that were 10 to 12-fold higher for both strains (Table 4.2). Similar to previous observations (Maarouf 1998), the resistant phenotype was maintained upon infection of macrophages, although not in a linear fashion. The growth rates of the PMM-S and PMM-R promastigotes were fully comparable (Figure 4.1). All clones showed comparable susceptibility to the selected parent strain representing a homogenous population (Table 4.3 & 4.4). The susceptibility profile for SAG and MIL remained unchanged (Table 4.5) but the stable selection of PMM resistance may indeed be a worrying observation. Therefore, it is necessary to understand

the mechanism of resistance to preserve the drug. The well defined PMM resistant lines were utilized to understand the mechanism of PMM resistance.

We observed increase in membrane fluidity of PMM-R isolate which indicated a change in the membrane composition of the parasite. Further, a decrease in the intracellular PMM content in the resistant parasite was observed in line with the previous findings which related the association of PMM resistance with decrease drug accumulation (Maarouf *et al.* 1998; Jhingran *et al.* 2009). In contrast to the previous study in PMM resistant *E. coli, we* did not observe any mutation in the *rRNA* gene of PMM-R isolate. ABC transporter genes are known to constitute the efflux system of the parasite and associated with drug resistance in diseases such as cancer and several infectious diseases (Higgins 1992; Gottesman *et al.* 2002;). The ABC transporters were found to be modulated in *Cryptosporidium parvum* in presence of PMM and cyclosporin A (Benitez *et al.* 2007); increased expression of these genes homologues of *Leishmania* (MDR1& MRPA) *was* found in PMM-R parasite. All these genes share a common fundamental structure composed of transmembrane domains and nucleotide binding domain. They are known to be involved in xenobiotic detoxification and they are several studies which suggest the role of MRPA and MDR1 in *Leishmania* drug resistance (Mookerjee *et al.* 2008; Purkait *et al.* 2012). Further, protein phosphatase 2A was also found to be up-regulated in PMM-R isolate whose increased expression confers PMM resistance in *Kluyveromyces lactis*. The bioinformatics' analysis of the *L. donovani* putative homolog of *Kluyveromyces lactis* protein phosphatase revealed it may modulate function of

transcriptional factors that activate expression of particular ABC transporter genes or may control the membrane targeting of drug transporter as well as regulate the drug pumping activity or substrate specificity of membrane transporter by dephosphorylating phosphor-serine/threonine residues. The design of modulators of transporters associated to drug resistance may provide a way to overcome PMM resistance in *Leishmania*.

We also investigated the proficiency of the PMM-R parasite against macrophage killing mechanisms. *Leishmania* parasites face numerous stresses when it reside in the gut of the sandfly or infect the macrophages and get transformed to amastigotes. Importance of reactive oxygen species and nitrogen species in the host defence system led to the hypothesis that resistance might be linked to tolerance to stresses imposed by the macrophage on the parasite as shown in other drug resistant isolates. Parasites are exposed to oxidative as well as nitrosative stresses upon phagocytosis by host macrophage, therefore, $H_2O_2$, SNAP and SIN-1 were used to impersonate the stress imposed by macrophages. The PMM-R promastigotes showed similar susceptibility towards oxidative stress in comparison to PMM-S isolate, with no difference in the tolerance level. In contrast, the tolerance of PMM-R parasite increased significantly against NO and NO + $O_2$ at promastigote as well as amastigote stage. The increased resistance of PMM-R strains to SNAP and SIN-1 may be a form of regulation of nitrosative stress response during promastigote growth. Overall, PMM-R isolate was tolerant to nitrosative stress which confers survival benefit for the parasite inside macrophages.

IFN-Ý secreted by macrophages, has leishmanicidal activity and it synergize to up-regulate production of reactive nitrogen intermediates. Therefore, we determined the mean% infectivity level of PMM-R and PMM-S parasite in macrophages incubated with different concentrations of IFN-Ý/LPS. We observed a dose dependent suppressive effect on both the PMM-R and PMM-S parasites however; PMM-R strain was significantly more resistant to the macrophage's antileishmanial killing mechanisms at higher stimulations (≥5 U/ml IFN γ, ng/ml LPS). IL-10 has a role in parasite persistance and inhibition of leishmanicidal activity, the supernatants of PMM-R parasite infected macrophages revealed an elevated level of IL-10. In addition, PMM-R isolate also resisted complement mediated lysis in comparison to PMM-S parasite.

The study showed that the PMM resistance in *Leishmania* is multifactorial and involves molecular as well as biochemical changes in the parasite. It led to the identification of intracellular targets such as efflux transporters and components of nitrosative stress defence mechanisms (reactive nitrogen species) ,which may be exploited to judiciously develop specific inhibitors. It also provided us with valuable information on association between drug resistance and fitness in *Leishmania*, which has been investigated in few organisms. In conclusion, these observations strongly endorse the need to adopt strong treatment policies to ensure long-term efficacy of PMM. Therefore, combination therapy is the policy to be developed in order to protect the novel antileishmanial agent from quickly becoming obsolete or if monotherapy is deployed then close monitoring would be essential in the field.

The surveillance of drug susceptibility and understanding the mechanism of resistance in the prevailing *Leishmania donovani* parasite population is a high priority due to the emergence of resistance to traditional antimony therapy and limited available alternatives for VL treatment. With successful completion of clinical trials of PMM confirming its safety and efficacy and recent introduction of MIL therapy for VL, our study focuses on determination of baseline data on the intrinsic sensitivity of prevailing *L.donovani* parasite population (unexposed to these drugs) and isolates obtained after MIL treatment (including relapse cases) towards PMM as well as MIL in order to correlate drug susceptibility with treatment outcome and study cross tolerance of the two drugs in parasite isolates. Additionally, PMM resistant lines were experimentally generated and characterized to understand the mechanism of PMM resistance and parasite fitness. These studies will help to steer decisions on epidemiological monitoring of drug resistance and future treatment approaches.

In the present study, we measured the *in vitro* susceptibility towards PMM and MIL in *L. donovani* isolated from VL and PKDL, pre- and post-treatment cases, using an amastigote-macrophage model. The present study revealed for the first time, the intrinsic *in vitro* sensitivity of Indian *L. donovani* isolates (at both pre treatment and post treatment stages) from a set of VL and PKDL patients treated with MIL. All VL/PKDL field isolates examined in the study (including both

responders and relapse cases) were found uniformly susceptible to PMM suggesting its potential efficacy in VL and PKDL therapy, whereas, VL/PKDL parasites obtained from the cases that relapsed after MIL treatment exhibited significantly reduced susceptibility to MIL. Both VL and PKDL isolates were found to be equally susceptible to PMM susceptibility. On the contrary, PKDL isolates were significantly more tolerant to MIL (~ 4 fold) as compared to VL isolates, representing a trend similar to SAG susceptibility of isolates from hyper-endemic regions.

Further, in an effort to understand the mechanism that contributes to PMM resistance in the parasite, we have generated and characterized two PMM resistant parasites by stepwise increase in drug pressure up to 97µM of PMM. The resistant lines showed up to approximately 10-12 fold decreased susceptibility to PMM compared to WT parasite at promastigote stage. The resistance induced at the promastigote stage was evident at the amastigote stage where PMM-R parasites exhibited more than 6 fold lower susceptibility than WT counterpart. We investigated mechanism of experimental PMM resistance in *Leishmania* such as drug accumulation, membrane fluidity, mutations in ribosomal rRNA gene and expression of various transporters genes involved in drug resistance using one PMM-R line. PMM-resistance in *Leishmania* leads to increased membrane fluidity accompanied by reduced drug accumulation. Full length gene sequencing of *L. donovani* rRNA gene failed to detect any mutations in the PMM-R strain of *L donovani*, unlike the reported observation in PMM resistant *E. coli*. Gene expression analysis in PMM-R

parasite revealed interesting changes in certain key molecules implicated in drug resistance. The expression of ATP binding cassette protein family genes (MDR1 & MRPA) responsible for efflux was significantly increased in PMM-R parasite. Additionally protein phosphatase 2A gene known to be responsible for activation of ABC transporter was also up-regulated.

Studies on the tolerance capacity of PMM resistant parasite towards host leishmanicidal mechanisms revealed that PMM resistant parasite were more tolerant to the nitrosative stress at both promastigote and amastigote stage, however, the tolerance to oxidative stress remained unaltered. The PMM resistant parasites also predicted a better persistence as indicated by resistance towards complement mediated lysis and increased IL-10 level in supernatants of macrophages infected with PMM-R strain. The susceptibility pattern of PMM resistant isolate towards other antileishmanial agents SAG and MIL remained unaltered. Therefore, the study concluded that increased membrane fluidity, decreased drug accumulation, increased expression of ATP binding cassette transporters and protein phosphatase 2A and better tolerance capacity of the parasite towards stresses imposed by host have a role in conferring PMM resistance in *Leishmania*.

## Future scope of work

The well characterized PMM-R parasite generated in the current study may be utilized for transcriptomic profiling, whole genome sequencing and proteomic/metabolomic analysis for an in depth understanding of the resistance mechanism. It will be of interest to undertake functional studies with genes modulated in resistance and pathways involved in the PMM resistance to understand the basis of PMM resistance in *Leishmania.*

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## ***Preparation of reagents***

### *10X MOPS*

0.2M MOPS pH 7.0, 20mM Sodium acetate, 10mM EDTA pH 8.0

Filter sterilized with 0.45μm filter.

### *DEPC water*

0.1% diethylpyrocarbonate was added to 1ltr double distilled water in a fume hood and mixed well. After incubating it for 1hr at 37°C it was autoclaved.

**20X SSC**

175.3gm of NaCl, 88.2gm of Sodium citrate were dissolved in 800ml distilled water. pH was set to 7.0 with HCl. Volume was adjusted to 1ltr, aliquots prepared and the solution was sterilized by autoclaving.

## Stock solution of commonly used reagents

1M Tris

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

**0.5M EDTA**

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

### *3M sodium acetate*

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

**10% SDS**

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

**Ethidium Bromide (10 mg/ml)**

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

**10 X TAE buffer (Tris acetate, EDTA)**

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

**10X TBE buffers (Tris borate, EDTA)**

8gm of Tris base, 55 gm of boric acid and 9.3gm $Na_2EDTA$. $H_2O$ were dissolved in 700ml dw and the final volume made up to 1 liter.

**Phosphate Buffer Saline (PBS)**

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

**DNA loading dye (6X)**

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

## Publications and Presentations

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### Publications

1. Kulshrestha A, **Bhandari V**, Mukhopadhyay R, V. Ramesh, Sundar S, Maes L, Dujardin J C, Roy S, Salotra P. Validation of a simple resazurin based promastigote assay for the routine monitoring of Miltefosine susceptibility in clinical isolates of *Leishmania donovani.* **Parasitol Res. 2013 Feb; 112(2):825-8**

2. Verma S, **Bhandari V**, Avishek K, Ramesh V, Salotra P. Reliable diagnosis of Post Kala-azar Dermal Leishmaniasis (PKDL) using slit aspirate specimen to avoid invasive sampling procedures. **Trop Med Int Health. 2012** doi:10.1111/tmi.12047

3. **Bhandari V**, Kulshrestha A, Deep DK, Stark O, Prajapati VK, Ramesh V, Sundar S, Schonian G, Dujardin JC, Salotra P. Drug susceptibility in Leishmania isolates following miltefosine treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis. **PLoS Negl Trop Dis. 2012**; 6(5):e1657.

4. Hendrickx S, Inocêncio da Luz RA, **Bhandari V**, Kuypers K, Shaw CD, Lonchamp J, Salotra P, Carter K, Sundar S, Rijal S, Dujardin JC, Cos P, Maes L. Experimental induction of paromomycin resistance in antimony-resistant strains of L. donovani: outcome dependent on in vitro selection protocol**. PLoS Negl Trop Dis. 2012**; 6(5):e1664.

**5.** Subba Raju BV, Gurumurthy S, Kuhls K, **Bhandari V**, Schnonian G, Salotra P. Genetic typing reveals monomorphism between antimony sensitive and resistant Leishmania donovani isolates from visceral leishmaniasis or post kala-azar dermal leishmaniasis cases in India. **Parasitol Res. 2012**; 111(4):1559-68.

**6.** Kumar D, Singh R, **Bhandari V**, Kulshrestha A, Negi NS, Salotra P. Biomarkers of antimony resistance: need for expression analysis of multiple genes to distinguish

resistance phenotype in clinical isolates of Leishmania donovani. **Parasitol Res. 2012**; 111(1):223-30.

7. **Bhandari V, Sundar S, Dujardin JC, Salotra P.** Experimental paromomycin resistance in *Leishmania donovani* is associated with increased membrane fluidity, drug efflux and parasite fitness. (Manuscript submitted)

**Presentations in National/International conferences**

1. Attended and participated in the Open dissemination meeting of European commission funded Kaladrug –R project on "New tools for monitoring drug resistance and treatment response in visceral leishmaniasis in the Indian subcontinent" held at Kathmandu, Nepal on 26th Sep, 2012.
2. Participated and presented work on "Molecular Diagnosis for Parasitic Diseases: Conventional and Real Time PCR Techniques" held at PGI, Chandigarh, India from 10-11 SEPTEMBER, 2012 Diagnostic at PGI Chandigarh, Sep9th 2012.
3. Presented work in Lab steering committee meeting of European funded Project Kaladrug-R in Institute of Tropical Medicine – Antwerp, Belgium on 19th June, 2012.
4. Invited participant at the Workshop on Metabolomics at Strathclyde University, Glasgow, UK, in June 2011.
5. Presented work in Lab steering committee meeting at Strathclyde University, Glasgow, UK in June, 2011.
6. Attended and presented poster in Symposium on "Neglected tropical diseases" held at Pasteur Institute, Paris from Sep 21-23rd, 2010.

**Abstracts in National/International conferences**

1. Kumar Avishek, Sandeep Verma, **Vasundhra Bhandari**, V Ramesh and Poonam Salotra. Use of Slit aspirate specimens to diagnose Post Kala-azar Dermal Leishmaniasis (PKDL): A non- invasive strategy. In Microcon 2012 **"36th National Conference of Indian Association of Medical Microbiologists"** held at Delhi, India from 22-25 Nov, 2012.
2. Deepak Kumar Deep, Arpita Kulshrestha, **Vasundhra Bhandari**, Vanila Sharma, Ruchi Singh, Poonam Salotra. Identification of Miltefosine resistance associated genes in *Leishmania donovani*. In Microcon 2012 **"36th National Conference of Indian Association of Medical Microbiologists"** held at Delhi, India from 22-25 Nov, 2012.
3. Poonam Salotra, Sandeep Verma, Kumar Avishek, **Vasundhra. Bhandari**, N.S. Negi, V. Ramesh. New tools for molecular diagnosis with simultaneous measurement of parasite burden in Kala Azar and Post Kala-azar Dermal Leishmaniasis. In PGI GOLDEN JUBILEE WORKSHOP on "Molecular Diagnosis for Parasitic Diseases: Conventional and Real Time PCR Techniques" held at PGI, Chandigarh, India from 10-11 Sep, 2012.
4. **Vasundhra Bhandari**, Arpita Kulshrestha, Dhiraj Kumar and Poonam Salotra. Drug susceptibility of Indian field isolates of L.donovani and development of experimental resistance towards Miltefosine and Paromomycin. In "Neglected Protozoan Diseases Conference" on held at Pasteur Institute, Paris, France from September 22nd -23rd 2010.
5. Arpita Kulshrestha, Lonchamp J., Inocêncio da Luz R., **Bhandari V**., Sundar S., Rijal S., Dujardin J.C. Maes L., Carter K.C., Coombs G. and Salotra P.

Natural susceptibility of Leishmania donovani isolates from Indian subcontinent towards Miltefosine and impact of SSG resistance background on development of Miltefosine resistance. In “Neglected Protozoan Diseases Conference” held at Pasteur Institute, Paris, France from September 22nd -23rd 2010.

## Biography

**Name** Vasundhra Bhandari

**Date of Birth** 01 Nov' 1984

**Educational qualification**

| Examination Passed | Board/University | Percentage | Year of passing |
|---|---|---|---|
| B.Sc (Life Sciences) | Delhi University | 65 | 2005 |
| M.Sc Biochemistry | Jiwaji University, Gwalior | 68 | 2007 |

**Publications**

**8.** Kulshrestha A, **Bhandari V**, Mukhopadhyay R, V. Ramesh, Sundar S, Maes L, Dujardin J C, Roy S, Salotra P. Validation of a simple resazurin based promastigote assay for the routine monitoring of Miltefosine susceptibility in clinical isolates of *Leishmania donovani.* **Parasitol Res. 2013**; 112:825-828.

9. Verma S, **Bhandari V**, Avishek K, Ramesh V, Salotra P. Reliable diagnosis of Post Kala-azar Dermal Leishmaniasis (PKDL) using slit aspirate specimen to avoid invasive sampling procedures. **Trop Med Int Health. 2012** doi:10.1111/tmi.12047.

10. **Bhandari V**, Kulshrestha A, Deep DK, Stark O, Prajapati VK, Ramesh V, Sundar S, Schonian G, Dujardin JC, Salotra P. Drug susceptibility in Leishmania isolates following miltefosine treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis. **PLoS Negl Trop Dis. 2012**; 6(5):e1657.

11. Hendrickx S, Inocêncio da Luz RA, **Bhandari V**, Kuypers K, Shaw CD, Lonchamp J, Salotra P, Carter K, Sundar S, Rijal S, Dujardin JC, Cos P, Maes L. Experimental induction of paromomycin resistance in antimony-resistant strains of L. donovani: outcome dependent on in vitro selection protocol**. PLoS Negl Trop Dis. 2012**; 6(5):e1664.

**12.** Subba Raju BV, Gurumurthy S, Kuhls K, **Bhandari V**, Schnonian G, Salotra P. Genetic typing reveals monomorphism between antimony sensitive and resistant Leishmania donovani isolates from visceral leishmaniasis or post kala-azar dermal leishmaniasis cases in India**. Parasitol Res. 2012**; 111(4):1559-68.

**13.** Kumar D, Singh R, **Bhandari V**, Kulshrestha A, Negi NS, Salotra P. Biomarkers of antimony resistance: need for expression analysis of multiple genes to distinguish resistance phenotype in clinical isolates of Leishmania donovani. **Parasitol Res. 2012**; 111(1):223-30.

14. **Bhandari V, Sundar S, Dujardin JC, Salotra P.** Experimental paromomycin resistance in *Leishmania donovani* is associated with increased membrane fluidity, drug efflux and parasite fitness. (Manuscript submitted).

## Academic achievements

1. Won the **Sri Ramachari Young scientist award** held at National Institute of Pathology, ICMR, New Delhi, India on May 15$^{th}$ 2012.

2. Won **Travel grant award** among all the research scholars working on European commission funded projects to attend and participate on the Symposium on Neglected Tropical diseases held at Pasteur Institute, Paris, France on 24$^{th}$ Sep, 2010.

3. Was among the 22 participants selected all over the world for the **Wellcome Trust Advance Course**, organized by Wellcome Trust Sanger Institute, UK and held at ICGEB, New Delhi between $16^{th}$ to $21^{st}$ Jan' 2011.

4. Was awarded **SRF** in a European Commission funded Kaladrug-R project on April, 2010.

5. Was awarded **JRF** in a European commission funded Kaladrug-R project on March, 2009.

6. Won **Gold medal** at the state level in junior science research talent program held at New Delhi, India in 2001.

## Workshops / Conferences/ meetings

7. Attended and participated in the Open dissemination meeting of European commission funded Kaladrug –R project on "New tools for monitoring drug resistance and treatment response in visceral leishmaniasis in the Indian subcontinent" held at Kathmandu, Nepal on $26^{th}$ Sep, 2012.

8. Presented work in Lab steering committee meeting of European project Kaladrug-R held at Kathmandu, Nepal from 23-$25^{th}$ Sep, 2012.

9. Attended workshop and presented work on "Molecular Diagnosis for Parasitic Diseases: Conventional and Real Time PCR Techniques" held at PGI, Chandigarh, India from 10-11 SEPTEMBER, 2012 Diagnostic at PGI Chandigarh, Sep9th 2012.

10. Presented work in Lab steering committee meeting of European funded Project Kaladrug-R in Institute of Tropical Medicine – Antwerp, Belgium on $19^{th}$ June, 2012.

11. Invited participant at the Workshop on Metabolomics at Strathclyde University, Glasgow, UK, in June 2011.

12. Presented work in Lab steering committee meeting at Strathclyde University, Glasgow, UK in June, 2011.

13. Was among the 22 participants selected all over the world for the Wellcome Trust Advance Course, organized by Wellcome Trust Sanger Institute, UK held at ICGEB, New Delhi between $16^{th}$ to $21^{st}$ Jan ' 2011.

14. Attended and presented poster in Symposium on "Neglected tropical diseases" held at Pasteur Institute, Paris from Sep 21-$23^{rd}$, 2010.

15. Attended 4th World Congress on Leishmaniasis (WL4) held during $3^{rd}$ to $7^{th}$ Feb'09 at Lucknow, India.

**Work experience**

1. Worked as ICMR-SRF from April 2010 to February 2013 towards my PhD problem "Studies on drug susceptibility of Indian *Leishmania donovani* isolates to Paromomycin" under the Supervision of Dr. Poonam Salotra, Deputy Director, National Institute of Pathology (ICMR), New Delhi.

2. Worked as ICMR -JRF from May 2008 to March 2010 towards my PhD problem under the Supervision of Dr. Poonam Salotra, Deputy Director, National Institute of Pathology (ICMR), New Delhi.

**Personal Particulars**


- Name Poonam Salotra
- Designation : Deputy Director
- Place of work : National Institute of Pathology (ICMR), Safdarjung Hospital Campus, New Delhi – 110 029, India.

- Tel. No: 26198402, 26166124
- Fax No: 26166124
- E. mail: salotra@vsnl.com, salotrap@icmr.org.in


**Academic Qualifications**

- B.Sc. Hons (Chemistry) from Delhi University in 1974.
- M.Sc. (Biochemistry) from P.G.I., Chandigarh in 1976.
- Ph.D. from V.P. Chest Institute, Delhi University in 1980.

**Professional Appointments:**

- Postdoctoral Fellow at Roche Institute of Molecular Biology, Nutley, New Jersey, U.S.A. (1980).
- Research Officer at Tuberculosis Research Centre, Madras (1982).
- Principal Investigator under Young Scientists scheme of Department of Science & Technology at Centre for Biotechnology, J NU, New Delhi (1991).
- Pool Research Officer (CSIR) at Centre for Biotechnology, JNU, New Delhi (1993).
- Senior Research Officer at Institute of Pathology (ICMR), New Delhi (1996).
- Assistant Director at Institute of Pathology (ICMR), New Delhi (2000).
- Deputy Director at Institute of Pathology (ICMR), New Delhi (2005 till date)

**Research Interests:**

- Worked mainly on the molecular basis of pathogenesis of infectious diseases such as Kala-azar, Anthrax, Tuberculosis and Cholera. Currently working on development of

diagnostic tests and attenuated vaccines for kala-azar, mechanism of drug resistance in Indian kala-azar, and characterization of immune responses in patients of kala-azar and post kala-azar dermal leishmaniasis.

**Awards/Honours**

1. Elected Fellow of the Indian National Science Academy, in the year 2011.
2. Member of the WHO expert committee meeting on "Control of Leishmaniaisis" in 2010.
3. Elected Fellow of the National Academy of Sciences, India, in the year 2008.
4. Basanti Devi Amir Chand Award conferred by ICMR in 2007.
5. Prof. BK Aikat Award conferred by Indian Council of Medical Research conferred by ICMR in 2007.
6. ICMR International fellowship for Senior Biomedical Scientists for the year 2006.
7. Awarded Courtesy Fellowship by CBER, FDA, USA in Sep 2005.
8. Awarded fellowship by National Foundation of Infectious Diseases, USA in 2005.
9. Granted ICMR Award for Excellent Research output in July 2004.
10. Silver Jubilee award by Indian Association of Medical Microbiology in 2003.
11. Awarded Courtesy Fellowship by CBER, FDA, USA in Dec 2003
12. Kshanika Oration Award, a National award for Eminent Woman Scientist, conferred by Indian Council of Medical Research in 2002.
13. National Science Talent Scholarship awarded by N.C.E.R.T. , New Delhi

**Patents**

1. Awarded US Patent No. 6,855,522, in 2005, for "Species-specific PCR assay for detection of Leishmania donovani in clinical samples of kala-azar and post kala-azar dermal leishmaniasis".
2. US patent no. 20060240046 for "Live attenuated *Leishmania* vaccines"

## Membership of professional associations

1. Society for Parasitology, India (Life member).

2. Society of Biological Chemists, India (Life member).

3. Association of Clinical Biochemists of India (Life member)

4. Indian Association of Medical Microbiologists, India (Life member

5. Indian Immunology Society, India (Life member).

**Publications**

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| Publications in indexed foreign journals | 76 |
| Publications in indexed Indian journals | 07 |
| Publications in Proceedings | 60 |
| Chapter in Books | 07 |
| Total | 157 |