Chapter 6: Screening of antimony -susceptible and -resistant isolates of
*L. donovani* from Visceral and Post-kala-azar dermal leishmaniasis
patients for potential biomarkers.
1.0 Introduction

The protozoan parasite *Leishmania* is the causative agent of kala-azar and is responsible for a variety of clinical manifestations. It causes a wide spectrum of diseases ranging from the simple self-healing cutaneous form to the debilitating visceral form. Visceral leishmaniasis (VL) is caused by *L. donovani* in the Indian sub-continent. Pentavalent antimonials (SbV) are the first line of drug used in the treatment against all forms of leishmanial infections (Guerin et al., 2002). Resistance to this drug is becoming a major barrier in the treatment of VL in many endemic regions particularly in India (Sundar et al., 2000). Kala-azar transmission in India is thought to be anthroponotic and post-kala-azar dermal leishmaniasis (PKDL) patients are considered to serve as a source for new outbreaks (Thakur and Kumar, 1992). The Post-kala-azar dermal leishmaniasis (PKDL) is a sequel to VL in India and Sudan; the disease develops months to years after the patient’s recovery from VL (Zijlstra et al., 2003).

The mechanism of action of sodium antimony gluconate (SAG) remained an enigma for more than 60 years of its effective use against all forms of leishmaniasis. It is generally agreed that pentavalent form (SbV) is reduced to a more toxic trivalent form (SbIII) which constitutes the active form of the drug against the parasite (Roberts et al., 1995). Molecules possibly implicated in reduction of SbV to SbIII include host and parasite thiols and two newly discovered parasite enzymes thiol-dependent reductase (TDR1) and arsenate reductase (ACR2) (Ferreira et al., 2003; Zhou et al., 2004; Denton et al., 2004). A loss of drug activation is reported to lead to resistance (Shaked-Mishan et al., 2001). The route of entry of SbV into *Leishmania* cells is still unknown but SbIII has been reported to be transported in *Leishmania* through aquaglyceroporin (AQP1) (Gourbal et al., 2004; Marquis et al., 2005). Recent evidence has suggested that part of the mode of action of SbV could be in depleting the cells of its reduced thiols (Wyllie et al., 2004). Trypanothione (TSH), a major reduced thiol of *Leishmania* is a N₁, N₈ bisglutathione spermidine conjugate. It is thought to bind to the active reduced form of the metal. These metal-trypanothione conjugates are either sequestered into an intracellular organelle by the ABC transporter MRPA or extruded outside the cell by an efflux pump (Fairlamb and Cerami, 1992; Dey et al., 1996; Legare et al., 2001; Wyllie et al., 2004). A number of candidate genes associated with the increased thiol concentration
have been described in Leishmania laboratory mutants. Resistance was induced in these mutants in vitro in the presence of metals such as arsenic or antimony (Mukhopadhyay et al., 1996; Legare et al., 1997; Haimeur et al., 2000).

However, till date it remains unclear as to whether similar mechanisms can be extrapolated to clinical isolates from geographical zones with a high incidence of primary antimony resistance. To address this question, we have characterized both the VL and the PKDL isolates from India and report that diverse mechanisms of resistance are operative in these isolates. This work aims at characterizing possible biomarkers for monitoring antimonial resistant Visceral leishmaniasis and Post-kala-azar dermal leishmaniasis in the field isolates.

In the present study, we report the role of thiols and also assessed the role of ABC transporter (MRPA), ornithine decarboxylase (ODC) and γ-glutamylcysteine synthetase (γ-GCS) genes as potential biomarkers for monitoring antimonial resistance in Indian leishmaniasis.

2.0 Materials and Methods
2.1 Parasite and culture conditions
Promastigotes of L. donovani clones, AG83 (MHOM/IN/80/AG83), 2001, MC4, MC7, MC8 and MC9 were isolated from patients with VL. Strains RK1, MS2, NR3A, RMP8 (HM/IN/RMP-8), RMP19 (HM/IN/RMP-19), RMP142 (HM/IN/RMP-142), RMP155 (HM/IN/RMP-155) and RMP240 (HM/IN/RMP-240) used in the present study were isolated from patients with PKDL (Dey and Singh, 2007). Clinical isolates obtained from VL and PKDL patients who responded to SAG chemotherapy were designated as SAG-S (SAG-sensitive) whereas isolates from VL and PKDL patients who did not respond to SAG were designated as SAG-R (SAG-resistant). SAG-sensitive strains, AG83-S, 2001-S, MC7-S, RK1-S, MS2-S and the nine SAG-R isolates, MC4-R MC8-R, MC9-R, NR3A-R, RMP8-R, RMP19-R, RMP142-R, RMP155-R and RMP240-R have been characterized earlier (Mandal et al., 2010). Clinical history of the patient infected with the strain RK1-S showed that the interval between the cure of VL and the onset of PKDL was 2.5 years where as in the case of PKDL patients infected with strains MS2-S and NR3A-R, the interval between the cure of VL and the onset of PKDL was 7 and 11 years respectively. The interval between the cure of VL and the onset of PKDL for the
remaining isolates is not known. The clinical isolates were maintained in vitro in the absence of the drug pressure. Promastigotes were routinely cultured at 22 °C in modified M-199 medium (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco/BRL, Life Technologies Scotland, UK) and 0.13 mg/mL penicillin and streptomycin. This study has the approval of the Institutional level ethics committee.

2.2 DNA and RNA manipulations
Chromosomes of the clinical isolates were separated by pulse field gel electrophoresis (PFGE) in which low melting agarose blocks, containing embedded cells (10^8/ml log phase promastigotes) were electrophoresed in a contour clamped homogeneous electric field apparatus (CHEF DRIII, Bio-Rad) as reported earlier (Mandai et al., 2010). Mid log phase promastigotes (~2 x 10^9 cells) of all the field isolates were used for isolation of genomic DNA. 5 µg of genomic DNA was digested with HindIII enzyme and subjected to electrophoresis. Total RNA was isolated from promastigotes (2 x 10^8 cells) using RNeasy Plus Mini Kit (Qiagen). Standard protocols were followed for Southern or northern hybridization (Sambrook et al., 1989). DNA probes used in the present study included a 400-bp MRPA fragment (released from plasmid PM12 that was digested with BamHI and PstI), a 2.3-kb γGCS fragment (derived from plasmid psphygroα-γGCS digested with HindIII and XbaI), a 2.0-kb ODC-full length probe (derived from plasmid psphygroα-ODC digested with HindIII and XbaI) and a 1.6-kb 5'-PTR1 probe derived from plasmid (psp72-Y-hygro-5'-PTR1).

2.3 cDNA synthesis and real time RT-PCR
Total RNA was isolated from 10^8 Leishmania promastigotes in the mid-log phase of growth using the RNaseasy Plus Mini Kit (Qiagen) as described by the manufacturer. Quality and quantity of the RNA were determined using the RNA 6000 Nano Lab chip kit on the Bio-analyzer 2100 (Agilent Technologies). The sequences of the primers for MRPA are forward 5’-GCGCAGCGCTTTGGCTTGTTGG-3’ and reverse 5’-TTGCCGTCGTCGATGGGTGC-3’ and for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control forward 5’-GAAGTACACGGTGGAGGCTG and reverse 5’-CGCTGATCACGACCTTCTTC primers. The sequences of the primers for ODC are forward 5’-GATGGTGCGCCCTTACTTTGC-3’ and reverse 5’-TTCCATCTCCAGCGGCTTG-3’, and for the γGCS, forward 5’-
CATGGGCTGGCGCGTTGAGTTC-3' and reverse 5'-ATGTGCGGGGCCCATATTCTCG-3' primer. Complementary DNAs from promastigotes were synthesized from 500 ng of total RNA using the AccuSuperscript High Fidelity RT-PCR kit (Stratagene, La Jolla) and Oligo (dT)$_{18}$ primers following manufacturer's instructions. Real-time PCR was performed in triplicate in 25 μl volumes using QuantiFast SYBR Green PCR Master Mix (Qiagen) in an Applied Biosystem 7500. Reactions were run using the following thermal profile: initial denaturation at 95 °C for 5 min followed by 40 cycles with denaturation at 95 °C for 30 s, annealing at 62 °C for 1 min and extension at 72 °C for 20 s. The PCR was followed by a melt curve analysis to ascertain that the expected products were amplified. The relative amount of PCR products generated from each primer set was determined based on the threshold cycle (Ct) value and amplification efficiencies and was normalized by dividing the values by the relative amount of the GAPDH gene used as a control.

2.4 Transfection and overexpression of the MRPA and γ-GCS gene

Episomal Leishmania expression vectors, pGL-αNEOαLUC containing luciferase encoding DNA and neomycin phosphotransferase selectable marker, pspαhygroα-γGCS containing coding sequence for heavy subunit of γGCS with hygromycin phosphotransferase as selectable marker and pGEM72f-aneα-MRP A containing MRPA coding DNA and neomycin phosphotransferase as selectable marker were gifted by Prof. Marc Ouellette, Centre de Recherche en Infectiologie du Centre de Recherche du CHUL, Universite Laval, Quebec, Canada. Twenty μg of each construct was transfected into L. donovani promastigotes by electroporation. Electroporation was done with a single pulse with the following parameters 450 V, 500 μF (Bio-Rad). Transfectants were selected for resistance to either G418 (40 μg/ml) or hygromycin B (80 μg/ml) as described in materials and methods section (Roy et al., 2000).

2.5 Chemosensitivity profiles of SAG-S and SAG-R strains in an amastigote macrophage model

Stationary phase Leishmania promastigotes expressing the luciferase gene (pGL-αNEOαLUC) and pspαhygroα-γGCS containing coding sequence for heavy subunit of γGCS with hygromycin phosphotransferase as selectable marker or pGEM72f-aneα-MRP A containing MRPA coding DNA and neomycin phosphotransferase as selectable
marker were infected into J774A.1 macrophages. Macrophage cell line J774A.1 (American Type Culture Collection) was maintained at 37°C, 5% CO₂ in RPMI-1640 medium (Sigma) containing 10% heat inactivated fetal bovine serum. Briefly, J774A.1 murine macrophages (1 x 10⁵ cells/ petri dish) were infected with 1 x 10⁶ promastigotes (expressing the luciferase gene (pGL-αNEOαLUC) which were maintained in M199 media with 10% FBS. After 3 h, the non-internalized parasites were washed off and SAG was added at different concentrations (10–100 µg/ml). After 5 days of drug exposure, plates containing adherent macrophages were washed and luciferase activity was determined (Roy et al., 2000). The 50% inhibitory concentration (IC₅₀) was determined from the graph representing different concentrations of SAG plotted against relative light units (RLU) produced by luciferase expressing parasites.

2.6 Thiol analysis
Thiols were derivatized with monobromobimane and separated by high-performance liquid chromatography (HPLC) as described earlier (Mukhopadhyay et al., 1996).

2.7 Statistical analysis
Data was analyzed by the Student’s t-test. The data is represented as mean ± S.D. The results are representative of three independent experiments. A p value of < 0.05 was considered statistically significant.

3.0 Results
3.1 Intracellular thiol levels in SAG-S and SAG-R clinical isolates
Resistance to antimonials in clinical isolates is not well defined. Understanding the mechanism of resistance to antimony in clinical isolates of L. donovani will aid in the development of biomarkers for antimony resistance and this in turn will enable the clinicians to monitor the treatment of the patients. With this background in mind, we used the previously characterized SAG-S and SAG-R, VL and PKDL clinical isolates for the present study (Mandal et al., 2010).

A total of fourteen field isolates were used to assess the putative role of the ABC transporter MRPA, ornithine decarboxylase and γ-glutamylcysteine synthetase in antimony -susceptible and -resistant clinical isolates of L. donovani from India. As reported earlier, SAG-S isolates, AG83-S, 2001-S, MC7-S, RK1-S and MS2-S coming from SAG responsive patients had IC₅₀ values 6.2 ± 1.8, 0.9 ± 0.12, 8 ± 3.3, 0.01 ± 0.02
and 4.75 ± 0.12 μM respectively whereas the SAG-R isolates, MC4-R, MC8-R, MC9-R, NR3A-R, RMP8-R, RMP19-R, RMP142-R, RMP155-R, RMP240-R coming from the SAG-unresponsive patients had IC_{50}s that were ~2 to >10 fold higher than that of the sensitive isolate, AG83-S (Mandal et al., 2010). RK1-S was the most sensitive of all the isolates with an IC_{50} of 0.01 ± 0.02 μM.

We had earlier reported polymorphism in these L. donovani clinical isolates as assessed by pulse field gel electrophoresis (PFGE) and PCR-RFLP of the ITS region (Mandal et al., 2010). Interestingly, restriction digestion pattern of the total genomic DNA from all these isolates with either HindIII or SalI showed a different pattern in RK1-S when compared to the restriction pattern in other isolates.

Intracellular thiol levels were quantified in the SAG-S and SAG-R isolates (Figure 1, 2 & 3). SAG-R isolates maintained significantly higher levels of cysteine and glutathione as compared to the SAG-S isolates. Cysteine levels in the SAG resistant strains MC4-R, MC8-R, MC9-R, NR3A-R, RMP8-R, RMP19-R, RMP142-R, RMP155-R and RMP240-R were ~1.7, ~1.6, ~1.5, ~2.7, ~2.0, ~1.8, ~1.8, ~2.0 and ~1.7 -fold higher respectively when compared to the SAG-S isolate, 2001-S (Figure 1). However, the levels of cysteine in RK1-S, a SAG-S isolate were ~1.6 fold more than the SAG-S isolate, 2001-S (Figure 1).

Similarly GSH levels showed significant increase in the SAG-R isolates, MC4-R (~2.7-fold), MC8-R (~3.6-fold), MC9-R (~3.0-fold), NR3A-R (~2.0-fold), RMP8-R (~3.2-fold), RMP19-R (~2.5-fold), RMP142-R (~3.1-fold), RMP155-R (~2.4-fold) and RMP240-R (~3.0-fold) when compared to the SAG sensitive isolate, 2001-S (Figure 2). A SAG-S strain, RK1-S had glutathione levels that were ~2.1 -fold higher when compared to the SAG sensitive isolate, 2001-S. Interestingly, no significant difference was observed in the trypanothione levels between the SAG-S and SAG-R isolates (Figure 3). Similar observation was made in our earlier study using a small set of clinical isolates (Mukherjee et al., 2007).
Screening of antimony-susceptible and -resistant isolates of *L. donovani*

**Figure 1:** Intracellular levels of cysteine in SAG-S and SAG-R *L. donovani* VL and PKDL clinical isolates. Thiols were derivatized with monobromobimane and separated by high-performance liquid chromatography (HPLC). Each value is the mean ± SD of triplicates from two independent experiments. a indicates *p* < 0.01; b indicates *p* < 0.001 when compared to 2001-S respectively.
Screening of antimony-susceptible and -resistant isolates of *L. donovani*

Figure 2: Intracellular levels of Glutathione in SAG-S and SAG-R *L. donovani* VL and PKDL clinical isolates. Thiols were derivatized with monobromobimane and separated by high-performance liquid chromatography (HPLC). Each value is the mean ± SD of triplicates from two independent experiments. a indicates *p* < 0.01; b indicates *p* < 0.001; c indicates *p* < 0.0004 when compared to 2001-S respectively.
Screening of antimony-susceptible and -resistant isolates of *L. donovani*

**Figure 3:** Intracellular levels of Trypanothione in SAG-S and SAG-R *L. donovani* VL and PKDL clinical isolates. Thiols were derivatized with monobromobimane and separated by high-performance liquid chromatography (HPLC). Each value is the mean ± SD of triplicates from two independent experiments.
Screening of antimony-susceptible and -resistant isolates of *L. donovani*

**Figure 4 A:** Southern blot analysis of MRPA and PTR1 genes in SAG-S and SAG-R *L. donovani* VL and PKDL clinical isolates. Total genomic DNA of isolates were digested with *Hind*III, electrophoresed, blotted and hybridized with a MRPA specific probe of 400-bp and 1.6 kb 5'-PTR1 specific probe. The size of the hybridizing bands was determined using *Hind*III digested lambda DNA. The blot was rehybridized with α-tubulin probe to monitor the amount of digested DNA layered on the gel. Quantitation of Southern blot was done by Image-quant software 5.2 (Molecular dynamics) and the fold difference in DNA copy number of MRPA is presented below each blot.

**Figure 4 B:** Southern blot analysis of the pulse field gel electrophoresis of SAG-sensitive (SAG-S) and SAG-resistant (SAG-R) isolates of *Leishmania donovani* chromosomes. Agarose blocks containing chromosomal DNAs of promatigotes of *L. donovani* clinical isolates were prepared and subjected to pulsed field gel electrophoresis for 24 hours run time and hybridized with a MRPA specific probe of 400-bp.
Screening of antimony-susceptible and -resistant isolates of L. donovani

Figure 5: Real time RT-PCR expression analysis of MRPA gene in L. donovani clinical isolates. MRPA RNA expression ratios in promastigotes of SAG-resistant isolates are relative to the SAG-sensitive isolate (2001-S). The graph represents mean of three independent experiments performed from three different RNA preparations.
3.2 Gene copy number and expression profiling of the ABC transporter MRPA in SAG-S and SAG-R clinical isolates

Our earlier work on the clinical kala azar L. donovani isolates from India showed MRPA overexpression as an important SAG resistance factor (Mukherjee et al., 2007). To further validate, the role of MRPA gene in antimony resistance phenotype, we checked the amplification of MRPA gene by Southern blot hybridization. Southern blot hybridization of total genomic DNA digested with HindIII followed by hybridization with MRPA specific probe was done. SAG-sensitive and SAG-resistant field isolates showed a single hybridizing fragment of 11-kb indicating that MRPA gene exist as a single copy gene in all the isolates (Figure 4A).

Quantitation of the Southern blot hybridization signal was done using Image-Quant software 5.2 (Molecular Dynamics) and the fold difference in DNA copy number of the isolates with the AG83-S or 2001-S was calculated. Amplification of MRPA gene was observed in the resistant isolates, MC4-R, MC8-R, MC9-R, RMP19-R, RMP142-R, RMP155-R and RMP240-R (Figure 4A). No amplification was observed in the resistant isolates, NR3A-R and RMP8-R (Figure 4A). The amplification observed in the resistant isolates was further analyzed by PFGE (Figure 4B). None of the resistant isolates showed circular amplification as was observed in our earlier studies with limited number of isolates (Mukherjee et al., 2007). Interestingly, two sensitive PKDL isolates, RK1-S and MS2-S and one resistant PKDL isolate, NR3A-R had the presence of MRPA on two chromosomes as indicated by their characteristic migration in PFGE (Figure 4B). We had earlier reported co-amplification of pterin reductase gene (PTR1) with MRPA in the clinical isolates (Mukherjee et al., 2007). In the present study, co-amplification of PTR1 gene with MRPA gene was not observed in any of the clinical isolates as determined by Southern-blot analysis using PTR1-specific probe (Figure 4A).

Comparison of MRPA gene expression in SAG-S versus SAG-R field isolates was done to verify if there is any correlation between MRPA gene expression and SAG susceptibility profile of the clinical isolates. Total RNA from promastigotes of the clinical isolates was isolated and complementary DNAs were synthesized. Real-time PCR using Quanti Fast SYBR Green PCR Master Mix (Qiagen) with MRPA (gene specific) and GAPDH (internal control) primers was performed. Up-regulation of MRPA
expression was observed in the resistant clinical isolates. *MRPA* expression in the resistant strains, MC4-R, MC8-R, MC9-R, NR3A-R, RMP8-R, RMP19-R, RMP142-R, RMP155-R and RMP240-R was ~4-, ~4.2-, ~9.6-, ~7.2-, ~6.1-, ~6.8-, ~6.0-, ~7.5- and ~4.4- fold more respectively when compared to the expression in the sensitive isolate, 2001-S (Figure 5).

3.3 Gene copy number and expression profiling of the ornithine decarboxylase (*ODC*) gene in SAG-S and SAG-R clinical isolates

Ornithine decarboxylase (*ODC*) is the rate-limiting enzyme of the polyamine biosynthetic pathway. In addition to *MRPA*, overexpression of the *ODC* gene has been reported in the antimony resistant mutants (Grondin et al., 1997; Haimeur et al., 1999; Mukherjee et al., 2007). These observations prompted us to determine if amplification of the *ODC* gene occurred in our clinical isolates. Southern blot analysis of the total genomic DNA digested with *HindIII* and hybridized with the *ODC* specific probe was done. SAG-S and SAG-R isolates showed a single copy of the *ODC* gene (Figure 6A) with the exception of three PKDL isolates, RK1-S, MS2-S and NR3A-R. Interestingly, two copies of the *ODC* gene were observed in these three PKDL isolates (Figure 6A).

Comparison of the *ODC* gene expression in SAG-S versus SAG-R field isolates was done to verify if there is any correlation between the gene expression and SAG susceptibility profile of the clinical isolates. Real-time PCR with the *ODC* (gene specific) and the *GAPDH* (internal control) primers was performed. Up-regulation of the *ODC* expression was observed in the resistant clinical isolates. *ODC* expression in the resistant strains, MC4-R, MC8-R, MC9-R, NR3A-R, RMP8-R, RMP19-R, RMP142-R, RMP155-R and RMP240-R was ~3.6-, ~7.4-, ~3.1-, ~2.7-, ~2.3-, ~3.3-, ~3.5-, ~2.8- and ~3.0- fold more respectively when compared to the sensitive isolate, 2001-S (Figure 6B). SAG-S isolate, RK1-S was an exception since it was the only sensitive isolate that showed 2.9-fold up-regulation when compared to the reference strain, 2001-S and also in comparison with all other SAG-S isolates (Figure 6B). No uniform correlation was observed between gene amplification and *ODC* gene expression in these isolates.
A

**Figure 6 A:** Characterization of *ODC* gene in SAG-S and SAG-R *L. donovani* VL and PKDL clinical isolates. Genomic DNAs were isolated and digested with *HindIII* and hybridized with a full-length *ODC* specific probe, derived from the *L. donovani* *ODC* gene. The sizes of the hybridizing bands were determined using *HindIII* digested lambda DNA marker. The blot was rehybridized with α-tubulin probe to monitor the amount of digested DNA layered on the gel. B: Real time RT-PCR expression analysis of *ODC* in *L. donovani* clinical isolates. *ODC* RNA expression ratios in the SAG-resistant isolates are relative to the SAG-sensitive isolate, 2001-S. Results are mean of three independent experiments performed from three different RNA preparations.
3.4 Gene copy number and expression profiling of the γ-glutamylcysteine synthetase (γ-GCS) gene in SAG-S and SAG-R clinical isolates

In addition to MRPA and ODC, another locus that has been reported to be amplified in the antimony-resistant isolates is the GSHI gene coding for the heavy subunit of γ-GCS. γ-GCS is the rate limiting enzyme for GSH synthesis (Dey et al., 1996; Mukherjee et al., 2007). We performed Southern blot analysis of the total genomic DNA digested with HindIII and hybridized with the γ-GCS specific probe. Southern blot analysis of the γ-GCS gene showed two copies in a resistant isolate MC4-R but in all other isolates, γ-GCS probe hybridized to a single hybridizing fragment of 10-kb (Figure 7A). SAG-S isolate, RK1-S was again an exception since it showed amplification of the γ-GCS gene (Figure 7A).

Comparison of the γGCS gene expression in SAG-S versus SAG-R field isolates was done to verify if there is any correlation between gene expression and SAG sensitivity profile of the clinical isolates. Real-time PCR using γ-GCS (gene specific) and GAPDH (internal control) primers was performed. Up-regulation of the γ-GCS expression was observed in the resistant clinical isolates. γ-GCS expression in the resistant strains, MC4-R, MC8-R, MC9-R, NR3A-R, RMP8-R, RMP19-R, RMP142-R, RMP155-R and RMP240-R was 4.3-, 5.2-, 6.4-, 5.5-, 3.0-, 5.0-, 5.3-, 4.2- and 8.8-fold more respectively compared to the expression in the sensitive isolate, 2001-S (Figure 7B). In the present study though up-regulation of γ-GCS was observed in all SAG-R isolates, interestingly one SAG-S isolate, RK1-S showed 5.5-fold up-regulation of γGCS expression when compared to a SAG-S isolate, 2001-S (Figure 7B). L-butathion sulfone-sulfoxamine (BSO), an inhibitor of γ-GCS was used to compare its effect on the SAG-S isolate, RK1-S and 2001-S. RK1-S promastigotes over-expressing γ-GCS were ~5 fold more resistant to BSO when compared to the IC_{50} of promastigotes of the 2001-S, the IC_{50} being 6.5 ± 0.5 mM and 1.3 ± 0.3 mM respectively.
Screening of antimony-susceptible and -resistant isolates of *L. donovani*

**Figure 7 A:** Southern blot analysis of the γ-GCS gene in the SAG-sensitive and the SAG-resistant *Leishmania donovani* VL and PKDL field isolates. Total genomic DNA was isolated and digested with *Hind*III. The digested DNA was electrophoresed, blotted and hybridized with γ-GCS probe. The sizes of the hybridizing bands were determined using *Hind*III digested λ- DNA. The blot was rehybridized with α-tubulin probe to monitor the amount of digested DNA layered on the gel. **B:** Real time RT-PCR expression analysis of γ-GCS gene in *L. donovani* clinical isolates. γ-GCS gene expression ratios in the SAG-resistant isolates are relative to the SAG-sensitive isolate, 2001-S. Results are mean of three independent experiments performed from three different RNA preparations.
3.5 Overexpression of MRPA or γ-GCS in an antimony-sensitive isolate conferred increased expression and resistance to antimony

To determine whether overexpression of MRPA and γ-GCS conferred antimony resistance in a sensitive isolate, we transfected MRPA or γ-GCS constructs into a SAG-S isolate, AG83-S.

**Figure 8:** Over-expression of MRPA and γ-GCS gene in a sensitive L. donovani isolate. A: Transfection of MRPA in SAG-sensitive isolate leads to resistance to antimony in amastigotes. AG83-S (■) was transfected with pGL-αNEOαLUC, AG83-S + LdMRPA (○) was transfected with pGEM72f-αneoαMRPA + pGL-αNEOαLUC and sensitivity to SAG in the J774A.1 line was measured as described in Materials and methods. Each data point represents the mean ± SD of three determinations. Northern blot analysis (insets in A) of vector transfected controls and MRPA transfected lines was performed as described under methods section. B: Transfection of γ-GCS gene in SAG-sensitive isolate leads to antimony resistance in intracellular amastigotes. AG83-S (■) transfected with pGL-αNEOαLUC, AG83-S + Ldγ-GCS (○) transfected with pspohygroγGCS + pGL-αNEOαLUC and sensitivity to SAG measured as described in Materials and methods. Each data point represents the mean ± SD of three determinations. Northern blot analysis (insets in B) of vector transfected controls and γ-GCS transfected lines was performed as described under methods section.
These MRPA and γ-GCS recombinant parasites were co-transfected with pGL-αNEOαLUC, encoding LUC. Intracellular amastigotes over-expressing MRPA (IC₅₀, 32.3 ± 6.4 µg/ml) were 3.6-fold more resistant to SAG when compared to amastigotes of the parent strain transfected with the control vector (IC₅₀, 9 ± 0.5 µg/ml) (Figure 8A). Intracellular amastigotes over-expressing γ-GCS were 3.3-fold resistant to SAG when compared to the sensitive L. donovani, the IC₅₀ value being (29.7 ± 1.5 µg/ml) respectively (Figure 8B). In our previous studies we had demonstrated that the ODC overexpressors exhibited significant resistance to Pentostam compared to the wild type cells (Singh et al., 2007). Intracellular amastigotes over-expressing ODC (IC₅₀ > 80 µg/ml) were >8.8-fold more resistant to SAG when compared to amastigotes of the parent strain transfected with the control vector (Singh et al., 2007).

4.0 Discussion
Currently, chemotherapy is the only effective way to control Leishmania infection. Pentavalent antimonials are the mainstay of therapy in the treatment of visceral leishmaniasis (den Boer et al., 2009). Increase in resistance to SAG has led to an upsurge in therapeutic failure and in the absence of limited chemotherapeutic alternatives, it is extremely necessary to identify biomarkers for monitoring antimony resistance.

Trivalent form of the antimonial drug (SbIII) is the prodrug that is formed by conversion of pentavalent antimony (SbV) by a putative metalloid reductase present in the macrophages (Sereno et al., 1998). Antimonial resistance in both laboratory mutants and clinical isolates has been associated with [a] decreased uptake of the drug through aquaglyceroporin (AQP1) that codes for the protein responsible for SbIII transport (Gourbal et al., 2004; Marquis et al., 2005; Maharjan et al., 2008; Mandal et al., 2010). [b] Over expression of ODC and γ-GCS enzymes of the trypanothione biosynthetic pathway (Haimeur et al., 1999; Haimeur et al., 2000). and [c] increased expression of the ABC transporter MRPA, which sequesters SbIII-thiol conjugate (Legare et al., 2001; Mukherjee et al., 2007).

We had earlier reported decreased uptake of antimony in all nine SAG-R isolates used in the present study. Down-regulation of SbIII influx pump; aquaglyceroporin (AQP1) was observed in seven out of the nine resistant isolates. Strains, MC8-R and
NR3A-R were an exception since they showed up-regulation of \textit{AQP1} gene expression (Mandal et al., 2010).

The ABC transporter gene \textit{MRPA} causes drug sequestration in \textit{Leishmania} promastigotes and amastigotes selected for SbIII resistance (Legare et al., 1997; El et al., 2005). Increased copy number of \textit{MRPA} has been reported in SbIII resistant mutant (Haimeur et al., 2000). Our earlier studies using limited number of SAG-S and SAG-R clinical isolates showed amplification of \textit{MRPA} gene as part of an extrachromosomal circle (Mukherjee et al., 2007). In the present study, we found distinct correlation between copy number of \textit{MRPA} gene and antimony sensitivity in a majority of the isolates (Figure 2A). However, two SAG-R strains, NR3A-R and RMP8-R were an exception. We have reported earlier that ABC transporter gene \textit{MRPA} was amplified in three out of four resistant VL isolates as part of the extrachromosomal circle and co-amplification of \textit{PTR1} along with \textit{MRPA} suggested amplification of the H locus in SAG-resistant clinical isolates (Mukherjee et al., 2007). In the present study, co-amplification of \textit{PTR1} gene with \textit{MRPA} gene was not observed in any of the clinical isolates. CHEF gel analysis of the SAG-S and SAG-R isolates did not show any circular amplification in any of the SAG-R isolates. \textit{MRPA} gene expression in SAG-S versus SAG-R field isolates showed correlation between \textit{MRPA} gene expression and SAG susceptibility profile of the clinical isolates. The present observation validated our earlier results where we have shown correlation between \textit{MRPA} gene expression with the antimony resistant clinical profile in the field conditions (Mukherjee et al., 2007).

In our previous study amplification of \textit{ODC} gene was noted in the resistant isolates but not that of the \textit{\gamma-GCS} (Mukherjee et al., 2007). In another study on \textit{L. donovani} isolates from Nepal, expression of \textit{\gamma-GCS} and \textit{ODC} was significantly decreased in the resistant isolates (Decuypere et al., 2005). In the present study we observed increased expression of \textit{ODC} gene and \textit{\gamma-GCS} in all SAG-R isolates.

It has been reported earlier that ABC transporter \textit{MRPA}, confers resistance to antimonials by sequestration of metal thiol conjugates in an intracellular organelle located close to the flagellar pocket (Legare et al., 2001). This model has been demonstrated in promastigotes of \textit{L. tarentolae}, in amastigotes and also in clinical isolates from India (Legare et al., 2001; El et al., 2005; Mukherjee et al., 2007). These
observations clearly highlighted the importance of intracellular thiol in MRPA mediated efflux of the antimony. An increase in cysteine and glutathione levels were reported in antimony resistant *L. donovani* clinical isolates (Mukherjee et al., 2007). In the present study also, we observed increase in cysteine and glutathione levels in all SAG-R isolates. However, no change in trypanothione levels were observed in SAG-R isolates in comparison to SAG-S isolates. Similar observation was made in our analysis of the mode of action of antimony in clinical isolates and earlier studies in *L. infantum* resistant to Sb(III) (Mukherjee et al., 2007). It was pointed out that antimony possibly depleted trypanothione by efflux of Sb-trypanothione conjugate (Legare et al., 2001). It is possible that the efflux system is increased in the resistant isolates thereby leading to increased trypanothione efflux. This would explain the constant levels of trypanothione in the present study in the resistant isolates.

RK1-S though a SAG-S isolate, was an exception to the multifactorial antimony resistant mechanisms reported in clinical isolates and also in the lab based resistant isolates. Clinical history of the RK1-S patient showed that the interval between the cure of VL and the onset of PKDL was 2.5 years where as MS2-S and NR3A-R, PKDL isolates the interval between the cure of VL and the onset of PKDL was 7 and 11 years respectively. It will be interesting to look at PKDL isolates with known clinical history in order to determine if this interval has a role to play in antimony susceptibility/resistance.

The data presented here in VL and for the first time in PKDL isolates, establishes the relevance of overexpression of \(\gamma\)-GCS and *ODC* and an increased expression of *MRPA* that may be responsible for an increased efflux of thiol-Sb-III conjugate. Our data further confirms that resistance mechanisms present in the laboratory strains can be found in the clinical isolates. Further work is presently going on the laboratory to get a global overview of the resistant mechanism by proteomics approach in order to find other possible resistant determinants. It will further help in proper selection of therapeutic regimen and improve treatment strategy against leishmaniasis.