

## ORIGINAL RESEARCH ARTICLE

# Assessing the Role of Potential Biomarkers in Antimony Susceptible and Resistant Clinical Isolates of *L. donovani* from India

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## Abstract

Failure of antimonial drugs, the mainstay therapy for leishmaniasis has become an escalating problem in the treatment of Indian leishmaniasis. Using 14 clinical isolates from both visceral (VL) and post-kala-azar dermal leishmaniasis (PKDL) patients, we have examined the role of ATP-binding cassette transporter (ABC transporter) gene, multidrug resistant protein A (MRPA) and two building blocks of the major thiol, trypanothione namely, ornithine decarboxylase gene (ODC) (a rate limiting enzyme in the polyamine biosynthesis) and  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS) (a rate limiting enzyme in glutathione biosynthesis) in antimony resistance. Amplification of these three genes was observed in some but not all clinical isolates. Increased expression of the three RNAs as determined by real-time PCR was observed in all SAG-R clinical isolates. Significant increase in cysteine and glutathione levels was observed in the resistant isolates. Our studies report the underlying mechanism of antimony resistance in the clinical isolates.

**Key words:** ABC transporter, ornithine decarboxylase,  $\gamma$ -glutamylcysteine synthetase, antimony resistance.

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## 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 [1,2]. Resistance to this drug is becoming a major barrier in the treatment of VL in many endemic regions particularly in India [3]. 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 [4]. 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 [5].

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 [6]. 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) [7-9]. A loss of drug activation is reported to lead to resistance [10]. 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) [11,12]. Recent evidence has suggested that part of the mode of

action of SbV could be in depleting the cells of its reduced thiols [13]. Trypanothione (TSH), a major reduced thiol of *Leishmania* is a N<sup>1</sup>, N<sup>8</sup> 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 [13-16]. A number of candidate genes associated with increased thiol concentration have been described in *Leishmania* laboratory mutants. Resistance was induced in these mutants *in vitro* in the presence of antimony related metals such as arsenic or antimony [2,17,18].

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  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS) genes as potential biomarkers for monitoring antimonial resistance in Indian leishmaniasis.

## Materials and Methods

### Parasite and culture conditions

Promastigotes of *Leishmania donovani* clones, AG83 (MHOM/IN/80/AG83), 2001, MC4, MC7, MC8 and MC9 were isolated from patients with VL and strains, RK1, MS2, NR3A, RMP8 (HM/IN/RMP-8), RMP-19 (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 [19]. 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 [20]. 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 whereas in the case of PKDL patients infected with the 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.

### 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 DRII, Bio-Rad) as reported earlier [20]. Mid log phase promastigotes ( $\sim 2 \times 10^9$  cells) of all the field isolates were used for isolation of genomic DNA. 5  $\mu$ g of genomic DNA was digested with *Hind*III enzyme and subjected to electrophoresis. Total RNA was isolated from promastigotes ( $2 \times 10^8$  cells) using RNeasy Plus Mini Kit (Qiagen). Standard protocols were followed for Southern hybridization [21]. DNA probes used in the present study included a 400-bp MRPA fragment (released from plasmid PM12 that was

digested with *Bam*HI and *Pst*I), a 2.3-kb  $\gamma$ GCS fragment - (derived from plasmid *psp* $\alpha$ hygro $\alpha$ - $\gamma$ GCS digested with *Hind*III and *Xba*I), a 2.0-kb *ODC*-full length probe (derived from plasmid *psp* $\alpha$ hygro $\alpha$ -*ODC* digested with *Hind*III and *Xba*I) and a 1.6-kb 5'-*PTR1* probe derived from plasmid (*psp*72-Y-hygro-5'-*PTR1*).

#### **cDNA synthesis and real time RT- PCR**

Total RNA was isolated from 10<sup>8</sup> *Leishmania* cells in the mid-log phase of growth using the RNeasy 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'-GCGCAGCCGTTTGTGCTTGTTGG-3' and reverse 5'-TTGCCGTACGTCGCGATGGTGC-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'-TTCCATCTCCAGCGGGTTGTCC-3', and for the  $\gamma$ GCS, forward 5'-CATTGGCTGGCGCGTTGAGTTC-3' and reverse 5'-ATGTGCGCGGCCCATATTCTCG -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)<sub>18</sub> primers following manufacturer's instructions. Real-time PCR was performed in triplicate in 25  $\mu$ l 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.

#### **Transfection and overexpression of the MRPA and $\gamma$ -GCS gene**

Episomal *Leishmania* expression vectors, pGL- $\alpha$ NEO $\alpha$ LUC containing luciferase encoding DNA and neomycin phosphotransferase selectable marker, *psp* $\alpha$ hygro $\alpha$ - $\gamma$ GCS containing coding sequence for heavy subunit of  $\gamma$ GCS with hygromycin phosphotransferase as selectable marker and pGEM72f- $\alpha$ neo $\alpha$ -MRPA containing MRPA coding DNA and neomycin phosphotransferase as selectable marker were obtained as gifts from Prof. Marc Ouellette, Centre de Recherche en Infectiologie du Centre de Recherche du CHUL, Universite Laval, Quebec, Canada. Twenty  $\mu$ g 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  $\mu$ F (Bio-Rad). Transfectants were selected for resistance to either G418 (40  $\mu$ g/ml) or hygromycin B (80  $\mu$ g/ml) as described earlier [22].

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

Stationary phase *Leishmania* promastigotes expressing the luciferase gene (pGL- $\alpha$ NEO $\alpha$ LUC) and *psp* $\alpha$ hygro $\alpha$ - $\gamma$ GCS containing coding sequence for heavy subunit of  $\gamma$ GCS with hygromycin phosphotransferase as selectable marker or pGEM72f- $\alpha$ neo $\alpha$ -MRPA 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 in RPMI-1640 medium (Sigma) containing 10% heat inactivated fetal bovine serum. Briefly, J774A.1 murine macrophages (1  $\times$  10<sup>5</sup> cells/ petridish) were infected with 1  $\times$  10<sup>6</sup> promastigotes (expressing the luciferase gene (pGL- $\alpha$ NEO $\alpha$ LUC) 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 [22]. The 50% inhibitory concentration (IC<sub>50</sub>) was determined from the graph representing different concentrations of SAG plotted against relative light units (RLU) produced by luciferase expressing parasites.

### Thiol analysis

Thiols were derivatized with monobromobimane and separated by high-performance liquid chromatography (HPLC) as reported earlier [18].

### 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.

## Results

### 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 [20].

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 IC<sub>50</sub> 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-

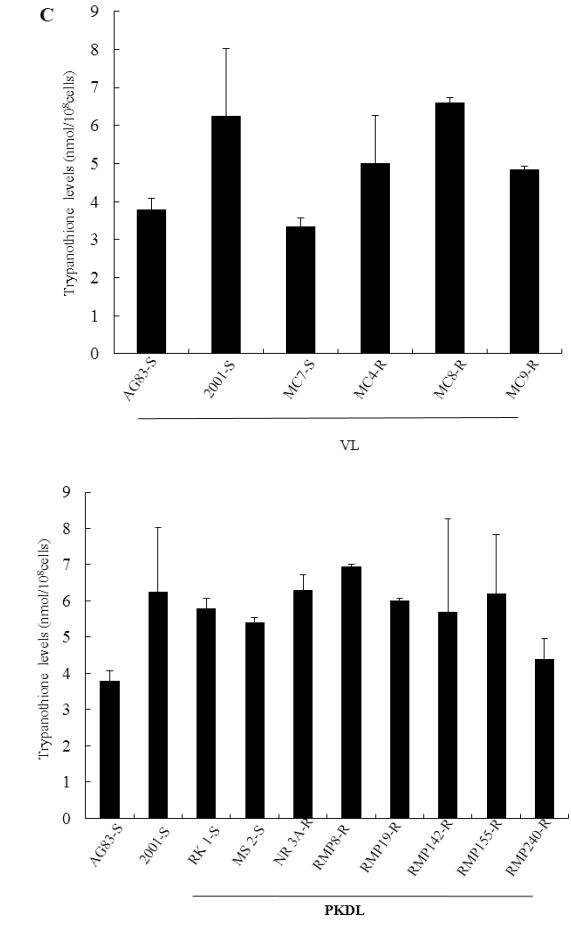
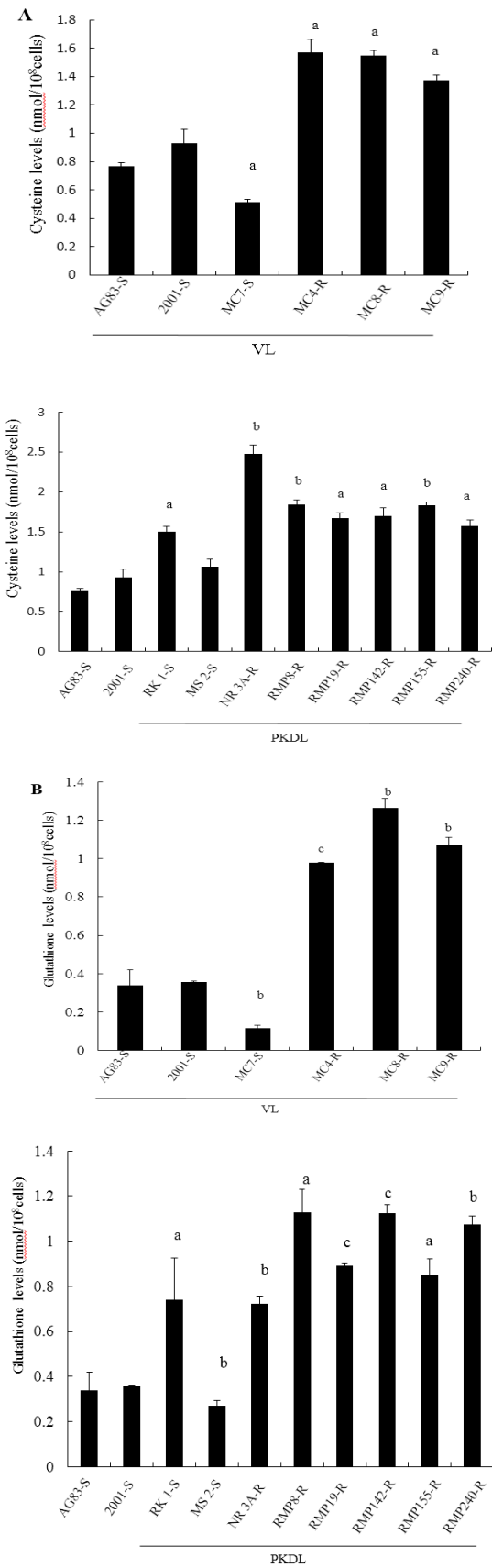
R, RMP8-R, RMP19-R, RMP142-R, RMP155-R, RMP240-R coming from the SAG-unresponsive patients had IC<sub>50</sub>s that were ~2 to >10 fold higher than that of the sensitive isolate, AG83-S. (20) RK1-S was the most sensitive of all the isolates with an IC<sub>50</sub> of 0.01 ± 0.02 µM.

Intracellular thiol levels were quantified in the SAG-S and SAG-R isolates (Figure 1). 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 1A).

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 1B). 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 1C). Similar observation was made in our earlier study using a small set of clinical isolates [23].

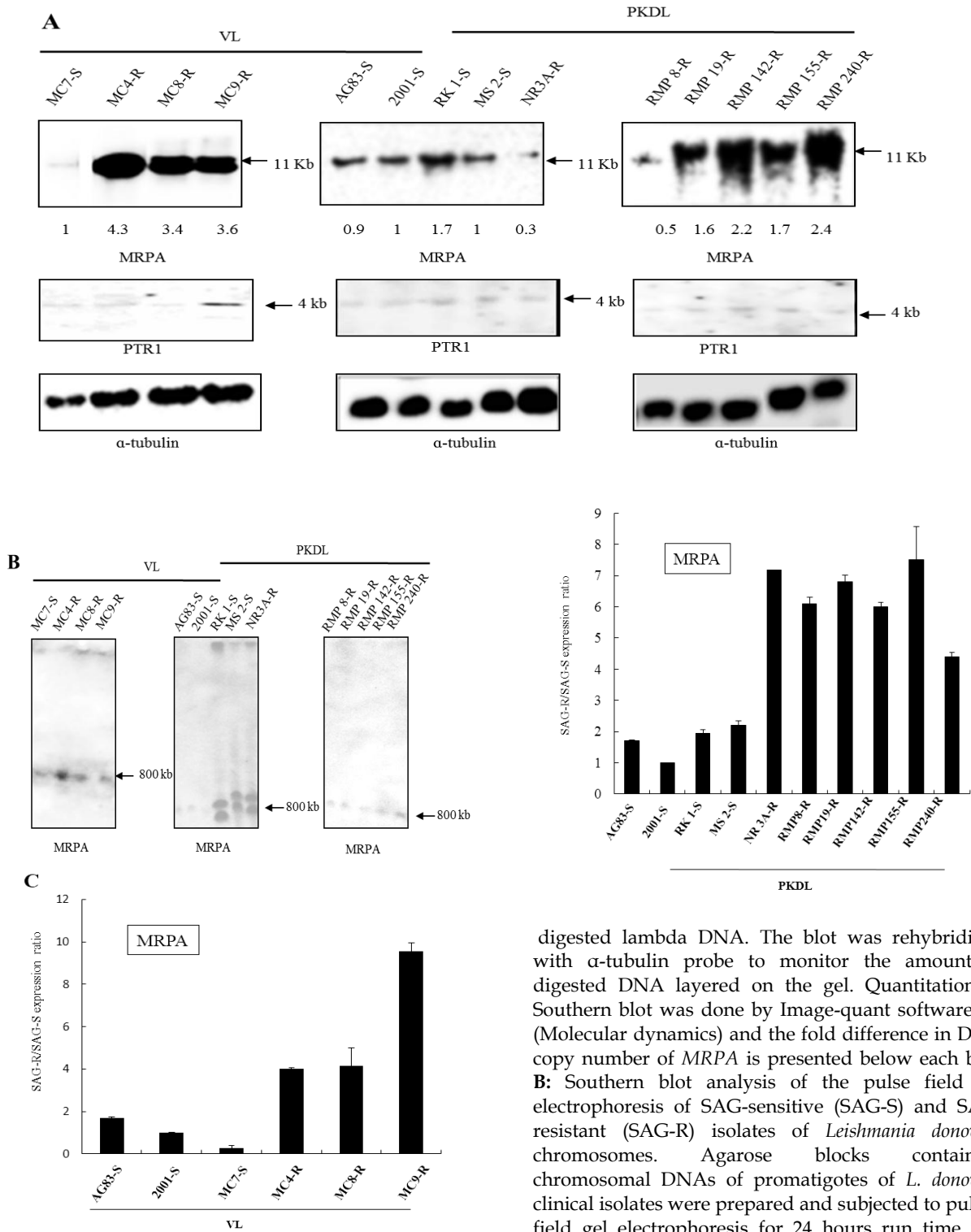
### Gene copy number and expression profiling of the ABC transporter MRPA in SAG-S and SAG-R clinical isolates

Our earlier work on the clinical kala azar *L. donovani* isolates from India showed MRPA overexpression as an important SAG resistance factor [23]. 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 *Hind*III followed by hybridization with



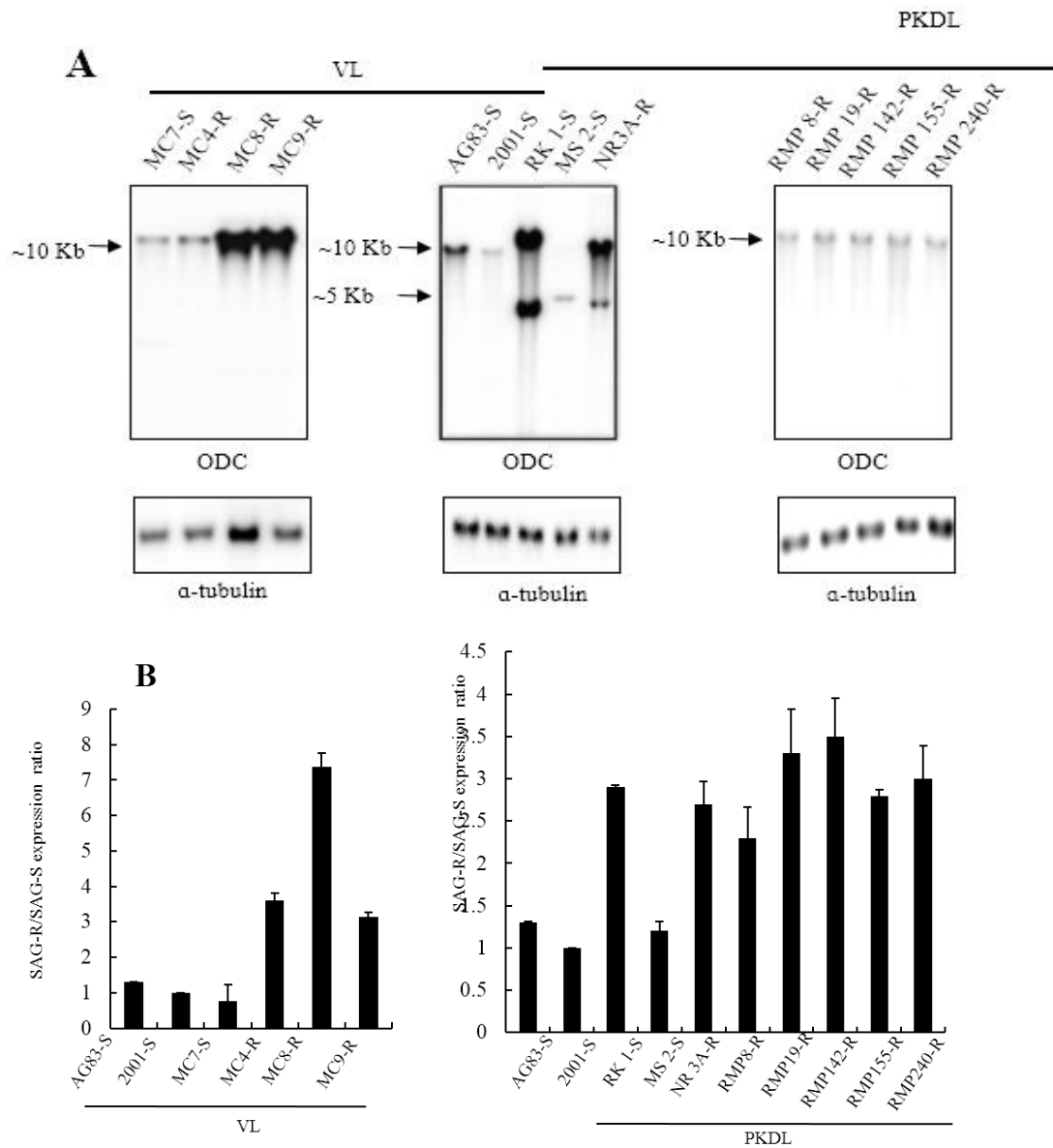
**Figure 1:** Intracellular levels of thiols, cysteine, glutathione and trypanothione in SAG-S and SAG-R *L. donovani* VL and PKDL clinical isolates. **A:** Cysteine levels **B:** Glutathione levels **C:** Trypanothione levels. Thiols were derivatized with monobromobimane and separated by high-performance liquid chromatography (HPLC). Each value is the mean  $\pm$  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. 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 2A). Quantitation of the Southern blot hybridization signal was done using ImageQuant 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





**Figure 2: 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 *HindIII*, electrophoresed, blotted and hybridized with a *MRPA* specific probe of 400-bp and 6 kb 5'-*PTR1* specific probe. The size of the hybridizing bands was determined using *HindIII*

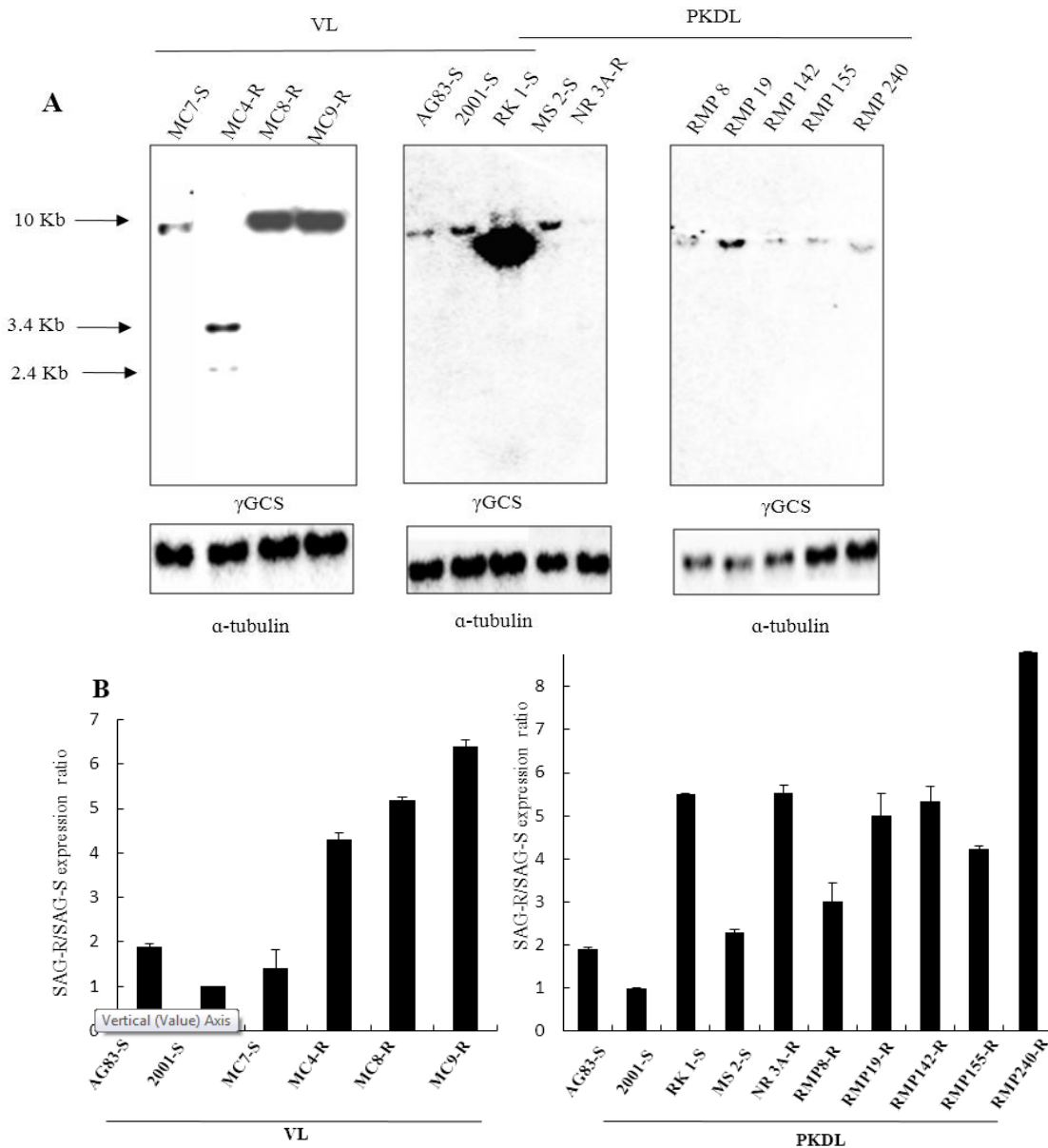
digested lambda DNA. The blot was rehybridized with  $\alpha$ -tubulin probe to monitor the amount of digested DNA layered on the gel. Quantitation of Southern blot was done by Image-quant software 5.2 (Molecular dynamics) and the fold difference in DNA copy number of *MRPA* is presented below each blot. **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 promastigotes 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. **C:** 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.



**Figure 3: A:** Characterization of *ODC* gene in SAG-S and SAG-R isolates *L. donovani* VL and PKDL clinical isolates. Genomic DNAs were isolated and digested with *Hind*III 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 *Hind*III digested lambda DNA marker. The blot was rehybridized with  $\alpha$ -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.

RMP240-R (Figure 2A). No amplification was observed in the resistant isolates, NR3A-R and RMP8-R (Figure 2A). The amplification observed in the resistant isolates was further analyzed by PFGE (Figure 2B). None of the resistant isolates showed circular amplification as was observed in our earlier studies with limited number of isolates [23]. 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 2B). We had earlier reported co-amplification of pterin reductase gene (*PTR1*) with *MRPA* in the clinical isolates [23]. In the present study, co-amplification of *PTR1* gene with *MRPA* gene was not observed in any of the clinical isolates as



**Figure 4:** **A:** Southern blot analysis of the  $\gamma$ -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  $\gamma$ -GCS probe. The sizes of the hybridizing bands were determined using *Hind*III digested  $\lambda$ -DNA. The blot was rehybridized with  $\alpha$ -tubulin probe to monitor the amount of digested DNA layered on the gel. **B:** Real time RT-PCR expression analysis of  $\gamma$ -GCS gene in *L. donovani* clinical isolates.  $\gamma$ -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.

determined by Southern-blot analysis using *PTR1*-specific probe (**Figure 2A**).

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 QuantiFast 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 2C).

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

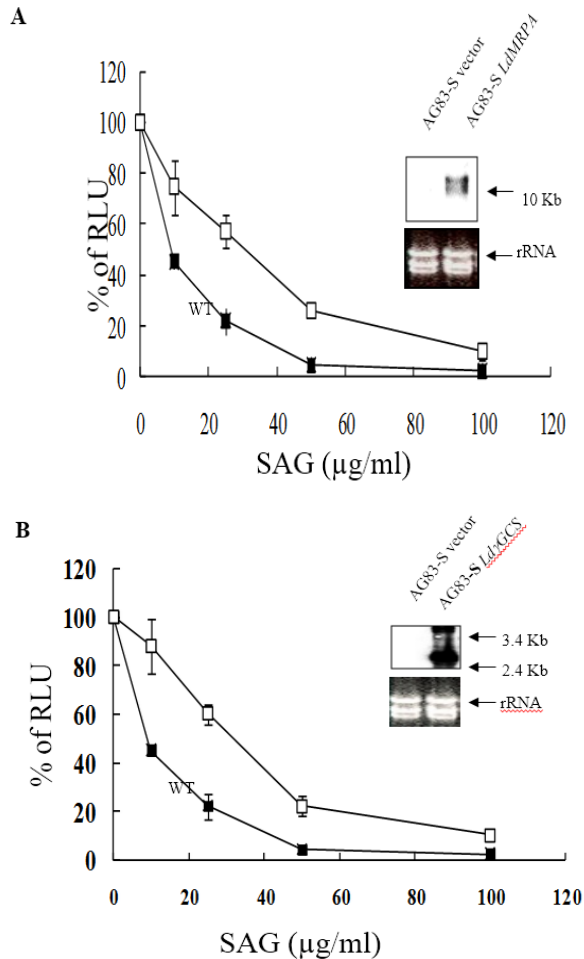
Ornithine decarboxylase (ODC) is the rate-limiting enzyme of the polyamine biosynthetic pathway. In addition to *MRPA*, overexpression of the *ODC* gene has been reported in the antimony resistant mutants [23-25]. These observations prompted us to determine if amplification of the *ODC* gene occurred 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 3A) 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 3A).

Comparison of *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 3B). 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 3B). No uniform correlation was observed between gene amplification and *ODC* gene expression in these isolates.

#### Gene copy number and expression profiling of the $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -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 *GSH1* gene coding for the heavy subunit of  $\gamma$ -GCS.  $\gamma$ -GCS is the rate limiting enzyme for GSH synthesis [14,23]. We performed Southern blot analysis of the total genomic DNA digested with *HindIII* and hybridized with the  $\gamma$ -GCS specific probe. Southern blot analysis of the  $\gamma$ -GCS gene showed two copies in a resistant isolate MC4-R but in all other isolates,  $\gamma$ -GCS probe hybridized to a single hybridizing fragment of 10-kb (Figure 4A). SAG-S isolate, RK1-S was again an exception since it showed amplification of the  $\gamma$ -GCS gene (Figure 4A).

Comparison of  $\gamma$ -GCS 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  $\gamma$ -GCS (gene specific) and *GAPDH* (internal control) primers was performed. Up-regulation of  $\gamma$ -GCS expression was observed in the resistant clinical isolates.  $\gamma$ -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 4B). In the present study though up-regulation of  $\gamma$ -GCS was observed in all SAG-R isolates, interestingly one SAG-S isolate, RK1-S showed 5.5-fold up-regulation of  $\gamma$ -GCS expression when compared to a SAG-S isolate, 2001-S (Figure 4B). L-butathione-sulfoxamine (BSO), an inhibitor of  $\gamma$ -GCS was used to compare its effect on the SAG-S isolate, RK1-S and 2001-S. RK1-S promastigotes over-expressing  $\gamma$ -GCS were ~5 fold more resistant to BSO when compared to the  $IC_{50}$  of promastigotes of the 2001-S, the  $IC_{50}$  being  $6.5 \pm 0.5$  mM and  $1.3 \pm 0.3$  mM respectively.



**Figure 5:** Over-expression of MRPA and  $\gamma$ -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- $\alpha$ NEO $\alpha$ LUC, AG83-S + LdMRPA (-□-) was transfected with pGEM72f- $\alpha$ neo $\alpha$ -MRPA + pGL- $\alpha$ NEO $\alpha$ LUC and sensitivity to SAG in the J774A.1 line was measured as described in Materials and methods. Each data point represents the mean  $\pm$  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  $\gamma$ -GCS gene in SAG-sensitive isolate leads to antimony resistance in intracellular amastigotes. AG83-S (-■-) transfected with pGL- $\alpha$ NEO $\alpha$ LUC, AG83-S +  $\gamma$ -GCS (-□-) transfected with psp $\alpha$ hygro $\alpha$ - $\gamma$ GCS + pGL- $\alpha$ NEO $\alpha$ LUC and sensitivity to SAG measured as described in Materials and methods. Each data point represents the mean  $\pm$  SD of three determinations. Northern blot analysis (*insets* in B) of vector transfected controls and  $\gamma$ -GCS transfected lines was performed as described under methods section.

**Overexpression of MRPA or  $\gamma$ -GCS in an antimony-sensitive isolate conferred increased expression and resistance to antimony**

To determine whether overexpression of MRPA and  $\gamma$ -GCS conferred antimony resistance in a sensitive isolate, we transfected MRPA or  $\gamma$ -GCS constructs into a SAG-S isolate, AG83-S. These MRPA and  $\gamma$ -GCS recombinant parasites were co-transfected with pGL- $\alpha$ NEO $\alpha$ LUC, encoding LUC. Intracellular amastigotes over-expressing MRPA (IC<sub>50</sub>, 32.3  $\pm$  6.4  $\mu$ g/ml) were 3.6-fold more resistant to SAG when compared to amastigotes of the parent strain transfected with the control vector (IC<sub>50</sub>, 9  $\pm$  0.5  $\mu$ g/ml) (**Figure 5A**). Intracellular amastigotes over-expressing  $\gamma$ -GCS were 3.3-fold resistant to SAG when compared to the sensitive *L. donovani* the IC<sub>50</sub> value being (29.7  $\pm$  1.5  $\mu$ g/ml) respectively (**Figure 5B**). In our previous studies we had demonstrated that the ODC overexpressors exhibited significant resistance to Pentostam compared to the wild type cells (26). Intracellular amastigotes over-expressing ODC (IC<sub>50</sub>, > 80  $\mu$ g/ml) were >8.8-fold more resistant to SAG when compared to amastigotes of the parent strain transfected with the control vector [26].

**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 [27]. 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 [28]. 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

[11,12,20,29] (b) Over expression of ODC and  $\gamma$ -GCS enzymes of the trypanothione biosynthetic pathway [2,25] and (c) increased expression of the ABC transporter MRPA, which sequesters SbIII-thiol conjugate [16,23].

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 *AQP1* gene expression [20].

The ABC transporter gene MRPA causes drug sequestration in *Leishmania* promastigotes and amastigotes selected for SbIII resistance [17,30]. Increased copy number of MRPA has been reported in SbIII resistant mutant [2]. Our earlier studies using limited number of SAG-S and SAG-R clinical isolates showed amplification of MRPA gene as part of an extrachromosomal circle [23]. In the present study, we found distinct correlation between copy number of 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 MRPA was amplified in three out of four resistant VL isolates as part of the extrachromosomal circle and coamplification of PTR1 along with MRPA suggested amplification of the H locus in SAG-resistant clinical isolates [23]. In the present study, co-amplification of PTR1 gene with 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. MRPA gene expression in SAG-S versus SAG-R field isolates showed correlation between MRPA gene expression and SAG susceptibility profile of the clinical isolates. The present observation validated our earlier results where we have shown correlation between MRPA gene expressions with the antimony resistant clinical profile in the field conditions [23].

In our previous study amplification of ODC gene was noted in the resistant isolates but not that of the  $\gamma$ -GCS [23]. In another study on *L. donovani* isolates from Nepal, expression of  $\gamma$ -GCS and ODC was significantly decreased in the resistant isolates [31]. In the present study we observed increased expression of ODC gene and  $\gamma$ -GCS in all SAG-R isolates.

It has been reported earlier that ABC transporter MRPA, confers resistance to antimonials by sequestration of metal thiol conjugates in an intracellular organelle located close to the flagellar pocket [16]. This model has been demonstrated in promastigotes of *L. tarentolae*, in amastigotes and also in clinical isolates from India [16,23,30]. 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 [23]. 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) [23]. It was pointed out that antimony possibly depleted trypanothione by efflux of Sb-trypanothione conjugate [16]. 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 the

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.

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