Chapter 4: Assessing aquaglyceroporin gene status and expression profile in antimony-susceptible and -resistant clinical isolates of *Leishmania donovani* from India.
1.0 Introduction

Visceral Leishmaniasis (VL) is a parasitic disease caused by the protozoan parasite *Leishmania donovani*. VL is thought to be anthroponotic in India and post-kala-azar dermal leishmaniasis (PKDL) patients are considered to serve as a source for new outbreaks (Thakur et al., 1991). Post-kala-azar dermal leishmaniasis (PKDL) is a cutaneous manifestation of visceral leishmaniasis (VL). In India and Sudan, the disease develops months to years after the patient recovery from VL (Zijlstra et al., 2003). Current chemotherapeutic agents are ineffective because of their high toxicity and emergence of drug resistance. Pentavalent antimonial, sodium antimony gluconate [SAG or SbV is the age old conventional therapy for visceral leishmaniasis (VL) (Sundar et al., 2000). However, increasing resistance to SAG has emerged as a major barrier in the treatment of VL. Resistance to SAG in the field isolates is less well defined. Recent reports show that determination of susceptibility using *in vitro* assays correlates well with the clinical response (Lira et al., 1999; Hadighi et al., 2006; Rojas et al., 2006). Our earlier studies showed that resistance mechanisms to antimony reported in laboratory strains are also operational in field isolates (Mukherjee et al., 2007). Variability in mechanism of resistance and varying degree of resistance was encountered in the field isolates (Mukherjee et al., 2007). Development of drug resistance has been a hindrance in chemotherapy of leishmaniasis and is of considerable importance to identify biomarkers responsible for resistance to antimonial drugs for visceral leishmaniasis. A number of candidate genes associated with resistance to antimonials have been described both in SAG-resistant laboratory mutants and clinical isolates of *L. donovani* (Mukhopadhyay et al., 1996; Legare et al., 1997; Haimeur et al., 2000).

Aquaglyceroporins (AQPs) are members of the aquaporin super family. They are membrane channels which permit transport of small neutral solutes such as glycerol or urea. Glycerol Facilitator (GlpF) and Glycerol Channel Protein (Fps1p), an aquaglyceroporin family member, have been reported to transport SbIII in *Escherichia coli* (Sanders et al., 1997) and in *Saccharomyces cerevisiae* (Wysocki et al., 2001) respectively. In *Leishmania* species, AQPI has been shown to facilitate SbIII transport (Gourbal et al., 2004). Overexpression of aquaglyceroporin in *L. major* (*LmAQP1*)
produces hypersensitivity to trivalent antimony (SbIII) whereas gene deletion renders the parasite resistant (Gourbal et al., 2004; Richard et al., 2004). This has provided a major insight into the uptake mechanism of drugs in *L. major* (Gourbal et al., 2004; Marquis et al., 2005). Aquaglyceroporin (AQP1) plays an important physiological role in water and solute transport, volume regulation and osmotaxis (Figarella et al., 2007). In *Plasmodium falciparum*, it has been shown that single amino acid Glu-125 in the extracellular C-loop plays an important role in reducing water but not glycerol permeability (Beitz, 2005). Crystal structure of *Leishmania* AQP1 is not available; Prediction of topology of *L. major* AQP1 showed that LmAQP1 consists of six membrane spanning helices containing the canonical Asn-Pro-Ala (NPA) motifs (Uzcategui et al., 2008). These helices are connected by five loops referred to as A-E (Uzcategui et al., 2008). Glutamate (Glu-152) present in the C-loop of LmAQP1 is reported to be critical for metalloid permeability. A single mutation in Glu-152 to alanine selectively abrogated the metalloid permeability (Uzcategui et al., 2008).

In this study, the role of aquaglyceroporin (AQP1) has been examined in monitoring antimonial resistance in Indian leishmaniasis. Susceptibility to sodium antimony gluconate (SAG) as determined *in vitro* with intracellular amastigotes from both Visceral (VL) and Post-kala-azar dermal leishmaniasis (PKDL) patients correlated well with the clinical response. A positive correlation between *AQP1* gene expression and SbIII accumulation in both VL and PKDL field isolates was observed. Cloning and sequencing of *AQP1* gene from both VL and PKDL isolates was also done to check the role of Glu-152 and Arg-230 in antimony resistance. Transfection of *AQP1* gene in the SAG-resistant field isolate conferred sensitivity to the resistant isolate. Our results demonstrate that down regulation of *AQP1* correlates well with the antimony drug resistance in a majority of Indian VL and PKDL isolates.

**2.0 Materials and Methods**

**2.1 Study Population**

Patients clinically diagnosed with VL (fever and hepatosplenomegaly) were recruited from the outpatient department of Medicine, Institute of Postgraduate Medical Education and Research, Kolkata, West Bengal. They gave a history of either residence or travel to Bihar, India. The diagnosis of VL was done by rk39 dip test and...
if found positive, was confirmed by bone marrow aspiration and microscopic examination of Giemsa stained smears that demonstrated presence of L.D. bodies. The bone marrow/splenic aspirations were collected aseptically and transferred to agar slants containing 1 ml of 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 (Medium A). Parasite transformation was checked and when a sufficient number of parasites was obtained (>5 x 10⁶); they were then adapted to Medium A.

Patients who were clinically diagnosed with PKDL were recruited from the outpatient unit of the Department of Dermatology, School of Tropical Medicine, West Bengal. The diagnosis was based on clinical features and a past history of VL which was corroborated by rK39 strip test and the presence of L.D. bodies in dermal lesions. The punch biopsy material collected aseptically, was cut into small pieces and placed in a 25 mm² tissue culture flask containing Schneider's medium (Sigma, USA) supplemented with 10% FCS, 0.13 mg/mL penicillin and streptomycin. After successful transformation, when cell number exceeded 5 x 10⁶, they were gradually adapted into Medium A. Once transformed, both VL and PKDL isolates were cloned on semisolid M199 medium containing 1% Bacto Agar (Difco) and 10% heat inactivated fetal bovine serum. Colonies were picked after 6-7 days and transferred to Medium A. Ethical clearance was obtained from the institutional review boards of the Institute of Post Graduate Medical Education and Research and School of Tropical Medicine, Kolkata, India and informed consent was obtained.

2.2 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) were isolated from patients with PKDL. Both VL and PKDL strains were from antimony resistant zone of Bihar, India. PKDL isolates, 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) were a gift from the Division of Clinical
Microbiology, All India Institute of Medical Sciences, New Delhi (Dey and Singh, 2007). SAG-sensitive strains, AG-83 and 2001 were used as reference strains and have been characterized earlier (Mukherjee et al., 2007). 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. There was no difference in the growth rate of these isolates. 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). Accordingly, SAG-S isolates used in this study include AG83-S, 2001-S, MC7-S, RK1-S, MS2-S whereas the nine SAG-R isolates were MC4-R MC8-R, MC9-R, NR3A-R, RMP8-R, RMP19-R, RMP142-R, RMP155-R and RMP240-R. These clinical isolates were maintained in the absence of drug pressure in vitro. The isolates were routinely passaged through BALB/c mice to retain their virulence. Institutional level ethics committee has approved this study.

2.3 DNA construct and transfection

The linearized β-lactamase expression vector (pIR1SAT-βLA) containing β-lactamase encoding gene used in the present study was kindly gifted by Dr. Frederick S. Buckner (Washington University, Seattle). This vector when linearized integrates into the *Leishmania* genome by replacing one copy of the small sub-unit (SSU) rRNA gene. The construct was linearized by *SwaI* restriction digestion (MBI, Fermentas) and 10 μg of the linearized construct having the β-lactamase gene was transfected into *L. donovani* log phase promastigotes (4 x 10⁷) by electroporation. Briefly, electroporation was done in 2 mm gap cuvettes at 450 V, 500 μF (Bio-Rad) as reported earlier (Mandal et al., 2009). The transfectants were selected for resistance to 50 μg/ml of nourseothricin (Sigma, USA) (Buckner and Wilson, 2005; Mandal et al., 2009).

2.4 Chemosensitivity profiles of SAG-S and SAG-R strains in an amastigote macrophage model using β-lactamase assay

Stationary phase *Leishmania* promastigotes expressing the β-lactamase gene were used to infect J774A.1 macrophages. Macrophage cell line J774A.1 (American Type Culture Collection) was maintained at 37°C in RPMI-1640 medium (Sigma, USA)
containing 10% heat inactivated FBS. Briefly, the intracellular amastigotes grown in macrophages were quantified for β-lactamase activity by removing the medium by gentle pipetting followed by addition of 50 μl of 50 μM substrate, CENTA (Calbiochem, La Jolla, CA) in PBS and 0.1% Nonidet P-40. The plates were incubated at 37°C for 4 h (Buckner and Wilson, 2005; Mandal et al., 2009). Catalysis of the substrate (CENTA) to its chrome yellow colour product was quantified at OD405 nm on a 96 well plate reader (Bio-Rad) (Buckner and Wilson, 2005). The 50% inhibitory concentration (IC₅₀) was determined from the graph representing different concentrations of the inhibitor plotted against % growth.

2.5 Determination of in vitro antileishmanial activity in promastigote cultures

In order to characterize the SAG sensitivity profile of L. donovani promastigotes, a modified MTT (Sigma, USA) assay was performed as described earlier (Dutta et al., 2005). Briefly, 25 μl of promastigotes (2.5x10⁴ cells/well) were cultured in a 96- well flat bottomed plate (Nunc, Roskilde, Denmark) and incubated with 25 μl of different drug concentrations at 26 °C. After 72 h, 10 μl of MTT (5 mg/ml) in 1 x PBS was added to each well and the plates were incubated at 37 °C for 3 h. The reaction was stopped by the addition of 50 μl of 50% isopropanol and 10% SDS. The plates were again incubated for 30 min at 37 °C with gentle shaking. Absorbance was measured at 570 nm in micro-plate reader. The 50% inhibitory concentration (IC₅₀) was determined from the graph representing different concentrations of the inhibitor plotted against % of cell growth.

2.6 Drug uptake assay

Uptake studies were done as described previously (Marquis et al., 2005). Briefly, log phase Leishmania promastigotes of SAG-S isolates (AG83-S, 2001-S, MC7-S, RK1-S and MS2-S) and SAG-R isolates (MC4-R, MC8-R, NR3A-R, RMP8-R, RMP19-R, RMP142-R, RMP155-R and RMP240-R) were washed twice with phosphate buffered saline (PBS, pH 7.4) and were resuspended in PBS containing 10 mM glucose at a density of 5 x 10⁷ cells/ml. Cells were then incubated at 27 °C with 100 μM SbIII for 30 minutes and an aliquot of 500 μL samples were collected at 0 and 30 minutes. Cells were pelleted down and washed twice with equal volume of ice cold PBS and
Accessing the role of \textit{AQP1}

centrifuged at 17,000 x g for 1 minute at room temperature. The pellet was dried and treated with 0.05% of 70% nitric acid for 2 h at 70 °C and then diluted with 3 ml of the high-pressure liquid chromatography grade water. Antimony levels were analyzed by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) as reported earlier (Shaked-Mishan et al., 2001; Marquis et al., 2005). Each uptake assay was repeated twice with triplicates in each set.

2.7 Nucleic acid isolation, pulse field gel electrophoresis (PFGE) and hybridization analysis

Genomic DNA was isolated from ~2 x 10^9 cells from 10-15 ml mid log phase promastigotes culture of all field isolates by standard procedure (Bellofatto and Cross, 1989). 5 µg of genomic DNA was digested with \textit{SalI} enzyme and subjected to electrophoresis. The fragments were transferred to Hybond™- N⁺ membrane (Amersham Pharmacia Biotech) and subjected to Southern blot analysis. 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) in 0.5x Tris-borate-EDTA, with buffer circulation at a constant temperature of 14°C. The gel running conditions were as follows: 6V/cm voltage gradient, initial switch time 60s and final switch time 120 s and run time of 24 h. \textit{Saccharomyces cerevisiae} chromosomes were used as size markers.

Promastigotes (2 x 10^8 cells/ml) were used for isolation of total RNA using TRI reagent™ (Sigma, USA). Total RNA (15 µg) was fractionated by denaturing agarose gel electrophoresis and transferred onto nylon membrane for northern blot analysis. The blots obtained above were pre-hybridized at 65 °C for 4 h in a buffer containing 0.5 M sodium phosphate; 7% SDS; 1mM EDTA, pH 8.0 and 100 µg/ml sheared denatured salmon sperm DNA. Hybridization was done with denatured α-[\textsuperscript{32}P]-dCTP-labelled DNA probe at 10^6 cpm/ml, which was labeled by random priming (NEB Blot\textsuperscript{®} Kit, New England Biolabs, Inc.). Labeling was done according to the manufacturer’s protocol. The DNA probe used in the present study included a 945-bp \textit{AQP1} probe (accession number, EF600686). Membranes were washed, air-dried and exposed to
imaging plate. The images were developed by PhosphorImager (Fuji film FLA-5000, Japan) using Image Quant software.

2.8 Molecular genotyping by PCR-RFLP of the ITS region of *L. donovani* clinical isolates

The entire internal transcribed spacer (ITS) in the ribosomal operon was amplified using the primers: LITSR (5'-CTGGATCATTTTCCGATG-3') and LITSV (5'-ACACTCAGGTCTGTAAAC-3') as reported earlier (El Tai, 2001), (Fig. 1B). Ten microlitre of the amplified ITS region were digested with *HaeIII* for 2 h at 37 °C using the conditions recommended by the manufacturer. Restriction fragments were separated on 2% agarose gel for 2-4 h in 1 x TBE (90 mM Tris borate, 0.2 mM EDTA) buffer and visualized under UV light after staining with ethidium bromide.

2.9 cDNA synthesis and real time RT-PCR

Total RNA was isolated from 10^5 *Leishmania* cells in the mid-log phase of growth using the RNeasy Plus Mini Kit (Qiagen) as described by the manufacturer. The RNAs were treated with RQ1 RNase-free DNase (Promega, Madison, USA) to avoid any genomic DNA contamination. 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 *AQP1* were forward 5'-CTGTGTCTTTGGTGCTTTCC and reverse 5'-GCCTTTTGGGCGTCGTC (Decuypere et al., 2005), and for the GAPDH control, forward 5'-GAAGTACACGGTGGAGGCTG and reverse 5'-CGCTGATCACGACCTTCTTC primer (Mukherjee et al., 2007). Complementary DNAs from promastigotes were synthesized from 500 ng of total RNA using the AccuSuperscript High Fidelity RT-PCR kit (Stratagene, La Jolla, CA) 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 20 s 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.

2.10 Cloning and sequencing of aquaglyceroporin (AQP1) gene from antimony sensitive and antimony resistant L. donovani isolates

Aquaglyceroporin (AQP1) gene from the VL and PKDL clinical isolates were PCR amplified using specific oligonucleotides. DNA fragments of 945-bp were amplified from the genomic DNA of 2001-S, AG83-S, MC7-S, MC4-R, MC8-R, MC9-R, RK1-S, MS2-S, NR3A-R and RMP142-R using a sense primer with a flanking XbaI site, 5'-GCTCTAGAATGAACTCTACAAGCACA-3', and an antisense primer with a flanking HindIII site, 5'-CCCAAGCTTCTAGAAGTTGGGTGGAATGA-3' (Maharjan et al., 2008). Polymerase chain reaction (PCR) was performed in a 50 μl reaction volume containing 100 ng of genomic DNA, 25 pmol each of the gene-specific forward and reverse primers, 200 μM of each dNTP, 2 mM MgCl2, and 5 U Taq DNA polymerase (MBI Fermentas). The conditions of the PCR were as follows: 94 °C for 10 min, 94 °C for 45 s, 60 °C for 30 s, 72 °C for 45 s and 30 cycles. Final extension was carried for 10 min at 72°C. Single band of 945-bp PCR product was obtained and subcloned in to pTZ57R/T (InsTAclone™ PCR cloning kit, MBI Fermentas) and subjected to automated sequencing. Multiple alignments of amino acid sequences were performed using CLUSTAL W program. Transmembrane domain of the sequences was identified using HMMTOP protein topology prediction online software (www.enzim.hu/hmmtop).

2.11 Transfection and overexpression of the AQP1 gene

Aquaglyceroporin (AQP1) gene from AG83-S L. donovani sensitive strain was PCR amplified. The amplified DNA fragment was cloned into the XbaI-HindIII site of the pspCneoα Leishmania shuttle vector (kindly provided by Dr. Marc Ouellette, Quebec, Canada) to create a psp-Cneoα-AQP1 gene construct containing the neomycin phosphotransferase gene (G418) antibiotic marker. Twenty μg of the construct was transfected into the promastigotes of AG83-S sensitive isolate and MC4-R resistant L. donovani promastigotes by electroporation using the following parameters 450 V, 500 μF, (Bio-Rad). Transfectants were selected for resistance to G418 (40μg/ml).
Transfectants with psp<sub>neo+</sub> vector alone were used as control. Drug uptake and susceptibility assays were performed as described above.

2.12 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 Susceptibility and characterization of SAG-S and SAG-R strains
A total of fourteen field isolates were collected from patients residing in the antimony resistant zone of Bihar state, India. The chemosensitivity profiles of the SAG-S and the SAG-R strains to SbIII were tested in intracellular-amastigotes by β-lactamase assay as described in the methods section. 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 (Table 1).

Molecular karyotyping of all <i>L. donovani</i> clinical isolates was carried out by Clamped Homogeneous Electric Field Apparatus (CHEF DR Ill. Bio-Rad) (Figure 1). Molecular karyotype of PKDL isolates when compared to that of the two VL reference strains, AG83-S and 2001-S, did not show any difference in the chromosome number and size. However, the VL isolates, MC7-S, MC4-R, MC8-R and MC9-R showed a slightly different pattern of chromosome size and number when compared to the other VL and PKDL isolates. Restriction fragment length polymorphism (RFLP) of the amplified internal transcribed spacer (ITS) has been used successfully as a genotyping marker in molecular epidemiology (El Tai, 2001). <i>HaeIII</i> restriction digestion of the entire ITS region was performed to further confirms genetic variation in the isolates (Figure 2 A and B). Comparison of the restriction digestion pattern of the amplified ITS region with <i>HaeIII</i>, indicated polymorphism among clinical <i>L. donovani</i> isolates (Figure 2B).
With the exception of NR3A-R, all PKDL isolates had the same banding pattern as that of the two VL reference strains, AG83-S and 2001-S. On the other hand, VL isolates, MC7-S, MC4-R, MC8-R and MC9-R had three different polymorphic patterns. MC7-R and MC8-R strains had a similar banding pattern.

### Table 1: Susceptibility of SAG-S and SAG-R field isolates to SbIII in intracellular amastigotes.

<table>
<thead>
<tr>
<th>S.N</th>
<th>Strain ID</th>
<th>Geographical location</th>
<th>Disease</th>
<th>Mean IC₅₀ ± S.D (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AG83-S</td>
<td>Bihar</td>
<td>VL</td>
<td>6.2 ± 1.8</td>
</tr>
<tr>
<td>2</td>
<td>2001-S</td>
<td>Bihar</td>
<td>VL</td>
<td>0.9 ± 0.12</td>
</tr>
<tr>
<td>3</td>
<td>MC7-S</td>
<td>Siwan, Bihar</td>
<td>VL</td>
<td>8 ± 3.3</td>
</tr>
<tr>
<td>4</td>
<td>MC4-R</td>
<td>Samastipur, Bihar</td>
<td>VL</td>
<td>32 ± 5.2 (5.16) *</td>
</tr>
<tr>
<td>5</td>
<td>MC8-R</td>
<td>Chapra, Bihar</td>
<td>VL</td>
<td>26 ± 4.6 (4.2) *</td>
</tr>
<tr>
<td>6</td>
<td>MC9-R</td>
<td>Samastipur, Bihar</td>
<td>VL</td>
<td>18 ± 2.1 (3.0) *</td>
</tr>
<tr>
<td>7</td>
<td>RK1-S</td>
<td>Bihar</td>
<td>PKDL</td>
<td>0.01 ± 0.02</td>
</tr>
<tr>
<td>8</td>
<td>MS2-S</td>
<td>Bihar</td>
<td>PKDL</td>
<td>4.75 ± 0.12</td>
</tr>
<tr>
<td>9</td>
<td>NR3A-R</td>
<td>Bihar</td>
<td>PKDL</td>
<td>52 ± 4.9 (8.4) *</td>
</tr>
<tr>
<td>10</td>
<td>HM/IN/RMP8</td>
<td>Bihar</td>
<td>PKDL</td>
<td>18.5 ± 1.06 (3.0) *</td>
</tr>
<tr>
<td>11</td>
<td>HM/IN/RMP19</td>
<td>Bihar</td>
<td>PKDL</td>
<td>14 ± 0.35 (2.3) *</td>
</tr>
<tr>
<td>12</td>
<td>HM/IN/RMP142</td>
<td>Bihar</td>
<td>PKDL</td>
<td>&gt;100.00 (&gt;10.0)*</td>
</tr>
<tr>
<td>13</td>
<td>HM/IN/RMP155</td>
<td>Bihar</td>
<td>PKDL</td>
<td>40 ± 3.5 (6.5) *</td>
</tr>
<tr>
<td>14</td>
<td>HM/IN/RMP240</td>
<td>Bihar</td>
<td>PKDL</td>
<td>30 ± 1.41 (5.0) *</td>
</tr>
</tbody>
</table>

All values presented are mean ± S.D. of triplicates from three independent experiments. The fold differences with respect to AG83-S strain are shown within bracket. * Statistically significant at \( p < 0.001 \) when compared to the corresponding values obtained for the AG83-S.
3.2 Accumulation of SbIII in *L. donovani* clinical isolates

Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) was used to show SbIII accumulation inside the cells at 30 min time point (Shaked-Mishan et al., 2001; Marquis et al., 2005). Promastigotes were incubated with 100 μM SbIII for 30 minutes at 27 °C as detailed in the methods section. Antimony resistant *Leishmania* isolates (SAG-R) had reduced accumulation of SbIII compared to the SAG-S isolates. In comparison to AG83-S, SAG-R field isolates MC4-R, MC8-R, MC9-R, NR3A-R, RMP8-R, RMP19-R, RMP142-R, RMP155-R and RMP240-R accumulated ~4.1, ~3.9, ~2.5, ~2.0, ~6.3, ~6.7, ~6.3, ~5.7 and ~5.6 - fold less SbIII respectively (Figure 3).

![Figure 1: Pulse field gel electrophoresis of SAG-sensitive (SAG-S) and SAG-resistant (SAG-R) VL and PKDL isolates of *Leishmania donovani* chromosomes. Agarose blocks containing chromosomal DNAs of promastigotes were prepared and subjected to pulsed field gel electrophoresis for 24 h at 14°C as reported in the methods section.](image-url)
3.3 Gene copy number and expression profiling of the aquaglyceroporin (AQP1) gene in SAG-S and SAG-R field isolates

Southern blot analysis showed variation in the gene copy number of AQP1 gene in both VL and PKDL isolates. Restriction enzyme, SalI which digests outside the gene producing a 10.8 kb restriction fragment (expected size) was used to check the copy number of the gene. A single hybridizing fragment of 10.8 kb was observed in the VL isolates, MC7-S, MC4-R, MC8-R, MC9-R, 2001-S and AG83-S. PKDL isolates, RMP8-R, RMP19-R, RMP142-R, RMP155-R, RMP240-R and MS2-S also showed a single hybridizing fragment of 7 kb indicating that AQP1 exists as a single copy gene in these strains (Figure 4). However, RK1-S had both 10.8-kb and 7-kb band representing two copies of AQP1.

![Diagram of ITS region](image)

Figure 2: [a] Schematic position of the internal transcribed space (ITS) in the ribosomal operon and position of the primers used to amplify ITS sequence. Sequence of the primers LITSR and LITSV are given in the methods section. [b] RFLP analysis of the amplified ITS from different clinical L. donovani isolates. Digestion with HaeIII of amplified ITS regions of different VL and PKDL isolates is shown. Fragments were separated on 2% agarose gel for 2 – 4 h to document differences in RFLP patterns. A 100 bp ladder was used as molecular size marker.
Figure 3: Accumulation of SbIII in VL and PKDL *Leishmania donovani* field isolates as measured by ICP-MS. Promastigotes were incubated with 100 μM SbIII for 30 minutes at 27°C as described in the materials and methods. Data are shown as mean ± standard deviation of two replicate experiments with triplicate in each set. * indicates $p < 0.01$ and ** indicates $p < 0.001$ when compared to AG83-S isolate values.

Interestingly, NR3A-R, a PKDL isolate that was ~8.4 -fold resistant to SbIII showed amplification of the *AQP1* gene. Comparison of aquaglyceroporin gene expression in SAG-S versus SAG-R field isolates was done to verify if there is any correlation between *AQP1* gene expression and SAG sensitivity 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 AQP1 (gene specific) and GAPDH (internal control) primers was performed.
Accessing the role of AQP1

Figure 4: Southern blot analysis of the aquaglyceroporin gene in the SAG-S and the SAG-R Leishmania donovani VL and PKDL field isolates. Total genomic DNA was isolated and digested with SalI. The digested DNA was electrophoresed, blotted and hybridized with a full length AQP1 specific probe of 945-bp. The sizes of the hybridizing bands were determined using HindIII digested λ DNA. The blot was rehybridized with α-tubulin probe to monitor the amount of digested DNA layered on the gel.

Downregulation of AQP1 expression was observed in the resistant clinical isolates. AQP1 expression in the resistant strains, MC4-R, MC9-R RMP8-R, RMP19-R, RMP142-R, RMP155-R and RMP240-R was 5.2-, 4.2-, 2.8-, 3.1-, 2.7-, 2.2- and 4.5-fold less respectively compared to the expression in the sensitive isolate AG83-S (Figure 5). In the present study though downregulation of AQP1 was observed in majority of the SAG-R isolates, there were two exceptions. Two SAG-resistant strains, NR3A-R and MC8-R showed upregulation of AQP1 gene expression. NR3A-R and MC8-R were 8.4- and 4.2- fold resistant to SbIII respectively as compared to the sensitive isolate, AG83-S. AQP1 gene expression levels of sensitive clinical isolates 2001-S, MC7-S, RK1-S and MS2-S were similar to that of the SAG-S isolate, AG83-S.
3.4 Comparison of sequence analysis of aquaglyceroporin gene of SAG-S and SAG-R field isolates

Downregulation of *L. major* aquaglyceroporin (*LmAQP1*) is reported to be linked to antimony resistance (Marquis et al., 2005). Introduction of point mutation in transporter proteins is yet another mechanism of drug resistance in *Leishmania* (Vasudevan et al., 2001; Perez-Victoria et al., 2003). Recently, point mutation at the extracellular loop of *LmAQP1* channel, Glu-152 (corresponding to Glu-185 in the full length sequence) was shown to confer SbIII resistance in *L. major* laboratory mutants (Uzcategui et al., 2008). Full length *L. donovani* *AQP1* gene coding sequence (945-bp) was cloned and sequenced from 6 VL and 5 PKDL isolates.

![Figure 5](image-url)  
**Figure 5:** Real time RT-PCR expression analysis of *AQP1* in *L. donovani* clinical isolates. *AQP1* RNA expression ratios in the SAG resistant isolates relative to the SAG sensitive isolate, AG83. Results are mean of three independent experiments performed from three different RNA preparations.
Figure 6: Aquaglyceroporin protein sequences from the SAG-S and the SAG-R *L. donovani* (VL and PKDL) isolates were aligned using CLUSTAL 2.0.11 multiple sequence alignment program. Transmembrane topology of the proteins was predicted using online transmembrane topology prediction server (HMMTOP) (www.enzim.hu/hmmtop). Six transmembrane domains (TM1-TM6) and five helical loops (A-E) connecting transmembrane domains were identified. Loop B and E containing conserved Asn-Pro-Ala (N-P-A) motifs form short α-helices that fold back into the membrane from opposite side forming a channel for glycerol transport. Two NPA motifs are boxed. Point mutations are marked with red and Glu-185 and Arg-263 (corresponding to Glu-152 and Arg-230 in the partial sequence) amino acid residues linked to ShIII susceptibility are shown in blue.
Figure 7: An hourglass model of aquaporin according to references (Jung et al., 1994; Zardoya, 2005), showing mutations in *L. donovani*. Mutations found in the extracellular C loop region of AQP1 sequence in strain, MC7-S is shown in the box. B: Conserved signature of the aquaglyceroporin (AQP1) protein of MC4-R, MS2-S and MC8-R that were deduced from the comparative analysis of the multiple alignment in Fig. 4. Asn-Pro-Ala (NPA) motifs are marked in red and amino acids mutations are marked with blue.
Transmembrane topology of the proteins was predicted using online transmembrane topology prediction server (HMMTOP) (www.enzim.hu/hmmtop). Six transmembrane domains (TM1-TM6) and five helical loops (A-E) connecting transmembrane domains were identified in the *L. donovani* sequence (Figure 6). Predicted protein sequence of the sensitive strains, AG83-S, 2001-S and RK1-S was similar to that of the SAG-resistant strains, MC9-R and NR3A-R (Figure 6). The hourglass model of aquaporin reported earlier (Jung et al., 1994; Zardoya, 2005), has been used to show mutations observed in the AQP1 protein of different *L. donovani* field isolates (Figure 7A). The model shows the presence of six transmembrane helices (1 – 6) connected by five loops (A – E). Loop B and E contain conserved Asn-Pro-Ala (N-P-A) motifs and form short α-helices that fold back into the membrane from the opposite side forming a channel for glycerol transport. Interestingly, AQP1 sequence polymorphism was observed in MC7-S, MS2-S, MC4-R and MC8-R with differences either in the transmembrane domains or in the helical loops (Figure 6 and 7) but none of the differences were uniform. Conserved signatures of the AQP1 protein were deduced from the multiple sequence alignments shown in Figure 6. Specific variation in the sequences of the isolates is shown in Figure 7B. Point mutation at Glu-185 and Arg-263 residues (corresponding to Glu-152 and Arg-230) (Uzcategui et al., 2008), could not be detected in any of the resistant field isolates, indicating that SAG resistance phenotype, at least in Indian *L. donovani* VL and PKDL field isolates, does not result from the point mutation of Glu-185 and Arg-263 residues of AQP1 gene coding sequence.

### 3.5 Overexpression of AQP1 in the antimony-resistant field isolate conferred increased expression, facilitated SbIII uptake and sensitivity to antimony.

To determine whether overexpression of AQP1 conferred sensitivity to resistant isolates, we transfected AQP1 construct into the promastigotes of both the antimony-sensitive isolate, AG83-S and the antimony-resistant isolate, MC4-R. Increased expression of AQP1 in transfected AG83-S and MC4-R strains was confirmed by comparison of northern blot analysis of vector transfected controls and AQP1 transfected lines (Figure 8A).
Figure 8: Accumulation of SbIII in \textit{AQP1} overexpressing \textit{Leishmania donovani} field isolates as measured by ICP-MS. Promastigotes were incubated with 100 \( \mu \)M SbIII for 30 minutes at 27\(^\circ\)C as described in the methods section. Data are shown as mean \pm standard deviation of two replicate experiments with triplicates in each set. * indicates \( p < 0.01 \) compared to respective controls. Northern blot analysis (insets in A) of vector transfected controls and \textit{AQP1} transfected lines was performed as described under methods section. B: Effect of different concentrations of SbIII on SAG-sensitive isolate, AG83-S and SAG-resistant field isolate MC4-R promastigotes, transfected with vector alone or psp-\( \alpha \)NEO\( \alpha \)-AQP1. Each data point represents the mean \pm SD of three determinations.
4.0 Discussion

Mechanism of antimony resistance in *Leishmania* has been studied extensively. Increase in resistance to SAG has led to an upsurge in therapeutic failure. In the absence of limited chemotherapeutic alternatives, it is necessary to identify biomarkers for antimony resistance to improve chemotherapeutic approach in field conditions. It has been reported earlier that the trivalent form of the antimonial drug, SbIII, is the prodrug that is formed by conversion of pentavalent antimony (SbV) to trivalent form (SbIII) by a putative metalloid reductase present in the macrophages (Sereno et al., 1998). Resistance to antimony in both the laboratory raised resistant strains and clinical isolates has been associated with: 1. overexpression of the enzymes of the thiol biosynthetic pathway (Fairlamb and Cerami, 1992; Haimeur et al., 1999), 2. increased expression of the ABC transporter, *MRPA*, which sequesters SbIII-thiol conjugate (Legare et al., 2001; Mukherjee et al., 2007), 3. decreased uptake of the drug by low expression of *AQP1* gene (Gourbal et al., 2004; Marquis et al., 2005; Maharjan et al., 2008), that codes for the protein responsible for the uptake of SbIII.

Disruption of one of the alleles of aquaglyceroporin gene in *L. major* has been reported to result in 10-fold increase in resistance to SbIII (Gourbal et al., 2004). However, increased expression of *AQP1* in antimony-resistant parasite resulted in reversal of resistance in *L. major* (Gourbal et al., 2004; Marquis et al., 2005). Gene expression analysis of natural SbV resistance in *L. donovani* isolates from Nepal showed that downregulation of *AQP1* led to reduced uptake of antimonite (Gourbal et al., 2004; Decuypere et al., 2005). Our earlier studies on a small subset of Indian clinical isolates however indicated that while downregulation of *AQP1* was one of the mechanisms of antimony resistance, it was however not a universal feature in all the isolates (Vasudevan et al., 2001), Furthermore, recent reports have shown that single mutation at Glu-152 to alanine in the extracellular C-loop of LmAQP1 abrogated metalloid permeability and is critical for the metalloid transport (Richard et al., 2004).

In the present study, we have used both VL and PKDL isolates that were either sensitive or resistant to SAG. Although there are several biological studies done on VL strains of *Leishmania*, studies on PKDL strains are few, primarily because of the difficulty in culture isolation of the parasite from dermal lesions. Molecular
genotyping of VL and PKDL isolates by PFGE and PCR-RFLP of the ITS region of *L. donovani* clinical isolates showed polymorphism in the isolates. Genetic heterogeneity between the VL and PKDL isolates observed in the present study and also among the VL isolates from the same geographic region is in concurrence with the previous studies (Dey and Singh, 2007; Thakur et al., 2008). However, it would be interesting to monitor the parasite population with other powerful genotyping tools in order to establish the differences between PKDL and VL isolates.

The *in vitro* susceptibility profiles obtained with SbIII correlated well with the clinical information and accordingly the isolates were classified as SAG-S or SAG-R. Out of eight PKDL isolates used in the present study, six were found to be refractory to antimony. In India, Post-kala-azar dermal leishmaniasis (PKDL), a complication of VL, occurs as a sequel to kala-azar after 1–7 years of treatment (Thakur et al., 2008; Croft SL, 2008). High incidence of refractoriness to SAG in these PKDL isolates may be because of the parasites previous exposure to SAG during treatment for VL. Alternatively, the higher doses of SAG that are required for treatment of PKDL may be necessitated by host factors. Recent clinical and experimental data shows that SAG has profound influence on the immune response (Saha S, 2007). Saha et al have recently reported that SAG had contrasting effect on the production of interleukin (IL)-10 and transforming growth factor (TGF)-β levels in PKDL and VL patients. It is therefore possible that these factors may also be responsible for susceptibility/resistance to antimony. However, further studies are required to verify this hypothesis.

We checked the differences in SbIII accumulation in the antimony sensitive and the resistant clinical isolates and its correlation with the SAG sensitivity profile. We found ~2-7-fold reduced accumulation of SbIII in the SAG-resistant field isolates when compared to the sensitive isolates.

We further characterized the role of *AQP1* in SbIII transport in the Indian clinical isolates. Southern blot hybridization showed polymorphism in the *AQP1* gene in both VL and PKDL isolates. While most strains had one copy of the *AQP1* gene, RK1-S, a SAG-sensitive isolate had two copies of the *AQP1* gene. To our surprise, amplification of *AQP1* gene was observed in the resistant strain, NR3A-R.
Interestingly, molecular karyotyping of NR3A-R showed variation in the genotype compared to other PKDL and VL isolates. No polymorphism was observed in the \textit{AQP1} gene in the VL isolates even though genetic heterogeneity was observed among the VL isolates, MC7-S, MC4-R, MC8-R and MC9-R.

Downregulation of \textit{AQP1} RNA expression as determined by real-time PCR was observed in the seven resistant isolates. Two isolates NR3A-R and MC8-R, however showed upregulation of \textit{AQP1} RNA. In the SAG-R strain, NR3A-R, increased \textit{AQP1} RNA expression correlated to the amplification to the \textit{AQP1} gene. In another resistant strain MC8-R, though upregulation of RNA levels similar to that of NR3A-R was detected, gene amplification of \textit{AQP1} gene was not present. A SAG-sensitive isolate, RK1-S, having two copies of \textit{AQP1} gene was found to have RNA expression level similar to that of the other sensitive isolates having a single copy of the \textit{AQP1} gene. We had reported similar exceptions in the \textit{AQP1} expression in the clinical isolates earlier (Maharjan et al., 2008).

Since we observed this discrepancy in some of the isolates and were unable to correlate the downregulation of \textit{AQP1} expression to SAG resistance, we decided to check if single mutation at Glu-152 to alanine (corresponding to Glu-185 in the full length sequence) in the extracellular C-loop of \textit{AQP1} abrogated metalloid permeability. This mutation has been reported to be critical for the metalloid transport (Uzcategui et al., 2008). In order to do so we decided to clone and sequence the \textit{AQP1} gene of these isolates. Although we found differences in the AQP1 sequences of the strains MC7-S, MC4-R, MC8-R and MS2-S, the observed changes were not at the positions Glu-152 and Arg-230 regions, which have been shown earlier by site directed mutagenesis to be responsible for metal (AsIII/SbIII) transport (Beitz, 2005; Uzcategui et al., 2008). It will be interesting to see if one or all these alterations in the \textit{AQP1} gene have a role in metalloid transport.

Analysis of \textit{L. major} and \textit{L. infantum} genomes shows the presence of five AQPs: AQP1, AQP\(\alpha\), AQP\(\beta\), AQP\(\gamma\) and AQP\(\delta\) (Beitz, 2005). While the role of \textit{L. major} AQP1 has been established in detail the role of other AQPs has not yet been worked out. It is possible that one of these AQPs may also have a role in metalloid transport. However, this needs to be established.
Earlier findings elucidated that resistance in the laboratory strains are also operational in the field isolates as the overexpression of AQP1 conferred sensitivity to the resistant isolates (Marquis et al., 2005). It has been reported earlier that transfection of LmAQP1 in a pentostam-resistant field isolate sensitized the parasite in the macrophage-associated amastigote form (Gourbal et al., 2004). In present study, we also found that transfection of AQP1 in the antimony-resistant field isolate conferred increased expression, facilitated SbIII uptake and sensitivity to antimony.

Drug resistance could be due to decreased influx and/or increased efflux of the drug. In Leishmania, AQP1 has been shown to mediate the uptake of trivalent antimony (Gourbal et al., 2004). Down regulation of AQP1 in antimony-resistant clinical isolates from Nepal and India has been reported earlier (Decuypere et al., 2005; Maharjan et al., 2008). Role of ABC transporter, MRPA, in conferring antimony resistance by sequestration of metal-thiol conjugates in Leishmania clinical isolates has also been reported earlier (Mukherjee et al., 2007). MRPA has been reported to decrease influx of antimony rather than increase efflux (Callahan HL, 1994). A metal efflux pump has also been reported in Leishmania plasma membrane. Like MRPA, this efflux pump also recognizes the metal conjugated to thiols and requires ATP (Mukhopadhyay et al., 1996). The identity of this efflux pump is not known. Earlier reports have shown no significant role of this efflux system in antimony resistance (Dey et al., 1996). Furthermore, an increased level of trypanothione (TSH), the main cellular thiol in Leishmania, has been observed in mutants selected for antimony resistance (Mukhopadhyay et al., 1996).

All the resistant clinical isolates used in the present study showed increased expression of MRPA, altered thiol levels (manuscript in preparation) and decreased uptake of SbIII. However, downregulation of AQP1 was not observed in all the resistant clinical isolates in the present study. As clearly seen from our present and earlier studies, resistance mechanisms found in the laboratory strains can be found in the clinical isolates but there are exceptions. This would indicate that reduced accumulation observed in these cells results from a channel or transporter different from AQP1 or from a more active efflux system (Dey et al., 1996).