

## Assessing aquaglyceroporin gene status and expression profile in antimony-susceptible and -resistant clinical isolates of *Leishmania donovani* from India

Swati Mandal<sup>1</sup>, Mahendra Maharjan<sup>1</sup>, Sarman Singh<sup>2</sup>, Mitali Chatterjee<sup>3</sup> and Rentala Madhubala<sup>1\*</sup>

<sup>1</sup>School of Life Sciences, Jawaharlal Nehru University, New Delhi, 110067, India; <sup>2</sup>Division of Clinical Microbiology, All India Institute of Medical Sciences, New Delhi, India; <sup>3</sup>Institute of Post Graduate Medical Education and Research, Kolkata, India

\*Corresponding author. Tel/Fax: +91-11-26742630; E-mail: madhubala@mail.jnu.ac.in

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**Objectives:** Clinical resistance to pentavalent antimonials results from an interplay between uptake, efflux and sequestration in *Leishmania*. Aquaglyceroporins (AQPs) have been shown to facilitate uptake of trivalent metalloids. Down-regulation of AQP1 in *Leishmania* results in resistance to trivalent antimony, whereas overexpression of AQP1 in drug-resistant parasites can reverse the resistance. The present work investigates the role of AQP1 in monitoring antimonial resistance in Indian leishmaniasis.

**Methods and results:** Susceptibility to trivalent antimony as determined *in vitro* with intracellular amastigotes from both visceral leishmaniasis (VL) and post-kala-azar dermal leishmaniasis (PKDL) patients correlated well with the clinical response. Higher accumulation of trivalent antimony (SbIII) was observed in all susceptible isolates compared with resistant isolates. Reduced accumulation of SbIII correlated, with a few exceptions, with down-regulation of AQP1 RNA as determined by real-time PCR. Cloning and sequencing of the AQP1 gene from both VL and PKDL isolates showed sequence variation in four of the clinical isolates. None of the isolates had an alteration of Glu152 and Arg230, which have been previously shown to affect metalloid transport. Transfection of the AQP1 gene in a sodium antimony gluconate-resistant field isolate conferred susceptibility to the resistant isolate.

**Conclusions:** Our studies indicate genetic variation in VL and PKDL isolates. Down-regulation of AQP1 correlates well with clinical drug resistance in a majority of Indian VL and PKDL isolates. AQP1 gene expression at both the genetic and transcriptional level showed positive correlation with SbIII accumulation, with some exceptions.

**Keywords:** AQP1, biomarker, antimony resistance, Indian isolates

### 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.<sup>1</sup> PKDL is a cutaneous manifestation of VL. In India and Sudan the disease develops months to years after the patient has recovered from VL.<sup>2</sup> Current chemotherapeutic agents are ineffective because of their high toxicity and emergence of drug resistance. The pentavalent antimonial, sodium antimony gluconate (SAG or SbV), is the age-old conventional therapy for VL.<sup>3</sup> However, increasing resistance to SAG has emerged as a major barrier in the treatment of VL.

Resistance to SAG in field isolates is less well defined. Recent reports show that determination of susceptibility using *in vitro* assays correlates well with the clinical response.<sup>4–6</sup> Our previous

studies showed that mechanisms of resistance to antimony reported in laboratory strains are also operational in field isolates.<sup>7</sup> Variability in the mechanism of resistance and a varying degree of resistance were encountered in field isolates.<sup>7</sup> Development of drug resistance has been a hindrance in chemotherapy of leishmaniasis and is of considerable importance to identify biomarkers responsible for resistance to antimonials for VL. A number of candidate genes associated with resistance to antimonials have been described in both SAG-resistant laboratory mutants and clinical isolates of *L. donovani*.<sup>8–10</sup>

Aquaglyceroporins (AQPs) are members of the aquaporin superfamily. They are membrane channels which permit transport of small neutral solutes such as glycerol or urea. In *Leishmania* species, AQP1 has been shown to facilitate trivalent antimony (SbIII) transport.<sup>11</sup> Overexpression of AQP in *Leishmania major* (*LmAQP1*) produces hypersusceptibility to SbIII, whereas gene deletion renders the parasite resistant.<sup>11,12</sup> This has provided

a major insight into the uptake mechanism of drugs in *L. major*.<sup>11,13</sup> Prediction of the topology of *L. major* AQP1 showed that LmAQP1 consists of six membrane-spanning helices containing the canonical Asn-Pro-Ala (NPA) motifs.<sup>14</sup> These helices are connected by five loops referred to as A–E.<sup>14</sup> Glutamate (Glu152) present in the C-loop of LmAQP1 is reported to be critical for metalloid permeability. A single mutation from Glu152 to alanine selectively abrogates metalloid permeability.<sup>14</sup>

In the present study we examined the role of AQP1 in monitoring antimonial resistance in Indian leishmaniasis. Susceptibility to SAG as determined *in vitro* with intracellular amastigotes from both VL and 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 the AQP1 gene from both VL and PKDL isolates was also performed to check the role of Glu152 and Arg230 in antimony resistance. Transfection of the AQP1 gene in an SAG-resistant field isolate conferred susceptibility 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.

## Materials and methods

### 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 in or travel to Bihar, India. The diagnosis of VL was performed by rK39 dip test and, if found positive, was confirmed by bone marrow aspiration and microscopic examination of Giemsa-stained smears that demonstrated the presence of *L. donovani* bodies. The bone marrow/splenic aspirates were collected aseptically and transferred to agar slants containing 1 mL of modified M199 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 ( $>5 \times 10^6$ ), 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. donovani* bodies in dermal lesions. The punch biopsy material, collected aseptically, was cut into small pieces and placed in a 25 mm<sup>2</sup> tissue culture flask containing Schneider's medium (Sigma, USA) supplemented with 10% FBS, 0.13 mg/mL penicillin and streptomycin. After successful transformation, when the cell number exceeded  $5 \times 10^6$ , they were gradually adapted to medium A.

Once transformed, both VL and PKDL isolates were cloned on semi-solid M199 medium containing 1% Bacto agar (Difco) and 10% heat-inactivated FBS. Colonies were picked after 6–7 days and transferred to medium A.

Ethical approval 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.

### 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, and strains RK1, MS2, NR3A, RMP8 (HM/IN/RMP8), RMP19 (HM/IN/RMP19),

RMP142 (HM/IN/RMP142), RMP155 (HM/IN/RMP155) and RMP240 (HM/IN/RMP240) were isolated from patients with PKDL. Both VL and PKDL strains were from the antimony-resistant zone of Bihar, India. PKDL isolates RMP8 (HM/IN/RMP8), RMP19 (HM/IN/RMP19), RMP142 (HM/IN/RMP142), RMP155 (HM/IN/RMP155) and RMP240 (HM/IN/RMP240) were a gift from the Division of Clinical Microbiology, All India Institute of Medical Sciences, New Delhi.<sup>15</sup> SAG-susceptible strains AG83 and 2001 were used as reference strains and have been characterized previously.<sup>7</sup> Promastigotes were routinely cultured at 22°C in modified M199 medium (Sigma, USA) supplemented with 10% heat-inactivated FBS 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 susceptible (SAG-S), whereas isolates from VL and PKDL patients who did not respond to SAG were designated as SAG resistant (SAG-R). Accordingly, SAG-S isolates used in this study included AG83-S, 2001-S, MC7-S, RK1-S and 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. This study was approved by the Institutional level ethics committee.

### DNA construct and transfection

The linearized  $\beta$ -lactamase expression vector (pIR1SAT- $\beta$ LA) containing a  $\beta$ -lactamase-encoding gene used in the present study was a kind gift of Dr Frederick S. Buckner (Washington University, Seattle, WA, USA). When linearized, this vector integrates into the *Leishmania* genome by replacing one copy of the small subunit rRNA gene. The construct was linearized by SmaI restriction digestion (MBI, Fermentas), and 10  $\mu$ g of the linearized construct having the  $\beta$ -lactamase gene was transfected into *L. donovani* log-phase promastigotes ( $4 \times 10^7$ ) by electroporation. Briefly, electroporation was done in 2 mm gap cuvettes at 450 V and 500  $\mu$ F (Bio-Rad) as reported previously.<sup>16</sup> The transfectants were selected for resistance to 50  $\mu$ g/mL nourseothricin (Sigma, USA).<sup>16,17</sup>

### Chemosensitivity profiles of SAG-S and SAG-R strains in an amastigote macrophage model using a $\beta$ -lactamase assay

Stationary-phase *Leishmania* promastigotes expressing the  $\beta$ -lactamase gene were used to infect J774A.1 macrophages. The macrophage cell line J774A.1 (ATCC) 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  $\beta$ -lactamase activity by removing the medium by gentle pipetting followed by addition of 50  $\mu$ L of 50  $\mu$ M CENTA (Cambiochem, La Jolla, CA, USA) in PBS and 0.1% Nonidet P-40. The plates were incubated at 37°C for 4 h.<sup>16,17</sup> Catalysis of the substrate (CENTA) to its chrome yellow colour product was quantified at an optical density of 405 nm using a 96-well plate reader (Bio-Rad).<sup>17</sup> The IC<sub>50</sub> was determined from the graph representing different concentrations of the inhibitor plotted against percentage growth.

### Determination of *in vitro* antileishmanial activity in promastigote cultures

In order to characterize the SAG susceptibility profile of *L. donovani* promastigotes, a modified MTT (Sigma, USA) assay was performed as described previously.<sup>18</sup> Briefly, 25  $\mu$ L of promastigotes ( $2.5 \times 10^4$  cells/well) were cultured in a 96-well flat-bottomed plate (Nunc, Roskilde, Denmark) and incubated with 25  $\mu$ L of different drug concentrations at 26°C. After 72 h, 10  $\mu$ L of MTT (5 mg/mL) in 1 $\times$  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 a microplate reader. The IC<sub>50</sub> (50% inhibitory concentration) was determined from the graph representing different concentrations of the inhibitor plotted against percentage cell growth.

### Drug uptake assay

Uptake studies were performed as described previously.<sup>13</sup> 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 PBS (pH 7.4) and were resuspended in PBS containing 10 mM glucose at a density of  $5 \times 10^7$  cells/mL. Cells were then incubated at 27°C with 100 µM SbIII for 30 min and 500 µL aliquots were collected at 0 and 30 min. Cells were pelleted down and washed twice with an equal volume of ice-cold PBS and centrifuged at 17000 g for 1 min 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 HPLC-grade water. Antimony levels were analysed by inductively coupled plasma mass spectroscopy (ICP-MS) as reported previously.<sup>13,19</sup> Each uptake assay was repeated twice with triplicates in each set.

### Nucleic acid isolation, PFGE and hybridization analysis

Genomic DNA was isolated from  $\sim 2 \times 10^9$  cells from 10–15 mL of a mid-log-phase promastigote culture of all field isolates by a standard procedure.<sup>20</sup> Genomic DNA (5 µg) was digested with the enzyme SalI and subjected to electrophoresis. The fragments were transferred to Hybond™-N<sup>+</sup> membrane (Amersham Pharmacia Biotech) and subjected to Southern blot analysis.

Chromosomes of the clinical isolates were separated by 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 0.5× Tris borate/EDTA, with buffer circulation at a constant temperature of 14°C. The gel running conditions were as follows: voltage gradient of 6 V/cm; initial switch time of 60 s; final switch time of 120 s; and run time of 24 h. *Saccharomyces cerevisiae* chromosomes were used as size markers.

Promastigotes ( $2 \times 10^8$ ) 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 a 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/1 mM EDTA (pH 8.0) and 100 µg/mL sheared denatured salmon sperm DNA. Hybridization was done with a denatured  $\alpha$ -[<sup>32</sup>P]dCTP-labelled DNA probe at  $10^6$  cpm/mL, which was labelled by random priming (NEB Blot® Kit, New England Biolabs, Inc.). Labelling was performed according to the manufacturer's protocol. The DNA probe used in the present study was a 945 bp AQP1 probe (accession number, EF600686). Membranes were washed, air dried and exposed to an imaging plate. The images were developed by PhosphorImager (Fuji film FLA-5000, Japan) using Image Quant software.

### Molecular genotyping by PCR-restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (ITS) region of *L. donovani* clinical isolates

The entire ITS in the ribosomal operon was amplified using the primers: LITSR, 5'-CTGGATCATTTCCGATG-3'; and LITSV, 5'-ACACTCAGGCTGTGA AAC-3'.<sup>21</sup> A 10 µL aliquot of the amplified ITS region was digested with HaeIII for 2 h at 37°C using the conditions recommended by the

manufacturer. Restriction fragments were separated on a 2% agarose gel for 2–4 h in 1× Tris borate/EDTA (90 mM Tris borate/0.2 mM EDTA) buffer and visualized under UV light after staining with ethidium bromide.

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

Total RNA was isolated from  $10^8$  *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, WI, USA) to avoid any genomic DNA contamination. The quality and quantity of the RNA were determined using the RNA 6000 Nano Lab Chip Kit on a Bio-analyzer 2100 (Agilent Technologies). The sequences of the primers for AQP1 were: forward, 5'-CTGTGCTTTGGTGCCTTTCC; and reverse, 5'-GCCTTTGGGCGTCGTC.<sup>22</sup> The sequences of the primers for the GAPDH (glyceraldehyde phosphate dehydrogenase) control were: forward, 5'-GAAGTACACGGTGGAGGCTG; and reverse, 5'-CGCTGATCACG ACCTTCTC.<sup>7</sup> Complementary DNAs from promastigotes were synthesized from 500 ng of total RNA using the AccuSuperscript High Fidelity RT-PCR Kit (Stratagene, La Jolla, CA, USA) and oligo(dT)<sub>18</sub> primers following the manufacturer's instructions. Real-time PCR was performed in triplicate in 25 µL volumes using the 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.

### Cloning and sequencing of the AQP1 gene from antimony-susceptible and antimony-resistant *L. donovani* isolates

The AQP1 gene from the VL and PKDL clinical isolates was 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'-GCTCTAGAATGAACCTCTCTACAAGCACA-3', and an antisense primer with a flanking HindIII site, 5'-CCCAAGCTTCTAGAAGTTGGGTG GAATGA-3'.<sup>23</sup> PCR was performed in a 50 µL reaction volume containing 100 ng of genomic DNA, 25 pmol of each of the gene-specific forward and reverse primers, 200 µM of each dNTP, 2 mM MgCl<sub>2</sub> and 5 U of 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 and 72°C for 45 s for a total of 30 cycles. Final extension was carried out for 10 min at 72°C. A single-band 945 bp PCR product was obtained and subcloned into pTZ57R/T (InsTAclone™ PCR Cloning Kit, MBI Fermentas) and subjected to automated sequencing. Multiple alignment of amino acid sequences was performed using the CLUSTAL W program. The transmembrane domain of the sequences was identified using HMMTOP protein topology prediction online software ([www.enzim.hu/hmmtop](http://www.enzim.hu/hmmtop)).

### Transfection and overexpression of the AQP1 gene

The AQP1 gene from the susceptible AG83-S *L. donovani* strain was PCR amplified. The amplified DNA fragment was cloned into the XbaI-HindIII site of the psp $\alpha$ neo $\alpha$  *Leishmania* shuttle vector (kindly provided by Dr Marc Ouellette, Quebec, Canada) to create a psp $\alpha$ neo $\alpha$ -AQP1 gene construct containing the neomycin phosphotransferase gene (G418) antibiotic marker. The construct (20 µg) was transfected into the promastigotes of the susceptible AG83-S isolate and resistant MC4-R *L. donovani* promastigotes by electroporation using the

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

Strain no.	Strain ID	Geographical location	Disease	Mean IC <sub>50</sub> ± SD (μM)
1	AG83-S	Bihar	VL	6.2 ± 1.8
2	2001-S	Bihar	VL	0.9 ± 0.12
3	MC7-S	Siwan, Bihar	VL	8 ± 3.3
4	MC4-R	Samastipur, Bihar	VL	32 ± 5.2 (5.16)*
5	MC8-R	Chapra, Bihar	VL	26 ± 4.6 (4.2)*
6	MC9-R	Samastipur, Bihar	VL	18 ± 2.1 (3.0)*
7	RK1-S	Bihar	PKDL	0.01 ± 0.02
8	MS2-S	Bihar	PKDL	4.75 ± 0.12
9	NR3A-R	Bihar	PKDL	52 ± 4.9 (8.4)*
10	HM/IN/RMP8	Bihar	PKDL	18.5 ± 1.06 (3.0)*
11	HM/IN/RMP19	Bihar	PKDL	14 ± 0.35 (2.3)*
12	HM/IN/RMP142	Bihar	PKDL	>100.00 (>10.0)*
13	HM/IN/RMP155	Bihar	PKDL	40 ± 3.5(6.5)*
14	HM/IN/RMP240	Bihar	PKDL	30 ± 1.41(5.0)*

All values presented are means ± SD of triplicates from three independent experiments. The fold differences with respect to the AG83-S strain are shown in parentheses.

\*Statistically significant at  $P < 0.001$  when compared with the corresponding values obtained for AG83-S.

parameters 450 V and 500 μF (Bio-Rad). Transfectants were selected for resistance to G418 (40 μg/mL). Transfectants with the psp $\alpha$ neo $\alpha$  vector alone were used as a control. Drug uptake and susceptibility assays were performed as described above.

### Statistical analysis

Data were analysed using the Student's *t*-test. The data are presented as means ± SD. The results are representative of three independent experiments. A *P* value of <0.05 was considered to be statistically significant.

## Results

### Susceptibility and characterization of SAG-S and SAG-R strains

A total of 14 field isolates were collected from patients residing in the antimony-resistant zone of Bihar, India. The chemosensitivity profiles of the SAG-S and the SAG-R strains to SbIII were tested in intracellular amastigotes by a β-lactamase assay as described in the Materials and methods section. SAG-S isolates AG83-S, 2001-S, MC7-S, RK1-S and MS2-S from SAG-responsive patients had IC<sub>50</sub> values of 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 and RMP240-R from SAG-unresponsive patients had IC<sub>50</sub> values that were ~2- to >10-fold higher than that of the susceptible isolate AG83-S (Table 1).

Molecular karyotyping of all *L. donovani* clinical isolates was carried out using a contour-clamped homogeneous electric field apparatus (Figure 1a). The molecular karyotype of PKDL isolates when compared with 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 with the other VL and PKDL isolates.

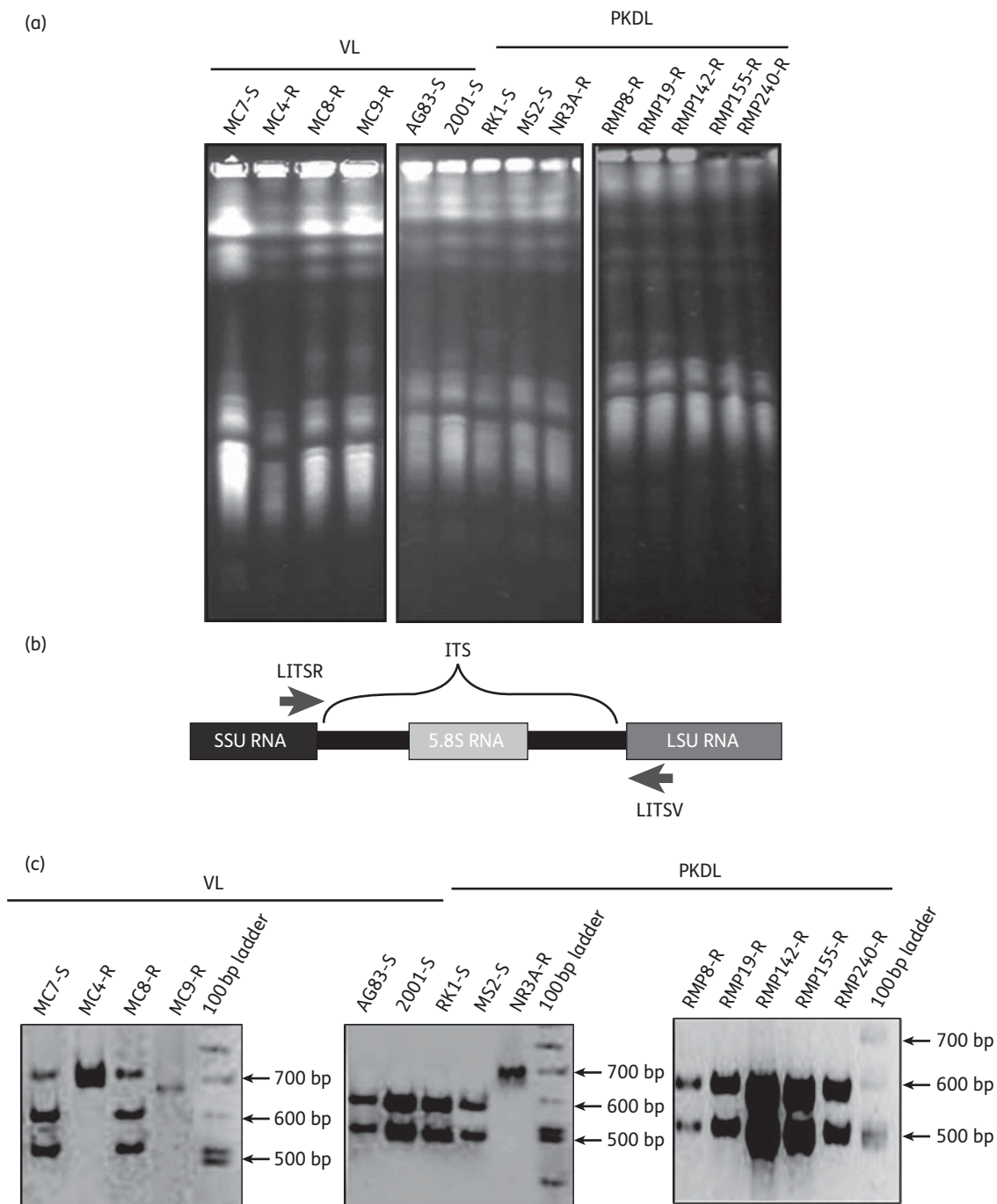
RFLP of the amplified ITS has been used successfully as a genotyping marker in molecular epidemiology.<sup>21</sup> HaeIII restriction digestion of the entire ITS region was performed to further confirm the genetic variation in the isolates (Figure 1b and c). Comparison of the HaeIII restriction digestion pattern of the amplified ITS region indicated polymorphism among clinical *L. donovani* isolates (Figure 1c). 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. Strains MC7-R and MC8-R had a similar banding pattern.

### Accumulation of SbIII in *L. donovani* clinical isolates

ICP-MS was used to show SbIII accumulation inside the cells at the 30 min timepoint.<sup>13,19</sup> Promastigotes were incubated with 100 μM SbIII for 30 min at 27°C as detailed in the Materials and methods section. Antimony-resistant *Leishmania* isolates (SAG-R) had reduced accumulation of SbIII compared with the SAG-S isolates. In comparison with 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 2).

### Gene copy number and expression profiling of the AQP1 gene in SAG-S and SAG-R field isolates

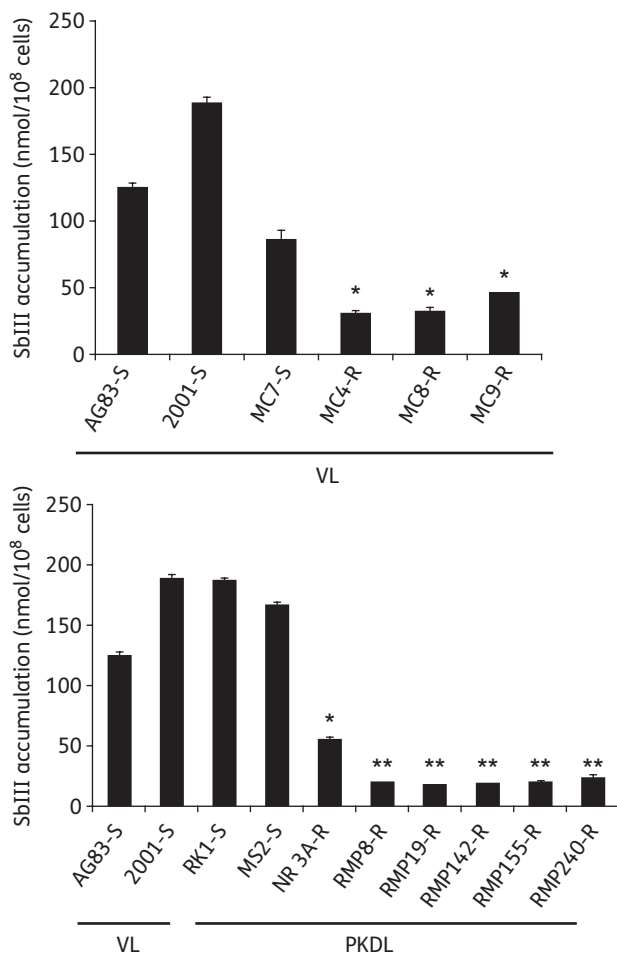
Southern blot analysis showed variation in the gene copy number of the AQP1 gene in both VL and PKDL isolates. The restriction enzyme SalI, which digests outside the gene producing a 10.8 kb restriction fragment (expected size), was used to check the copy number of the gene. A single hybridizing fragment of



**Figure 1.** (a) PFGE of SAG-S and SAG-R VL and PKDL isolates of *L. donovani* chromosomes. Agarose blocks containing chromosomal DNAs of promastigotes were prepared and subjected to PFGE for 24 h at 14°C as reported in the Materials and methods section. (b) Schematic position of the internal transcribed spacer (ITS) in the ribosomal operon and position of the primers used to amplify the ITS sequence. Sequences of the primers LITSR and LITSV are given in the Materials and methods section. SSU, small subunit; LSU, large subunit. (c) 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 a 2% agarose gel for 2–4 h to document differences in RFLP patterns. A 100 bp ladder was used as a molecular size marker.

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 3a). However, RK1-S had 10.8 and 7 kb bands representing two copies of *AQP1*. Interestingly, NR3A-R, a PKDL isolate that was ~8.4-fold resistant to SbIII, showed amplification of the *AQP1* gene.



**Figure 2.** Accumulation of SbIII in VL and PKDL *L. donovani* field isolates as measured by ICP-MS. Promastigotes were incubated with 100  $\mu$ M SbIII for 30 min at 27°C as described in the Materials and methods section. Data are shown as means  $\pm$  SD of two replicate experiments with triplicates in each set. \* $P$ <0.01 and \*\* $P$ <0.001 when compared with AG83-S isolate values.

Comparison of *AQP1* gene expression in SAG-S versus SAG-R field isolates was carried out to verify whether there was any correlation between *AQP1* gene expression and the 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 *AQP1* (gene-specific) and *GAPDH* (internal control) primers was performed. Down-regulation 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 with the expression in the susceptible isolate AG83-S (Figure 3b). In the present study, although down-regulation of *AQP1* was observed in the majority of the SAG-R isolates, there were two exceptions. Two SAG-resistant strains, NR3A-R and MC8-R, showed up-regulation of *AQP1* gene expression. NR3A-R and MC8-R were 8.4- and 4.2-fold resistant to SbIII, respectively, as compared with the susceptible

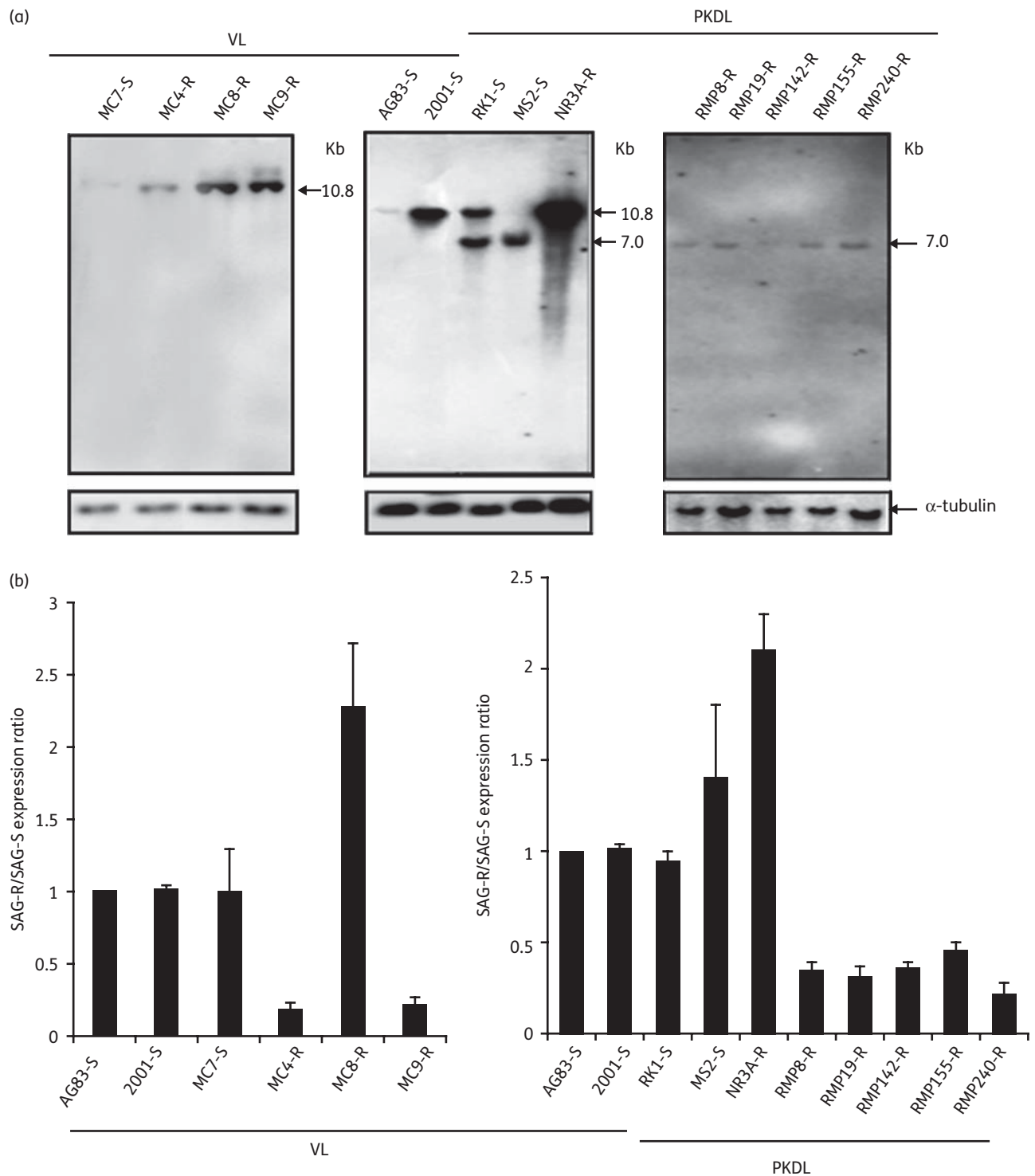
isolate AG83-S. *AQP1* gene expression levels of susceptible clinical isolates 2001-S, MC7-S, RK1-S and MS2-S were similar to that of the SAG-S isolate, AG83-S.

### Comparison of sequence analysis of the *AQP1* gene of SAG-S and SAG-R field isolates

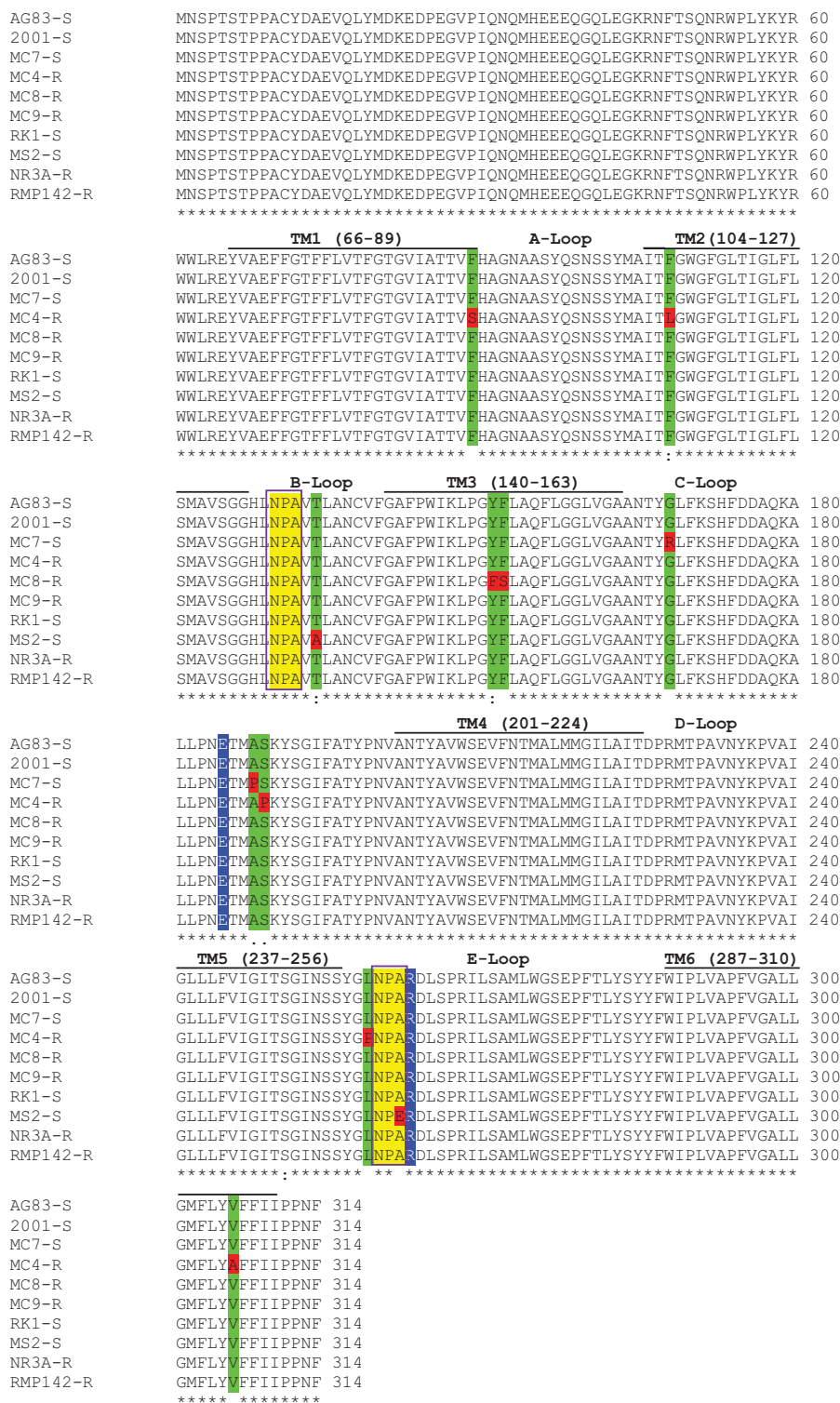
Down-regulation of *LmAQP1* is reported to be linked to antimony resistance.<sup>13</sup> Introduction of point mutations in transporter proteins is yet another mechanism of drug resistance in *Leishmania*.<sup>24,25</sup> Recently, a point mutation in the extracellular loop of the *LmAQP1* channel, at Glu152 (corresponding to Glu185 in the full-length sequence), was shown to confer SbIII resistance in *L. major* laboratory mutants.<sup>14</sup> The full-length coding sequence (945 bp) of the *L. donovani* *AQP1* gene was cloned and sequenced from six VL and five PKDL isolates. The transmembrane topology of the proteins was predicted using an 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 4). The predicted protein sequence of the susceptible strains AG83-S, 2001-S and RK1-S was similar to that of the SAG-resistant strains MC9-R and NR3A-R (Figure 4). The hourglass model of aquaporin reported previously<sup>26,27</sup> has been used to show mutations observed in the *AQP1* protein of different *L. donovani* field isolates (Figure 5a). The model shows the presence of six transmembrane domains (1–6) connected by five helical loops (A–E). Loops B and E contain conserved Asn-Pro-Ala (NPA) motifs and form short  $\alpha$ -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 (Figures 4 and 5), but none of the differences was uniform. Conserved signatures of the *AQP1* protein were deduced from the multiple sequence alignments shown in Figure 4. Specific variation in the sequences of the isolates is shown in Figure 5(b). Point mutation at Glu185 and Arg263 residues (corresponding to Glu152 and Arg230)<sup>14</sup> could not be detected in any of the resistant field isolates, indicating that the SAG resistance phenotype, at least in Indian *L. donovani* VL and PKDL field isolates, does not result from the point mutation of Glu185 and Arg263 residues of the sequence encoded by the *AQP1* gene.

### Overexpression of *AQP1* in an antimony-resistant field isolate conferred increased expression and susceptibility to antimony, and facilitated SbIII uptake

To determine whether overexpression of *AQP1* conferred susceptibility to resistant isolates, we transfected the *AQP1* construct into the promastigotes of both the antimony-susceptible 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 6a). The role of *AQP1* in the uptake of SbIII was investigated. *LdAQP1*-transfected strains AG83-S and MC4-R showed rapid uptake of SbIII compared with vector-control transfectants (Figure 6a).

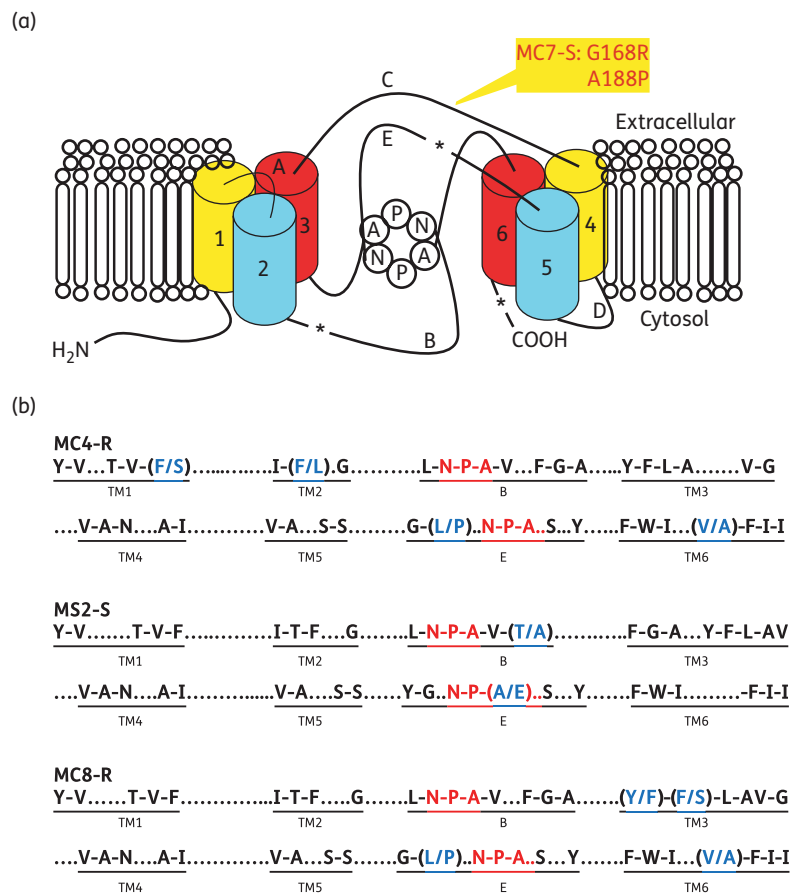


**Figure 3.** (a) Southern blot analysis of the *AQP1* gene in the SAG-S and the SAG-R *L. donovani* VL and PKDL field isolates. Total genomic DNA was isolated and digested with *Sal*I. The digested DNA was electrophoresed, blotted and hybridized with a full-length *AQP1*-specific probe of 945 bp. The sizes of the hybridizing bands were determined using *Hind*III-digested  $\lambda$  DNA. The blot was re-hybridized with an  $\alpha$ -tubulin probe to monitor the amount of digested DNA layered on the gel. (b) 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-susceptible isolate AG83. Results are the means of three independent experiments performed using three different RNA preparations.



**Figure 4.** AQP1 protein sequences from the SAG-S and the SAG-R *L. donovani* (VL and PKDL) isolates were aligned using the CLUSTAL 2.0.11 multiple sequence alignment program. Transmembrane topology of the proteins was predicted using an 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. Loops B and E containing conserved Asn-Pro-Ala (NPA) motifs form short  $\alpha$ -helices that fold back into the membrane from the opposite side, forming a channel for glycerol transport. Two NPA motifs are boxed. Point mutations are marked in red, and Glu185 and Arg263 (corresponding to Glu152 and Arg230 in the partial sequence<sup>14</sup>) amino acid residues linked to SbIII susceptibility are shown in blue. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.





**Figure 5.** (a) An hourglass model of aquaporin according to Jung *et al.*<sup>26</sup> and Zardoya *et al.*,<sup>27</sup> showing mutations in *L. donovani*. Mutations found in the strain MC7-S are shown in the box. (b) Conserved signatures of the AQP1 protein of MC4-R, MS2-S and MC8-R that were deduced from the comparative analysis of the multiple alignment in Figure 4. Asn-Pro-Ala (NPA) motifs are marked in red and amino acid mutations are marked in blue. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

Promastigotes of AG83-S, an antimony-susceptible isolate overexpressing *AQP1*, became  $\sim 3.1$ -fold more susceptible to SbIII when compared with the promastigotes of the parent strain (transfected with the control vector), the  $IC_{50}$  values being  $25 \pm 2.5$  and  $8 \pm 3 \mu M$ , respectively (Figure 6b). MC4-R promastigotes overexpressing *AQP1* were  $\sim 4.2$ -fold more susceptible to SAG when compared with the  $IC_{50}$  of promastigotes of the parent strain (transfected with the control vector), the  $IC_{50}$  values being  $68 \pm 4.0$  and  $16 \pm 2.7 \mu M$ , respectively. Increased uptake of SbIII by the resistant isolate MC4-R is possibly responsible for the re-sensitization of this drug-resistant mutant.

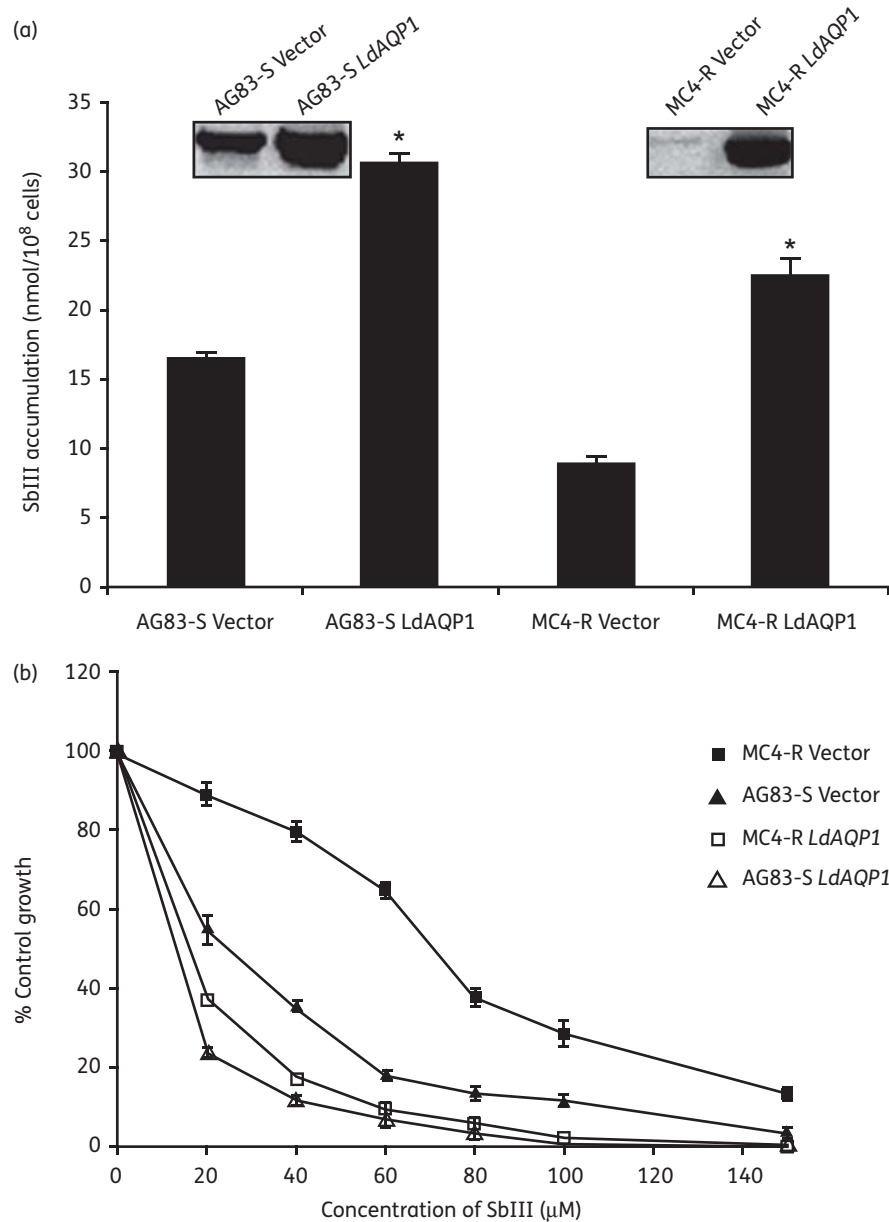
## Discussion

The mechanism of antimony resistance in *Leishmania* has been studied extensively. An 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 the chemotherapeutic approach in field conditions. It has been reported previously that the 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.<sup>28</sup>

Resistance to antimony in both the laboratory-raised resistant strains and clinical isolates has been associated with: (i) overexpression of the enzymes of the thiol biosynthetic pathway;<sup>29,30</sup> (ii) increased expression of the ABC transporter, MRPA, which sequesters the SbIII–thiol conjugate;<sup>7,31</sup> and (iii) decreased uptake of the drug by low expression of the *AQP1* gene,<sup>11,13,23</sup> which codes for the protein responsible for the uptake of SbIII.

Disruption of one of the alleles of the *AQP1* gene in *L. major* has been reported to result in a 10-fold increase in resistance to SbIII.<sup>11</sup> However, increased expression of *AQP1* in an antimony-resistant parasite resulted in reversal of resistance in *L. major*.<sup>11,13</sup> Gene expression analysis of natural SbV resistance in *L. donovani* isolates from Nepal showed that down-regulation of *AQP1* led to reduced uptake of antimonite.<sup>22</sup> Our previous studies on a small subset of Indian clinical isolates, however, indicated that while down-regulation of *AQP1* was one of the mechanisms of antimony resistance, it was not a universal feature in all the isolates.<sup>23</sup> Furthermore, recent reports have shown that a single mutation at Glu152 (to alanine) in the extracellular C-loop of LmAQP1 abrogated metalloid permeability, showing that Glu152 is critical for metalloid transport.<sup>14</sup>

In the present study, we have used both VL and PKDL isolates that were either susceptible or resistant to SAG. Although there



**Figure 6.** (a) Accumulation of SbIII in AQP1-overexpressing *L. donovani* field isolates as measured by ICP-MS. Promastigotes were incubated with 100 μM SbIII for 30 min at 27°C as described in the Materials and methods section. Data are shown as means ± SD of two replicate experiments with triplicates in each set. \* $P < 0.01$  compared with respective controls. Northern blot analysis (insets) of vector-transfected controls and AQP1-transfected lines was performed as described in the Materials and methods section. (b) Effect of different concentrations of SbIII on SAG-susceptible 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 ± SD of three determinations.

are several biological studies that have been performed 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 agreement with previous studies.<sup>15,32</sup> 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, PKDL, a complication of VL, occurs as a sequel to kala-azar after 2–7 years of treatment.<sup>33,34</sup> The high incidence of refractoriness to SAG in these PKDL isolates may be because of the previous exposure of the parasites 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 show that SAG has a profound influence on the immune response.<sup>35</sup> Saha et al.<sup>35</sup> have recently reported that SAG had a varying effect on the production of interleukin (IL)-10 and transforming growth factor (TGF)- $\beta$  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-susceptible and -resistant clinical isolates and its correlation with the SAG susceptibility profile. We found ~2- to 7-fold reduced accumulation of SbIII in the SAG-resistant field isolates when compared with the susceptible 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, an SAG-susceptible isolate, had two copies of the *AQP1* gene. To our surprise, amplification of the *AQP1* gene was observed in the resistant strain NR3A-R. Interestingly, molecular karyotyping of NR3A-R showed variation in the genotype compared with other PKDL and VL isolates. No polymorphism was observed in the *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.

Down-regulation of *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 up-regulation of *AQP1* RNA. In the SAG-R strain NR3A-R, increased *AQP1* RNA expression correlated with the amplification to the *AQP1* gene. In another resistant strain, MC8-R, although up-regulation of RNA levels similar to that of NR3A-R was detected, gene amplification of the *AQP1* gene was not present. An SAG-susceptible isolate, RK1-S, having two copies of the *AQP1* gene, was found to have an RNA expression level similar to that of the other susceptible isolates having a single copy of the *AQP1* gene. We have reported similar exceptions in *AQP1* expression in clinical isolates previously.<sup>23</sup>

Since we observed this discrepancy in some of the isolates and were unable to correlate the down-regulation of *AQP1* expression to SAG resistance, we decided to check if a single mutation at Glu152 (corresponding to Glu185 in the full-length sequence) to alanine in the extracellular C-loop of *AQP1* abrogated metalloid permeability. This mutation has been reported to be critical for metalloid transport.<sup>14</sup> In order to do so we decided to clone and sequence the *AQP1* gene of these isolates. Although we found differences in the *AQP1* sequences of strains MC7-S, MC4-R, MC8-R and MS2-S, the observed changes were not at positions Glu152 and Arg230 which previously have been shown by site-directed mutagenesis to be responsible for metal (AsIII/SbIII) transport.<sup>14,36</sup> It will be interesting to see if one or all these alterations in the *AQP1* gene have a role in metalloid transport.

Analysis of *L. major* and *Leishmania infantum* genomes shows the presence of five AQPs: *AQP1*; *AQP $\alpha$* ; *AQP $\beta$* ; *AQP $\gamma$* ; and *AQP $\delta$* .<sup>36</sup> While the role of *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.

Previous findings elucidated that resistance in laboratory strains is also operational in field isolates as the overexpression

of *AQP1* conferred susceptibility to the resistant isolates.<sup>13</sup> It has been reported previously that transfection of *LmAQP1* in a pentostam-resistant field isolate sensitized the parasite in the macrophage-associated amastigote form.<sup>11</sup> In the present study, we also found that transfection of *AQP1* in an antimony-resistant field isolate conferred increased expression and susceptibility to antimony, and facilitated SbIII uptake.

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 SbIII.<sup>11</sup> Down-regulation of *AQP1* in antimony-resistant clinical isolates from Nepal and India has been reported previously.<sup>22,23</sup> The role of the ABC transporter, MRPA, in conferring antimony resistance by sequestration of metal-thiol conjugates in *Leishmania* clinical isolates has also been reported previously.<sup>7</sup> MRPA has been reported to decrease influx of antimony rather than increase efflux.<sup>37</sup> A metal efflux pump has also been reported in the *Leishmania* plasma membrane. Like MRPA, this efflux pump also recognizes the metal conjugated to thiols,<sup>10</sup> and requires ATP. The identity of this efflux pump is not known. Previous reports have shown no significant role for this efflux system in antimony resistance.<sup>38</sup> Furthermore, an increased level of trypanothione (TSH), the main cellular thiol in *Leishmania*, has been observed in mutants selected for antimony resistance.<sup>10</sup>

All the resistant clinical isolates used in the present study showed increased expression of MRPA, altered thiol levels (S. Mandal, M. Maharjan and R. Madhubala, unpublished results) and decreased uptake of SbIII. However, down-regulation of *AQP1* was not observed in all the resistant clinical isolates in the present study. As clearly seen from our present and previous studies, resistance mechanisms found in laboratory strains can be found in 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.<sup>38</sup>

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## Transparency declarations

None to declare.

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