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Increased parasite surface antigen-2 expression in clinical isolates of *Leishmania donovani* augments antimony resistance

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ABSTRACT

Resistance to sodium antimony gluconate (SAG) is a major cause of therapeutic failure in a large proportion of visceral leishmaniasis (VL) cases. Determinants of SAG resistance have been widely studied; however, the mechanism operating in clinical isolates is poorly understood. In the present study, expression of parasite surface antigen-2 (PSA-2) gene was studied in clinical isolates of *Leishmania donovani* comprising of antimony resistant ($n = 10$) and sensitive ($n = 4$) parasites. The expression of PSA-2 gene was found to be consistently high in SAG resistant clinical isolates ($\geqslant 1.5$-fold) at both transcript and protein level. Further, over-expression of PSA-2 in *L. donovani* isolates (*Ld*PSA-2^{++}) resulted in conversion of SAG sensitive phenotype to resistant. The *Ld*PSA-2^{++} parasites showed significantly decreased susceptibility towards SAG (>12-fold), amphotericin B (>4-fold) and miltefosine (>2.5-fold). Marked decrease in antimony accumulation and enhanced tolerance towards complement mediated lysis was evident in *Ld*PSA-2^{++} parasites. The study established the role of PSA-2 gene in SAG resistance and its potential as a biomarker to distinguish resistant and sensitive clinical isolates of *L. donovani*.

1. Introduction

Visceral leishmaniasis (VL) is a potentially fatal parasitic disease caused by *Leishmania donovani*. The disease is endemic in 70 countries with an estimated 500,000 new infections annually and a total of 200 million people at risk [1]. More than 90% of the estimated VL cases occur in India, Bangladesh, Nepal, Sudan, Ethiopia and Brazil, with India alone sharing almost 50% of the world's total disease burden [1,2]. In the absence of an effective anti-leishmanial vaccine, chemotherapy remains the mainstay of VL control strategy. Widespread resistance against antimonials has been observed in many parts of the world, especially in Bihar, India, where it is reported in above 60 percent of cases [3]. Further, reports of relapse cases have surfaced against the oral drug miltefosine which was recently introduced for treatment of VL under the VL elimination programme [4]. Therefore, there is a need to develop surveillance tools to monitor treatment efficacy as well as emergence of drug resistance in the field.

Majority of the knowledge on antimony resistance in *Leishmania* spp. has been derived from laboratory mutants developed by adapting to increasing drug concentration. Among the suggested mechanisms of antimony resistance are gene amplification and the parasite's inability to convert SbV to SbIII. It is established that the trivalent antimony Sb(III) is the active form of the drug which is generated by reduction of Sb(V) by thiols either by the parasites or the macrophages, or both [5,6].

Earlier studies revealed the modulation of several genes in antimony resistant *Leishmania* parasites such as increased expression of multidrug resistance protein A (MRPA), thiol biosynthetic enzymes, HSP70 protein, a leucine rich repeat (LRR) superfamily protein, histone H2A gene and the SbIII/thiol conjugate sequestering pump, and decreased expression of Aquaporin-1 (AQP1) [5,7–10]. Several glycoproteins located on the cell surface, including gp63 and parasite surface antigen-2 (PSA-2), play a vital role in determining parasite infection and survival [11–14]. PSA-2, detected in all *Leishmania* species except *L. braziliensis*, belongs to the superfamily of leucine rich repeats (LRR) proteins implicated in protein–protein interactions [15,16]. It is shown to be involved in various processes like host invasion by CR-3 receptor of macrophages and evasion of complement mediated lysis [17,18]. Further, we observed an increased expression of PSA-2 (2.5-fold) in sodium antimony gluconate (SAG) resistant parasite by microarray analysis [19].

In the present study, we investigated the role of PSA-2 in antimony resistance by episomal expression of PSA-2 gene in antimony sensitive *L. donovani*.

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2. Material and methods

2.1. Parasite culture

Standard parasites strains, *L. donovani* 1S (*Ld*S) and *L. donovani* *AG*83 as well as clinical isolates ($n = 14$) prepared from bone-marrow aspirates of VL patients were used for this study. The study was approved by the Ethical Committee of Safdarjung Hospital, New Delhi, India. Written informed consent was obtained from all patients based on the guidelines of the Ethical Committee. The clinical isolates were described as SAG sensitive (IC_{50} range, 2.14 ± 0.28 to 5.63 ± 0.57 μg/ml) or resistant (IC_{50} range, 11.82 ± 1.28 to 20.30 ± 0.84 μg/ml) based on *in vitro* susceptibility at amastigote stage that correlated well with the patients' response to SAG treatment, as described in our previous studies [20,21]. Promastigotes were cultured at 24 °C in M199 medium with 25 mM HEPES (pH 7.4) supplemented with 10% FBS, 100 IU penicillin G and 100 μg/ml streptomycin [8].

2.2. RNA isolation and real time PCR

Total RNA was isolated from stationary phase promastigotes using Trizol reagent (Invitrogen, USA) following instructions recommended by the manufacturer. All samples were treated with Deoxyribonuclease I (Fermentas, USA). Total RNA (5 μg/reaction) was reverse transcribed at 42 °C with M-MLV Reverse transcriptase (Invitrogen, USA) using conditions recommended by the manufacturer with oligo (dT) primer. Real time PCR reactions were performed in triplicate in 25 μl volumes using SYBR Green for detection in an ABI Prism 7000 Sequence Detection System (Applied Biosystem) using PSA-2 gene (For-CGT GCG ATC CCT GAG CTT and Rev-CCG GCA TAC TTT GGC TGA AA). We used the $2^{-\Delta\Delta Ct}$ method to calculate relative changes in gene expression. The data was presented as the fold change in the expression of PSA-2 in *L. donovani* isolates normalized to the internal control gene (GAPDH) and relative to the *Ld*AG83 reference strain of *L. donovani* [22].

2.3. Western blot analysis

Cell lysates (100 μg) from 14 *L. donovani* isolates comprising of 4 sensitive and 10 resistant parasites were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 12% polyacrylamide gel and transferred to nitrocellulose membranes. The membrane was blocked and incubated sequentially with the anti-PSA-2 antibody and subsequently with anti mouse IgG conjugated with HRP. The blots were developed using ECL reagent and visualized on X-ray film. The images were scanned and quantitative assessment carried out with "Image J Software" (NIH IMAGE).

2.4. Sequence analysis

For comparative analysis, *L. donovani* PSA-2 protein sequences were aligned with PSA-2 from other *Leishmania* species. Phylogenetic relationship was deduced using a Phylogeny.fr available on http://www.phylogeny.fr/version2_cgi/index.cgi. The gene sequences for the analysis were retrieved from TriTrypDB genome database (http://tritrypdb.org/tritrypdb/). The protein structure and transmembrane domain analysis was performed using LRR finder web based tool and TMHMM v. 2.0 profile (http://www.cbs.dtu.dk/services/TMHMM/).

2.5. Generation of Leishmania parasite over expressing PSA-2 (LdSPSA-2^{++})

The fragment encoding PSA-2 was obtained by amplifying *L. donovani* DNA and sub cloned into pCR®2.1-TOPO TA cloning vector. This recombinant DNA construct was termed pCR®2.1-*Ld*PSA-2. Sequence confirmed plasmid DNA of pCR®2.1-*Ld*PSA-2 was used as a template using respective gene primers with *SpeI* site and HA tag for sub cloning into the *Leishmania* expression plasmid pKSNeo [23]. The primer sequence used for the amplification of full ORF of PSA was as below.

Forward: 5′ GG ACTAGT A TGG CGC TGT GCG TGC GTC GGC-3′
Reverse: 5′ CC ACTAGT C TA C GCG TAG TCC GGC ACG TCG TAC GGG TAC GCC GCC AGCCCC ACG CTC AG-3′

SpeI site is underlined and HA tag is in bold. The amplified product was ligated using T4 DNA ligase in pKSNeo vector and transformed in *Escherichia coli* TOP 10F' cells. The recombinant ampicillin resistance colonies were screened and plasmid prepared was digested with *SpeI* enzyme for confirming the presence of gene of interest. The orientation of the insert was checked by digestion with *HindIII* and *XhoI*.

2.6. Transfection and selection of LdPSA-2^{++}

A clinical isolate [K133 (MHOM/IN/2000/K133) obtained from SAG-responsive patient and a reference *Leishmania* isolates *L. donovani* Sudan [LdS (MHOM/SD/62/1S-C12D] were used for generation of PSA-2 over expressing parasites. Mid-log phase promastigotes ($2–4 \times 10^7$cells/ml) were electroporated with the plasmid constructs in 2 mm gap cuvettes at 450 V, 500mF. Transfected promastigotes were selected with minimal doses of G418 (50 μg/ml) as described previously [8]. Parasites transfected with the empty vector pKSNeo were used as controls. The G418 selected cells were used in all subsequent experiments. The expression of PSA-2 was validated by western blotting using anti HA antibody (Sigma) or anti-PSA-2 antibody. The *Ld*PSA-2^{++} parasites were cloned using limited dilution.

2.7. In vitro drug susceptibility assay

The antileishmanial drug susceptibility of vector transfected parasite and *Ld*PSA-2^{++} was determined for SAG (Albert David Ltd, India), amphotericin B (Sigma) and miltefosine (Cayman Chemical Company, USA) at intracellular amastigote stage [8,21]. Briefly, mouse-macrophage–adherent cell line J774A.1 (2×10^5 - cells/well) in 8-well chamber slides was infected with stationary-stage promastigotes at a 10:1 (parasite:macrophage) ratio and incubated in 5% CO_2 for 4 h at 37 °C. After washing, the cells were incubated for 12–18 h. Infected cells were re-incubated, for 48 h, with SAG (0, 3, 10, 30, 60 & 100 μg/ml), miltefosine (0, 0.5, 1.25, 2.5, 5, 10 & 30 μg/ml) and, amphotericin B (0, 0.25, 0.5, 1.0, 2.0, 3.0 & 4.0 μg/ml). After staining with Diff-Quik (Dade Behring Inc.), the numbers of amastigotes per cell were counted in 100 macrophages. The percent killing was calculated by sigmoidal regression analysis (Origin6.0).

2.8. Infectivity of LdPSA2^{++} parasites

The mean % infectivity of wild type and *Ld*PSA2^{++} parasites was determined *in vitro* using mouse macrophages infected with promastigotes at 10:1 (parasite:macrophage) ratio. After 24 h of infection, non-internalized parasites were washed off and plates were incubated for 48 h at 37 °C. Slides were fixed with methanol and stained with Diff-Quik. The number of amastigote per cell was counted in 100 macrophages to calculate the mean % infectivity.

2.9. Complement mediated cell lysis (CML)

Promastigotes (10^6/ml in M199 + 20% FBS) were added to 96 well plates containing doubling dilutions of freshly isolated human serum (50–0.78%) and incubated at 37 °C for 60 min. Plates were incubated for 24 h at 25 °C after addition of 40 μl of cold EDTA and 24 μl of resazurin, before fluorescence was measured using fluorimeter (Tecan) [24]. The percent killing was calculated by sigmoidal regression analysis (Origin6.0).The results were expressed as the percentage of live parasites compared to controls which were not exposed to serum.

Quantification of intracellular antimony accumulation studies were performed as described previously [25]. Briefly, mouse macrophage adherent cell line J744A.1(5×10^6) was infected with parasites at a ratio of 10:1 (promastigote:macrophage) in 24 well tissue culture plates, excess promastigotes washed off after 6 h and cells incubated further for 24 h. SAG (20 μg/ml) was added to the infected cells. Cells were harvested at specific time points (0, 30, 60 & 90 min) by gentle scrapping to remove adherent cells from the plate and washed three times using PBS. Cells (10^5) were resuspended in 50 μl of PBS, digested with conc. HNO3 (150 μl) by overnight incubation at room temperature. Digested cell content was diluted with 1 ml PBS and centrifuged at 12,000 rpm × 10 min. Antimony content was measured in supernatant by inductively coupled plasma mass spectroscopy (ICP-MS).

3. Results

3.1. Sequence analysis of PSA-2 gene

The sequence of PSA-2 gene amplified from *L. donovani* was found identical to *L. infantum gene, LinJ.12.0666* which has orthologs present in other *Leishmania* species *(L. major, L. tarentolae and L. mexicana*). It encodes for a putative protein of 417 amino acids with a predicted molecular weight of 44 kDa, having predominance of hydrophobic and hydrophilic amino acids and two predicted transmembrane domains at N and C terminal end. The primary amino acid sequence comparison revealed that *Ld*PSA-2 protein had the consensus sequence observed in all known LRR containing proteins.

3.2. PSA-2 is up-regulated in SAG resistant VL isolates

The PSA-2 transcripts were analyzed in 14 clinical isolates, including 4 SAG sensitive and 10 resistant isolates. There was ⩾1.5-fold (range: 1.5 ± 0.14 to 8.9 ± 0.76) increase in expression level of PSA-2 in 10/10, resistant isolates with respect to *Ld*AG83 (Fig. 1A). We observed a strong positive correlation between gene expression and SAG IC_{50} of the isolates ($r = 0.79$, $P = 0.0008$). Further, high expression of PSA-2 in antimony resistant isolates at protein level (fold change: 1.5× to 4.1×) (10/10) was confirmed by immuno-blotting with anti-PSA-2 antibody (Fig. 1B).

3.3. Characterization of LdPSA-2^{++} parasites

To investigate the role of PSA-2 in antimony resistance, parasites were genetically manipulated by over-expression of PSA-2 gene in antimony sensitive laboratory strain (*Ld*1S) as well as in a clinical isolate of *L. donovani* (K133). Total lysates were isolated from the transfected parasites and analyzed by western blot using anti-HA antibody (Fig. 2A) and anti-PSA-2 antibody (Fig. 2B). Quantitative analysis revealed that the expression of PSA-2 was 6- to 7-fold high in *Ld*SPSA-2^{++} and K133PSA-2^{++} in comparison to controls. The *Ld*PSA2^{++} parasites showed a similar growth pattern as that of wild type parasites (Fig. 2C).

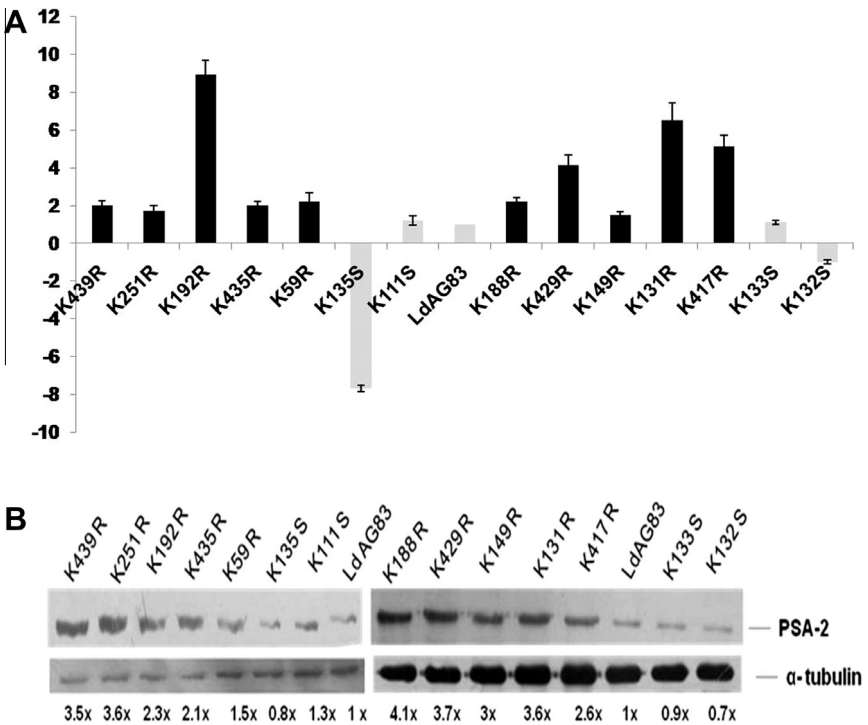


Fig. 1. Expression analysis of PSA-2 gene in *L. donovani* clinical isolates. (A) Gene expression was analyzed by quantitative real time PCR using GAPDH as internal control. Graph shows the expression index, defined as the ratio of gene expression relative to that of *Ld*AG83. Values given are mean ± SD of three different experiments. (B) Western blot analysis for expression of PSA-2 protein was performed using 100 μg cell lysates of promastigotes from sensitive (S) or resistant (R) isolates. Proteins were separated on a 12% SDS–PAGE gel and transferred to nitrocellulose membranes. The membrane was probed with anti-PSA-2 antibody followed by HRP-conjugated antibody and developed using ECL.

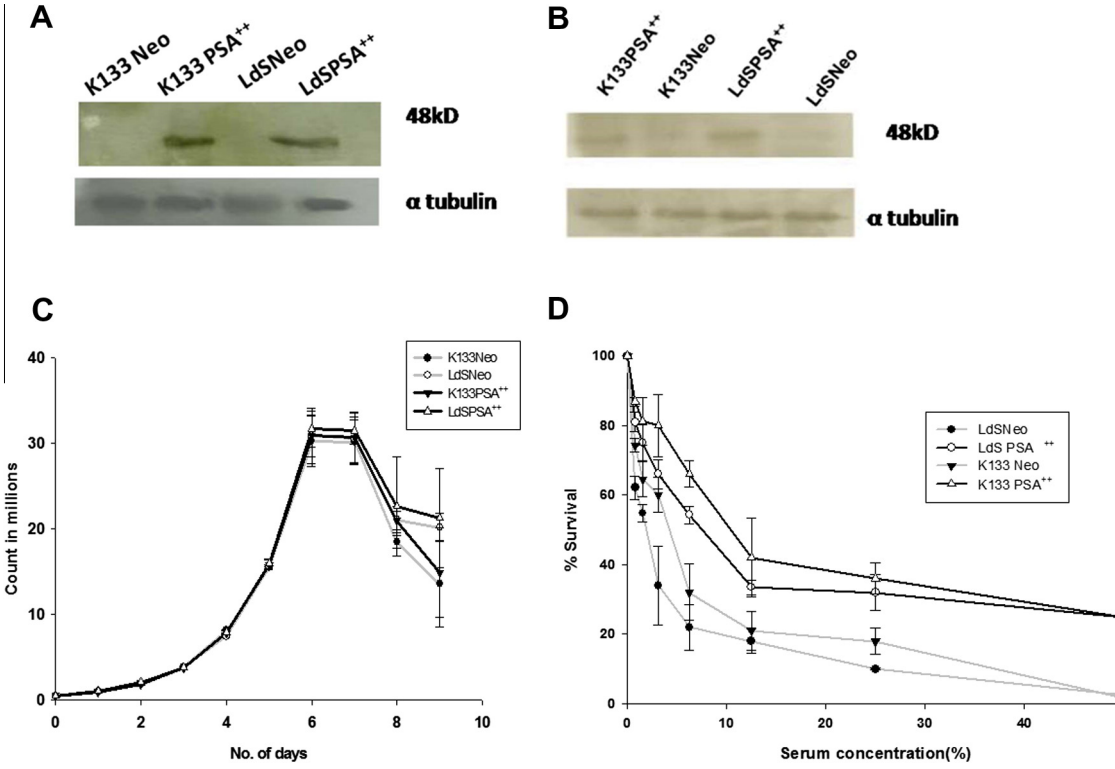


Fig. 2. Characterization of PSA-2 over expressing *L. donovani* isolates. (A) Western blot analysis for protein expression of PSA-2 in parasites over-expressing PSA-2 as compared to control. 100 μg total promastigotes lysates were separated on a 12% SDS–PAGE gel and transferred to nitrocellulose membranes. The blot was probed with anti HA antibody followed by rabbit IgG conjugated with HRP and developed using ECL. The blot was rebound with an α-tubulin antibody to monitor the amount of protein lysates loaded on the gel. (B) Western blot analysis for protein expression of PSA-2 in parasites over-expressing PSA-2 as compared to control. Total promastigotes lysates (100 μg) were separated on a 12% SDS–PAGE gel and transferred to nitrocellulose membranes. The blot was probed with anti PSA-2 antibody followed by HRP conjugated antibody and developed using ECL. The blot was rebound with an α-tubulin antibody to monitor the amount of protein lysates loaded on the gel. (C) Growth curve of K133 PSA-2^{++} and *Ld*SPSA-2^{++} in comparison with the control K133 Neo and *Ld*SNeo. Each data point on the curve represents the mean ± SD of 3 separate assays. (D) Resistance to complement mediated lysis by *Ld*PSA-2^{++} parasites. Parasites were incubated with fresh human serum and percentage parasite survival was measured at different serum concentrations. The assay was performed thrice in triplicate. Values given are mean percentage survival ± SD.

The mean % infectivity of K133PSA-2^{++} *and* *Ld*SPSA-2^{++} was 85.0 ± 3.0% and 77.5 ± 0.5% respectively, comparable to the corresponding wild type, K133 Neo (81.0 ± 2.82 %) and *Ld*SNeo (80.0 ± 2.0%). The *Ld*PSA-2^{++} parasite clones (n = 4) displayed comparable growth and infectivity levels (ranging from 73.0 ± 2.0% to 80.5 ± 1.5%), to the corresponding parental strain, indicating a homogenous population of transfected *Ld*PSA2^{++} parasites.

3.4. *LdPSA-2^{++} parasites resist complement mediated lysis*

Towards understanding the role of this surface protein in parasite virulence, we investigated complement mediated lysis and macrophage infectivity with *Ld*PSA-2^{++}. The data showed that *Ld*SPSA-2^{++}-parasite (mean IC_{50} = 7.03 ± 0.32%) resisted complement lysis by 3.5-fold and K133PSA-2^{++} (mean IC_{50} = 9.92 ± 1.22%) resisted it by 2.5-fold as compared to the respective control parasites *Ld*SNeo (mean IC_{50} = 2.05 ± 0.10%) and K133Neo (mean IC_{50} = 3.99 ± 1.10%) (Fig. 2D).

3.5. *LdPSA-2^{++} parasites show decreased susceptibility towards SAG*

We analyzed the susceptibility of *Ld*PSA-2^{++} parasites towards SAG at intracellular amastigote stage using J774A.1. The IC_{50} of K133PSA-2^{++} and *Ld*SPSA-2^{++} for SAG was 70.19 ± 3.56 and 80.57 ± 5.01 μg/ml, respectively, significantly higher ($P < 0.001$) than the corresponding controls K133Neo (IC_{50} 6.28 ± 0.92 μg/ml) and *Ld*SNeo (IC_{50} 6.37 ± 0.26 μg/ml) (Fig. 3A). The SAG susceptibility of clonal populations of *Ld*PSA-2^{++} was similar to the parental population (data not shown).

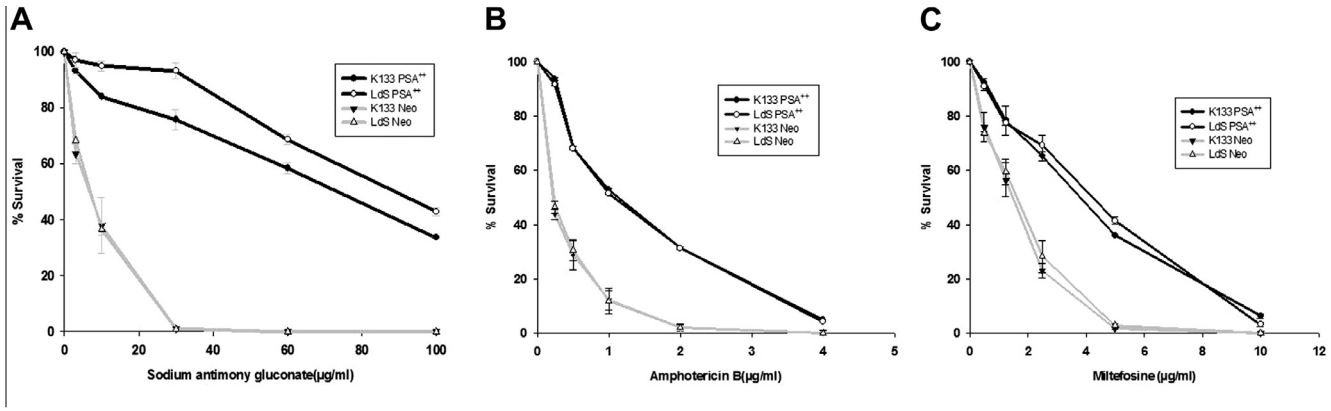
Further, we observed a significantly lower susceptibility of *Ld*PSA-2^{++} parasites towards both amphotericin B (>4-fold) and miltefosine (>2.5-fold) as compared to controls (Fig. 3B and C). The IC_{50} values of the parasites towards the various antileishmanial drugs are given in Fig. 3D.

3.6. *Reduced accumulation of intracellular antimony in LdPSA-2^{++} parasites*

Reduced accumulation of drug within the parasite cell is well reported to be an important mechanism of drug resistance. Here, we estimated the total intracellular antimony (Sb) content in macrophages infected with *Ld*SPSA-2^{++} and K133 PSA-2^{++} parasites at various time points. The Sb content increased up to 60 min post SAG treatment but not thereafter in *Ld*PSA-2^{++} parasite while it increased up to 90 min in the control parasite. At 60 min the intracellular Sb content was significantly lower (2–3-fold) in *Ld*PSA-2^{++} in comparison to the control (Fig. 4).

4. Discussion

In view of widespread antimony resistance in VL, it is important to understand the mechanism of resistance and develop molecular tools for monitoring drug resistance in the field. A number of genes associated with antimony resistance have been identified by transcriptomic and proteomic approaches, however, none of them



D

Parasite ID	IC_{50} Sodium antimony gluconate	IC_{50} Amphotericin B	IC_{50} Miltefosine
K133 Neo	6.28 ± 0.92	0.24 ± 0.007	1.40± 0.25
LdS Neo	6.37 ± 0.26	0.265 ± 0.007	1.46 ± 0.19
K133 PSA-2^{++}	70.19 ± 3.56	1.11 ± 0.11	3.53 ± 0.14
LdS PSA-2^{++}	80.57 ± 5.01	1.08 ± 0.07	3.95 ± 0.11

Fig. 3. Drug susceptibility of *Ld*SPSA-2^{++} and K133PSA-2^{++}*L. donovani* isolates. Susceptibility of *Ld*SPSA-2^{++} and K133PSA-2^{++} was determined towards (A) Sodium antimony gluconate, (B) Amphotericin B and (C) Miltefosine as intracellular amastigotes assay using J774 A.1 macrophage cells. Cells transfected with plasmid alone (*Ld*S Neo and K133 Neo) were used as controls. (D) IC_{50} values for each of the 4 drugs based on sigmoidal regression analysis by Origin 6.0 software. Each data point represents the mean ± SD of 3 separate assays.

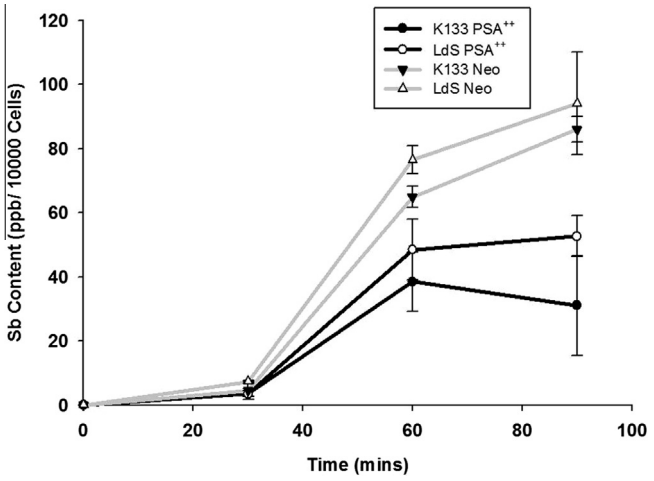


Fig. 4. Intracellular accumulation of antimony (Sb) in *Ld*PSA-2^{++} parasites. Antimony accumulation was analysed using ICP-MS in *Ld*PSA-2^{++} and wild type parasites after incubating with 20 μg/ml Sb at different time points 0, 30, 60, 90 min. Values given are mean ± SD of two different experiments.

individually represents a biomarker for monitoring drug resistance [22]. The present study characterized the role of PSA-2 in antimony resistance and suggested its potential as a biomarker of antimony resistance.

PSA-2 gene, a virulence factor of *Leishmania,* consists of LRRs involved in protein–protein interaction and signal transduction [16,26,27]. Lysis by complement is one of the first immune mechanisms encountered by promastigotes upon inoculation by sandfly bite into the vertebrate host. We observed that *Ld*SPSA-2^{++} parasite resisted complement mediated lysis as compared with the wild type, indicating enhanced capability to establish infection in host cells.

Recently, another novel leucine-rich repeat (LRR) protein, LinJ34.0570, was implicated in antimony resistance of *Leishmania* since parasites over-expressing this LRR protein were resistant to SbIII as axenic amastigotes and to SbV as intracellular parasites [10]. In the present study, episomal expression of PSA-2 in two distinct drug sensitive strains of *L. donovani* resulted in a marked decrease in susceptibility not only towards SAG (more than 12-fold) but also towards amphotericin B (~4-fold) and miltefosine (>2.5-fold). Thus, consistent up-regulation of PSA-2 in SAG resistant clinical isolates may also predict higher tolerance for amphotericin B and miltefosine. Indeed, some degree of cross tolerance between these drugs has been reported in field isolates of *L. donovani* complex [20,28,29]. We observed a decreased antimony uptake in *Ld*PSA-2^{++} parasites in comparison to the wild type. Topology analysis of PSA-2 suggested presence of two transmembrane domains which may help to span through the membrane and integrate to cell surface, a structural similarity to that observed in ABC efflux pumps that modulate antimony uptake [9].

It has been suggested that expression analysis of multiple genes may be necessary to mark SAG resistance since none of the genes studied showed absolute correlation with the phenotype [22]. In the present study, the expression of PSA-2 was modulated at both transcript level and protein level, being consistently high in the resistant isolates and low in all sensitive isolates, indicating its potential for discriminating SAG sensitive and resistant isolates.

Overall, the current study demonstrated for the first time the functional importance of PSA-2 in antimony resistance. In view

of the emerging threat of drug resistance in VL, the application of molecular markers to distinguish the sensitive and resistant parasite is of foremost importance. We recommend expression analysis of PSA-2 to be tested at large scale for monitoring the spread of drug resistance.

Author contributions

Conceived and designed the experiments: V.B., D.K., R.S., P.S. Performed the experiments: V.B., D.K., S.V., G.S., R.S. Analyzed the data: V.B., D.K., G.S., R.S., P.S. Contributed reagents/materials/analysis tools: N.S.N., R.S., P.S. Wrote the paper: V.B., R.S., P.S.

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