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Short communication

Possibility of membrane modification as a mechanism of antimony resistance in *Leishmania donovani*

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Abstract

Resistance to antimonials has become a clinical threat in the treatment of visceral leishmaniasis (VL). Unravelling the resistance mechanism needs attention to circumvent the problem of drug resistance. In one of the resistant isolates, we earlier identified a gene (PG1) implicated in antimony resistance whose localization in the present study was confirmed on the pellicular plasma membrane of the parasite thereby indicating towards membrane modification as a mechanism of resistance in this resistant isolate.

Keywords: *Leishmania*; Visceral leishmaniasis (VL); Clinical isolates; Drug resistance

Pentavalent antimonial Sb(V) drugs have remained the first line of treatment for visceral leishmaniasis (VL) worldwide for more than six decades [1]. However, the clinical value of antimony therapy is now threatened. With emergence of drug resistance at an alarming rate and reaching up to 65–70% in North Bihar, India [2], its use has almost been abandoned by the clinicians and replaced by alternative therapy such as amphotericin B and miltefosine.

To be active against *Leishmania*, Sb(V) has to be converted to a trivalent form Sb(III), thus leading to the definition of Sb (V) as a prodrug [3]. Different studies have suggested various modes of action of antimony [4–8]. Though there has been expansion of the available treatments but the problem of drug resistance has bypassed this success. The parasite has evolved to survive under drug pressure by expressing various mechanisms of resistance. Known determinants of drug resistance in the laboratory mutants selected for Sb(III) have been found to be associated with over expression of thiol biosynthetic enzymes [9–11] and the Sb(III)/thiol conjugate sequestering pump MRPA [12,13] and a decreased Sb(III) accumulation, which seems to be caused by a lower level of expression of the gene AQP1 [14,15]. Other studies on *in vitro* induced Sb(V) resistance have described a deficient intracellular reducing activity of Sb(V) to Sb(III) [3]. The mechanism of resistance to antimony in clinical isolates is beginning to be unraveled [16–19].

In our earlier study [16] we had identified a novel gene (PG1) implicated in antimonial resistance from a clinical isolate (R-5). Our present study is an attempt to understand the mechanism of resistance imparted by the gene PG1 (Accession No. AF273843). *L. major* and *L. infantum* genome sequence database (www.genedb.org/) showed homologue of PG1 gene on chromosome 18 with 80% and 79% identity respectively. The percentage identity found seems rather low as normally one would expect 98–99% identity for any two homologues in *L. donovani* and *L. infantum* and probably 95% identity for *L. donovani* and *L. major*. Various explanations are possible for this low level of identity, perhaps the ORF is a *donovani*-specific gene; perhaps it is a paralogue of the true homologue of *L. infantum* ORF in *L. donovani*; perhaps it is a mutant form of the gene, selected by and part of the drug resistance phenotype.

Isolate R-5 (resistant) and 2001 (sensitive) used in the present study were obtained from patients with visceral leishmaniasis, maintained in culture and *in vitro* and *in vivo* drug susceptibility to antimony determined as described previously [16,20].

Logarithmic phase promastigote cultures of *L. donovani* clinical isolates were used to isolate total RNA using TRIZOL reagent (Gibco BRL) by standard procedures. All RNA samples

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were tested for purity and integrity. For Northern hybridizations (10 μg) of the promastigote total RNA of R-5 and transfectants (P6.5+C) [16] was transferred to nylon membrane. The PCR amplified entire 1254 bp fragment of R-5 nDNA encompassing the PG1 ORF [16] and ~900 bp *L. donovani* á-tubulin gene were used as probes. For RT-PCR analyses cDNA was prepared using 100 U of reverse transcriptase (Amersham). To ensure exclusion of DNA contaminants, total RNA was treated with DNase (MBI Fermentas) and enzyme was inactivated by phenol:chloroform extraction. 3 μl of this cDNA was then used as template in the PCR amplification reaction. The gene specific primers (F-5′-cgcaagcttaaatgtcaaatcgc-3′ and R-5′-ggcaagcttt-tagagctggccca-3′) were used to get the RT product for PG1 gene. PCR conditions used for amplification were the same as described [21]. As an internal control, primers specific for α-tubulin were used. Probes were made by labeling 25 ng of the DNAs with [α-^{32}P]dCTP by random priming method (BRIT/BARC, India).

By Northern blot analysis a ~300 bp gene transcript was detected in total RNA samples from the transfectants (p6.5+C) (Fig. 1). The corresponding transcript could not be detected in the R-5 isolate. The undetectable expression of the gene in the wild-type promastigotes by Northern can be attributed to the fact that in the clinical condition there is a very modest over expression of resistance, only about 2–3 folds as opposed to many folds higher resistance found in laboratory mutants [16]. The PG1 transcript could be detected by RT-PCR (Fig. 2) in both R-5 and 2001 isolates.

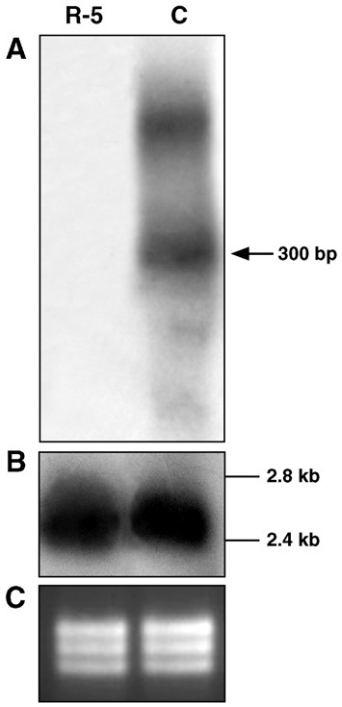

Fig. 1. Northern blot analysis. Total RNA was isolated from (R-5) promastigotes and transfectants (P6.5+C) maintained in tunicamycin at 20 μg/ml. The RNA samples were electrophoresed on 1.5% RNA denaturing gel, transferred to nylon membrane and hybridized with [α-^{32}P] labeled (A) PG1 gene and (B) α-tubulin gene probe (C) EtBr stained rRNA bands showing equal loading.

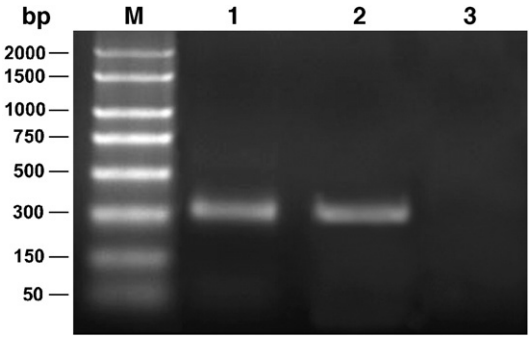

Fig. 2. Reverse transcriptase PCR showing PG1 transcript. (M) PCR marker (Sigma), (1) R-5 isolate, (2) 2001 isolate (3) negative control (without template).

In order to further gain insight into the mechanism of resistance of PG1 gene, it was cloned, over expressed and purified by affinity chromatography [21] and polyclonal antibodies to the recombinant protein were raised in New Zealand white rabbit by Bangalore Genei, India. The specificity of the polyclonal antiserum to PG1 protein was confirmed by Western blot analysis (Fig. 3) [21]. In the present study using these antibodies confocal microscopy was done to study the subcellular localization of PG1 protein. Sensitive (2001) and resistant (R-5) *L. donovani* promastigotes were washed two times for 5 min in PBS and washed cells were allowed to adhere on poly-L-lysine coated coverslips and fixed by adding 4% paraformaldehyde for 30 min at RT. Fixed cells were permeabilized with 0.5% (v/v) Triton X-100 and washed for 5 min (3×) with PBS containing 0.05% (w/v) glycine. Blocking was done with 1% BSA (Sigma) in PBS at RT for 30 min. The promastigotes were incubated with the antiserum raised against the recombinant protein at a dilution of (1:200) for 1 h at RT and washed with 1% BSA for 5 min (6×). Incubation with the FITC conjugated anti-rabbit goat antibody (1:50 dilution) was done for overnight at 4 °C in dark. Coverslips were washed for 5 min (6×) with chilled PBS and mounted in the mounting media (Oncogene) and confocal images were collected using 60×1.4 NA (oil) Plan Apochromate lenses on Bio-Rad Radiance 2100 Confocal system equipped with Eclipse TE300 Nikon inverted microscope. FITC was excited with 488 nm laser line.

Antiserum against PG1 predominantly stained the pellicular plasma membrane, surrounding the cell body and flagellum in R-5 (Fig. 4). 2001 showed a very faint stain. No fluorescence was observed with rabbit preimmune serum (data not shown). In

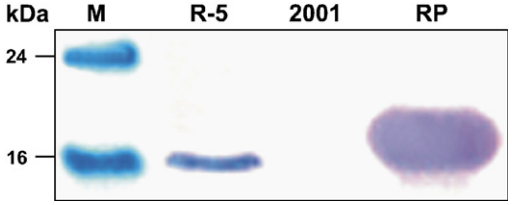

Fig. 3. Western blot analysis using polyclonal antisera against recombinant PG1 protein. Proteins were separated on 12% acrylamide gel. (M) Prestained molecular mass marker (MBI Fermentas), (R-5) total cell lysate of *L. donovani* resistant strain, (2001) total cell lysate of *L. donovani* sensitive strain, (RP) column purified recombinant protein.