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Short Communication

P-glycoprotein A Gene Expression in Glucantime-Resistant and Sensitive *Leishmania major* (*MRHO/IR/75/ER*)

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Abstract

Background: Leishmaniasis is a parasitic disease caused by different species of *Leishmania* parasites with a wide range of clinical manifestations. Antimonial compounds such as meglumine antimoniate (glucantime) are the first line drugs for the treatment of leishmaniasis. However, according to reports of the drug resistance of parasites, the efficacy of antimonial compounds is low. The ATP-binding cassette (ABC) proteins are present in all organisms and mediate the transport of vital elements through biological membranes. One of the important mechanisms of resistance in *Leishmania* parasites is the overexpression of ABC efflux pumps. P-glycoprotein A (pgpA) is a related gene for ABC transporter in *Leishmania* species. The aim of this study was to compare the pgpA expression in laboratory-induced resistant *L. major* (*MRHO/IR/75/ER*) and sensitive parasites.

Methods: RNA extraction of promastigotes of sensitive and resistant clones was performed and total RNA was reverse transcribed. The real-time quantitative polymerase chain reaction (PCR) was used to assess RNA expression profiles and the expression levels were calculated using $2^{-\Delta Ct}$ method.

Results: The mean expression level of pgpA mRNA was 2.70 ± 0.51 in in sensitive *Leishmania* clone and 6.08 ± 1.50 in resistant *Leishmania* clone (P = 0.021).

Conclusion: The expression of pgpA gene in resistant strains of *L. major* was almost fivefold higher than those in susceptible strains. Therefore, this can be used in field isolates, i.e. overexpression of the gene can prove resistance in wild type field isolates.

Introduction

Leishmaniasis is a range of diseases caused by different obligate intracellular parasites of *Leishmania* species and transmitted by the bite of infected sand flies (1). While cutaneous leishmaniasis (CL) due to *L. tropica* is known as anthroponotic CL (ACL) or urban form of the disease, the zoonotic CL (ZCL), which is caused by *L. major*, is endemic in many rural areas of Iran (1).

Although antimonial compounds such as meglumine antimoniate (glucantime) have been the first line drugs for the treatment of leishmaniasis, they are now proved not to have adequate efficacy and to cause resistance in long-term use (2-4). However, the main reasons for such problems have not been well explained. On the other hand, while it is difficult to justify the development of natural resistance of wild *L. major* in the field, laboratory-induced resistance can be used to assess ATP-binding cassette (ABC) proteins (5), which are present in all organisms as a frequent mechanism by which *Leishmania* responds to drug selection (6, 7). Most of these proteins mediate transport across biological membranes (5). Over-expression of ABC efflux pumps may be an important way to produce resistant *Leishmania* parasites (8). One of the genes for an ABC transporter in *Leishmania* is a P-glycoprotein A (pgpA) (9) which is commonly amplified in metal-resistance in *Leishmania* (9).

The amount of trypanothione (TSH), a glutathione-spermidine conjugate (10), is increased in several *Leishmania* species which are resistant to metal. TSH is the main reduced thiol in *Leishmania* and the metal-TSH conjugate might be sequestered to an intracellular compartment using pgpA. The metal-TSH conjugate may leave cells by exocytosis generally through the flagellar pocket (11, 12).

In this study, pgpA expression was compared between laboratory-induced glucantime-resistant *L. major* and unchanged susceptible reference parasite. In our lab glucantime resistant model of *L. major* strain was transformed from natural intact line with the aim of comparison pgpA gene expression in wild type isolates from patients with no response to chemotherapy with glucantime in the future studies. We hypothesized that resistant and susceptible parasites would have different pgpA gene expressions. The results of this study would promote our understanding of not only mechanisms through which *Leishmania* evades the killing effects of drugs but also different pathways used by *Leishmania* to neutralize the therapeutic effects of chemical formulations such as glucantime.

Materials and Methods

Parasite and in-vitro culture

L. major promastigotes (MRHO/IR/75/ER) were used for the current study. In order to avoid using over-passaged parasites and to maintain virulence, 0.1 ml phosphate buffered saline (PBS) containing 2×10^6 *L. major* promastigotes was inoculated subcutaneously in the base of the tail of BALB/c mice. Three-four weeks after the injection, the lesions appeared and the infected mice were autopsied. The animals' spleens were removed, homogenized under sterile conditions, and transferred to modified NNN medium. Mass production of promastigotes was performed via sub-passaging of the isolates from the NNN medium in RPMI 1640 medium.

Drug sensitivity of the proliferated isolates was proved using the half maximal inhibitory concentration (IC50) method since 50% of the promastigotes died in the presence of glucantime concentrations of 10 μg/ml (4).

Induction of resistance in cultured promastigotes

L. major parasites were grown in RPMI 1640 medium supplemented with 10% heat-inacti-

vated fetal calf serum (FCS) at 23-25°C. In order to induce resistance, the parasites were then pulsed by gradually increasing meglumine antimoniate concentration from 1 mg/ml to 5, 10, 15, 30, 60, and finally 120 mg/ml. The upper limit of 120 mg/ml was selected based on the toxicity for the promastigotes. Each 5-ml ampoule of glucantime contained 1.5 g meglumine antimoniate corresponding to 0.405 g of pentavalent antimony (13).

RNA isolation

Total RNA was extracted using RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. Centrifugation was then employed to pack 5×10^6 alive promastigotes from the media containing sensitive and resistant parasites. The harvested promastigotes were washed three times and RNA extraction was performed. The samples were treated with a DNA-free kit (Fermentas, USA) to remove possible contaminated genomic DNA. RNA concentration was determined using absorbance at 260 nm in a spectrophotometer (NanoDrop, USA).

cDNA synthesis and Real-time quantitative PCR

Total RNA was reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA) with a random hexamer and according to the manufacturer's instructions. Briefly, 1 µl of random hexamer was added to 11 µl of extracted RNA and incubated at 65°C for five minutes. After adding 4 µl of reaction buffer, 1 µl of RiboLock RNase Inhibitor (Life Science Research, USA), 2 µl of Revert Aid, and 1 µl (200 U/µl) of transcriptase, a thermal cycler (Bio-Rad, USA) was used to incubate the samples at 42ºC for 60 minutes and then at 70ºC for five minutes.

Subsequently, 1.5 µl of the resulting cDNA was used as the template and added to 18.5 µl of SYBR Green quantitative PCR reactions (Sigma-Aldrich, USA) and entered into the ABI 1 plus model of real-time PCR for 40 cycles. Forward and reverse primers were designed by the National Center for Biotechnology Information (NCBI) (Table 1).

PCR amplification consisted of an initial denaturation at 95ºC for 10 minutes, followed by 40 cycles of denaturation at 95ºC for 15 seconds, and annealing and extension at 60ºC for one minute. Melting curve analysis (60ºC → 95ºC increment at 0.3ºC) was used to determine the melting temperature of specific amplification products and primer dimers. These experiments were independently repeated for at least three times. Quantitative real-time RT PCR was performed to quantitatively estimate the RNA expression of sensitive and resistant *L. major*.

Two genes were analyzed: first pgpA involved in metal resistance and 18s rRNA (internal control) for normalization purposes. Following the generation of the standard curve, the PCR efficiency of genes was determined. For real time reverse transcribed PCR data analysis, $2^{-\Delta Ct}$ method was used. Data was analyzed using t-tests.

Results

Expression of pgpA mRNA of antimony [Sb (V)] sensitive and resistant mutants was verified as mentioned. The extracted RNA samples were checked with agarose gel electrophoresis. Sharp ribosomal RNA bands of 18s and 28s without smears represented the intact and adequately qualified RNA. Moreover, cDNA was synthesized from 0.495 µg of the extracted RNA. The total extracted RNA concentration was 45 µg/ml and the OD260/280 ratio was 1.9.

The mean expression of pgpA mRNA normalized to 18s rRNA endogenous gene was significantly higher in resistant parasites than in sensitive samples (6.08 ± 1.50 vs. 2.70 ± 0.51; $P = 0.021$) (Figure 1). The efficacy of the primer set and PCR was 95%. In addition, pgpA expression with $2^{-\Delta Ct}$ method showed fivefold expression in resistant line when compared to the susceptible line.

Table 1: Forward and reverse primers

Gene	Forward primer	Reverse Primer	Amplicon size
18s rRNA	CCAAAGTGTGGAGATCGAAG	GGCCGGTAAAGGCCGAATAG	187
PGPA	GGTCTCATCTCGCTG TCACT	ACGCTCCACGCTGTTCAT	108

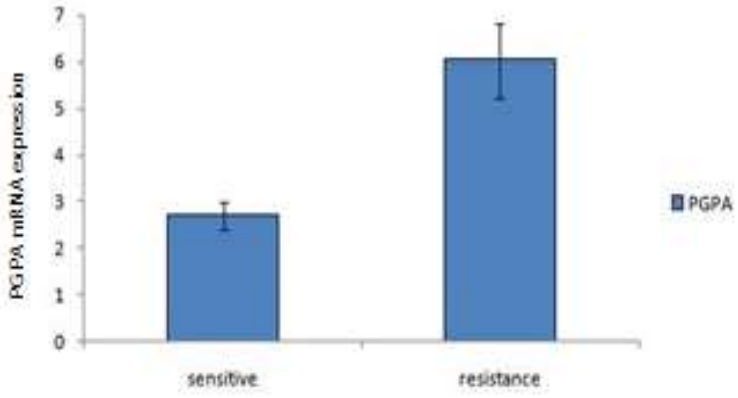

Fig. 1: Comparison of different relative pgpA mRNA expression in sensitive and resistant *Leishmania major* promastigotes

Discussion

Pentavalent antimonials have been the sole and first choice treatment of CL in the past decades. Glucantime concentration and the duration of therapy have recently increased in order to maintain the strong therapeutic effects of treatment (14). However, treatment efficacy is still low and cases of resistance have been reported (2-4, 14). It is strongly suspected that the parasite has acquired mutations to induce resistance to glucantime (15). Thus, it seems necessary to investigate molecular mechanism(s) involved in non-responsiveness of clinical treatment and possibly drug resistance, not only to track drug resistance spread, but also for designing new and efficient drugs for the treatment of refractory leishmaniasis.

North Bihar in India is an area of anthroponotic visceral leishmaniasis with increasing cases of drug-resistant leishmaniasis. Several studies have reported that in this area, the disease is poorly responding to treatment with antimony and that 34%-65% of the cases have actually failed to respond to treatment (16). In vitro studies with amastigotes have demonstrated that treatment failure is a result of decreased sensitivity of the parasite to antimony (17). Reports of unresponsiveness to treatment have also come from Iran. A cross-sectional research in Mashhad (a north-eastern city of Iran) indicated that 13% of patients did not respond to treatment with glucantime (4).

In order to obtain more information about parasite resistance, we compared pgpA gene expression from *L. major* with laboratory-induced resistance to treatment and homogenous sensitive *L. major*. Among all pathways for metal resistance, overexpression of energy-dependent transporters seems to play a major role (18). For instance, pgpA are large membrane proteins that act as ATP-dependent extrusion pumps. They are responsible for effluxing the drug from the parasite or collection that in vesicles and are hence crucial in the protection of parasites from the killing effects of the drug (19). These proteins have

been shown to amplify as an extrachromosomal circle (H-circle) in a methotrexate resistant *L. tarentolae* promastigotes cell line (20).

Quantitative RT-Real-time PCR is a highly sensitive technique enabling the amplification and quantification of a specific nucleic acid sequence with detection of the target gene and its expression. In recent years, this method has been widely used to assess the expression of different genes of *Leishmania*. A typical example is calcineurin which is a protein phosphatase with a critical role in the apoptosis of resistant and sensitive *L. infantum* isolates from endemic areas of Iran (21).

Heather et al. studied two different lines of *L. major* (the wild type and the pgpA gene transfected *L. major*). They evaluated antimonial accumulation using electrothermal atomic absorption spectrophotometry and found a fourfold reduction in the accumulation of antimony in resistant pgpA-transfectants (22). In another study to determine the role of genetic variation in resistant phenotypes, Alizadeh et al. assessed samples collected from endemic areas of Iran. The mutation of multidrug resistant (MDR) gene was evaluated with PCR method. They found that 11.1% of the patients showed changes in the MDR gene sequences (20).

Genomic variation in sodium stibogluconate resistance has also been reported in patients with anthroponotic CL (13). The overexpression of pgpA has been suggested as a major reason of drug resistant in Indian visceral leishmaniasis due to *L. donovani* (23). Similarly, the overexpression of pgpA has been found in resistant axenic amastigotes of *L. infantum*. The transfection of this gene shows antimony-resistance upon promastigotes and amastigotes of *L. infantum* (24).

Conclusion

According to the results of the present study, the expression of pgpA gene in resistant strains of *L. major* was almost fivefold higher than those in susceptible strains. Therefore, the rate of pgpA expression can be used as an indication in field isolates, i.e. overexpression of the gene can prove resistance in wild type field isolates.

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Reference

1. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, et al. Leishmaniasis Worldwide and Global Estimates of Its Incidence. PLoS ONE. 2012; 7(5):e35671.
2. Firooz A, Khamesipour A, Ghoorchi MH, Nassiri-Kashani M, Eskandari SE, Khatami AR, Hooshmand B, Gorouhi F, Rashighi-Firoozabadi M, Dowlati Y. Imiquimod in combination with meglumine antimoniate for cutaneous leishmaniasis: A randomized assessor-blind controlled trial. Arch Dermatol. 2006; 142:1575-1579.
3. Shazad B, Abbaszadeh B, Khamesipour A. Comparison of topical paromomycin sulfate (twice/day) with intralesional meglumine antimoniate for the treatment of cutaneous leishmaniasis caused by *L. major*. European J Dermatol. 2005; 15(2):85-7.
4. Hadighi R, Mohebali M, Boucher P, Hajjaran H, Khamesipour A, Ouellette M. Unresponsiveness to Glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites. PLoS Med. 2006; 3:162.
5. Légaré D, Cayer S, Singh A K, Richard D, Papadopoulou B, Ouellette M. ABC Proteins of *Leishmania*. J Bioener Biomem. 2001; 33:469-74.
6. Haldar, Kumar Sen A, Roy P, Syamal Use of Antimony in the Treatment of Leishmaniasis: Current Status and Future Directions. Mol Biol Int. 2011; 10:1-23.

7. Higgins CF. ABC transporters: from microorganisms to man. Annu Rev Cell Biol. 1992; 8:67- 113.
8. Leandro C, Campino L. Leishmaniasis: efflux pumps and chemo resistance. Int J Antimicrob Agents. 2003; 22:352- 57.
9. Ouellette M, Fase-Fowler F, Borst P. The amplified H circle of methotrexate-resistant *Leishmania* tarentolae contains a novel Pglycoprotein gene. EMBO J. 1990; 9:1027- 33.
10. Fairlamb AH, Blackburn P, Ulrich P, Chait BT, Cerami A. Trypanothione: a novel bis (glutathionyl) spermidine cofactor for glutathione reductase in trypanosomatids. Science. 1985; 227:1485–7.
11. Mukhopadhyay R, Dey S, Xu N. Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*. Proc Natl Acad Sci USA. 1996; 93:10383–7.
12. Le´gare´ D, Papadopoulou B, Roy G. Efflux systems and increased trypanothione levels in arsenite-resistant *Leishmania*. Exp Parasitol. 1997; 87:275–82.
13. Esmaeili J, Mohebali M, Edrissian G. H, Rezayat S. M, Ghazi-Khansari M,. Charehdar S. Evaluation of miltefosine against *Leishmana major* (MRHO/IR/75/ER): in vitro and in vivo studies. Acta Med Iranica. 2008; 46(3):191-196.
14. Pourmohammadi B, Motazedian MH, Handjani F, Hatam GH, Habibi S, Sarkari B. Glucantime efficacy in the treatment of zoonotic cutaneous leishmaniasis. Southeast Asian J Trop Med Public Health. 2011; 42:502-8.
15. Croft SL. Monitoring drug resistance in leishmaniasis. Trop Med Int Health. 2001; 6:899–905.
16. Sundar S. Drug resistance in Indian visceral leishmaniasis. Trop Med Int Health. 2001; 6:849-854.
17. Lira R, Sundar S, Makharia A, Kenney R, Gam A, et al. Evidence that the high incidence of treatment failures in Indian Kala-Azar is due to the emergence of antimony-resistant strains of *Leishmania donovani*. J Infect Dis. 1999; 180:564–567.
18. Mary C, Faraut F, Deniau M, Dereure J, Aoun K, Ranque S, et al. Frequency of Drug Resistance Gene Amplification in Clinical Leishmania Strains. Int Microbiol. 2010; 5:1-8.
19. Ouellette M, Papadopoulou B. Mechanisms of Drug Resistance in *Leishmania*. Parasitol Today. 1993; 9:150-53.
20. Alizadeh R, Hooshyar H, Bandehpor M, Arbabi M, Kazemi F, Talari A et al. Detection of Drug Resistance Gene in Cutaneous Leishmaniasis by PCR in Some Endemic Areas of Iran. Iran Red Crescent Med J. 2011; 13(12):863-867.
21. Bagher Khadem E M, Mohebali M, Kazemirad E, Hajjaran H, Edrissian G, Mamishi S et al. Downregulation of Calcineurin Gene Is Associated with Glucan-time®Resiatance in *Leishmania infantum*. Iranian J Parasitol. 2013; 8(3):359-366.
22. Heather L. Callahan A, William L. Roberts B, Petrie M, Rainey b, Stephen M, Beverley A, The *PGPA* gene of *Leishmania major* mediates antimony (SbIII) resistance by decreasing influx and not by increasing efflux. Mol Biochem Parasitol. 1994; 68:145-49.
23. Maharjan M, Singh S, Chatterjee M, Rentala Madhubala. Role of Aquaglyceroporin (AQP1) Gene and Drug Uptake in Antimony-resistant Clinical Isolates of *Leishmania donovani*. Am J Trop Med Hyg. 2008; 79:69–75.
24. Ashutosh, Sundar S, Goyal N. Molecular mechanisms of antimony resistance in *Leishmania*. J Med Microbiol. 2007; 56:143–153.