Original Article

The study of P-glycoprotein A, G-glutamylcysteine synthetase 1, and aquaglyceroporin 1 genes expression in non-healing zoonotic cutaneous leishmaniasis cases

Seyed Hossein Hejazi[1] , Sedigheh Saberi[1] , Reza Arjmand[2] , Simindokht Soleimanifard[3*]

[1]Skin Disease and Leishmaniasis Research Center, Department of Parasitology, School of Medicine Isfahan University of Medical Sciences and Health Services, Isfahan, Iran
[2]Department of Parasitology, School of Medicine, Jundishapur University of Medical Sciences, Ahvaz, Iran
[3]Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences and Health Services, Isfahan, Iran

*Corresponding Author: Simindokht Soleimanifard, Parasitology and Mycology Department, School of Medicine, Isfahan University of Medical Science, Hezar Jerib Street, Isfahan, Iran. Tel: +989132943369, Email: Soleimanifard@med.mui.ac.ir

Abstract

Background and aims: Regarding the increasing numbers of clinical antimonial-resistant Leishmaniasis, understanding the reasons for drug resistance is helpful. This study aimed to find the expression level of the genes related to resistance, P-glycoprotein A (PgpA), G-glutamylcysteine synthetase 1 (Gsh1), and aquaglyceroporin 1 (Aqp1) in antimonial-resistant clinical isolates.
Methods: Samples were isolated from leishmaniasis ulcers of 10 non-healing patients and the species were identified by the nested-polymerase chain reaction (PCR) method. In vitro experiments were performed using the amastigote-macrophage model by J774 cell line, and in vivo studies were conducted by animal model, the Balb/c mice. Finally, the values of genes expression were determined by quantitative-reverse transcription (q-RT) real-time PCR method and then compared with non-resistant *Leishmania major* (MRHO/IR/75/ER).
Results: Molecular identification showed that all isolated protozoa were *L. major*. The isolated samples from clinical resistant patients represented no increase in expression in the tested resistance genes ($P > 0.05$). Finally, it was found that the lack of improvements in patients was not associated with the increased expression of resistance genes.
Conclusion: In general, no inherent resistance was observed in the tested samples neither a correlation between the healing of lesions and the level of genes expression.
Keywords: Drug resistance, Gene expression, Zoonotic cutaneous leishmaniasis, q-RT PCR

Introduction

Leishmaniasis is a parasitic disease caused by *Leishmania* genus of the Trypanosomatidae family in the form of cutaneous (CL), mucocutaneous (MCL), visceral (VL), and post-kala-azar dermal leishmaniasis. The World Health Organization (WHO) identified leishmaniasis as one of the most neglected diseases. Leishmaniasis is endemic in more than 88 countries. Approximately 700 000-1 000 000 new cases are annually reported and ~26 000 to 65 000 deaths occur every year. Different species of *Leishmania* cause cutaneous leishmaniasis, including *Leishmania major*, *Leishmania tropica*, and *Leishmania aethiopica*. In addition, Iran has been identified as an important focus of zoonotic cutaneous leishmaniasis (ZCL) caused by *L. major* (1,2).

Treatment is generally recommended in spite of the self-limiting feature of CL. Treatment can prevent not only scarring but also necrosis and rapid development of lesions following secondary bacterial infections. The pentavalent antimonial compounds Glucantime® (meglumine antimoniate) and Pentostam® (sodium stibogluconate) have been the first-line treatment for all forms of leishmaniasis (1). Variations in therapeutic responses to glucantime in previous decades and numerous cases of treatment failure around the world have complicated the role of the first-line drugs in leishmaniasis (3). In the clinical manifestation, resistance to glucantime has been proven by some researchers such Hadighi et al in Iran and Kumar et al in India (4,5). In North Bihar in India, an area with various anthroponotic visceral leishmaniasis, many cases of glucantime resistance have been observed so that up to 60% of newly diagnosed VL cases in this area do not respond to pentavalent antimonial compounds (6). Previous evidence demonstrated that treatment failure is the result of reduced parasite sensitivity to the antimony compounds (3).

Some studies discovered that the prevalence of parasites becoming resistant to the first-line drug pentavalent antimonial (Sb^V) is increasing in several parts of the world and there are wide differences in the sensitivity of various

CL isolates to Sb^V compounds (3). Gene variation and drug resistance in leishmaniasis caused by *L. tropica* have been reported in Iran (7). According to a cross-sectional research study in Mashhad (Iran), 13% of patients did not respond to glucantime (4).

One of the mechanisms of drug resistance in the parasite is the decrease of drug density. To achieve this goal, the parasite does some mechanisms such as a decrease in receiving the drug, sequestrate, and efflux of the received drug, inhibiting the activation of the drug or degradation of the activated drug by metabolism or analysis (3). Adenosine triphosphate (ATP)-binding cassette proteins (ABC) are present in all organisms and known as the largest superfamily of efflux pumps. Different ABC classes are explained in *Leishmania*, one of which is P-glycoprotein A (PgpA), which is a gross membrane protein that acts as an ATP-dependent extraction pump and controls the mechanism of drug efflux and its sequestration (3).

It has been proven that glucantime-resistant *Leishmania*, as a multidrug resistance protein (MDR) which is a homolog class of ABC, is amplified in an external chromosomal shape. It has been demonstrated that the overexpression of PgpA is an important reason for resistance against glucantime (8).

On the other hand, oxidative stress is one of the Sb^V mechanisms of action against *Leishmania*, and the existent of Thiol, as an internal reduction, is important for neutralizing the oxidative action. The laboratory mutants of Sb^V-resistant *Leishmania* species have shown a clear rise in the intracellular thiols level, including trypanothione (TSH), which is the only major thiol in trypanosomatidae (9). The synthesis of G-glutamyl cysteine synthetase (Gsh), encoding gamma-glutamylcysteine synthetase (γ-Gcs), determines the TSH level. Therefore, the level of TSH increases due to the high expression of Gsh. In a study on therapeutic failure isolates, *Leishmania (Viannia) guyanensis* L. represented the overexpression of Gsh1 (9).

Aquaglyceroporin 1 (Aqp1), as another studied gene, is a member of the superfamily of aquaporin. These are membrane channels that allow the entry of small neutral salts such as glycerol or urea (10). In *Leishmania* species, Aqp1 is the major route of trivalent antimony entry. It is shown that the rate of Aqp1 expression is in relationship with resistance, thus the overexpression of Aqp1 can affect sensitivity to Sb^V in *Leishmania* (11).

Resistance against glucantime in some species of *Leishmania* such as *L. tropica* has been proven, and the prevalence of no response of *L. infantum* against glucantime has been reported in recent years (12). In this study, the expression rate of PgpA, Gsh1, and Aqp1in phenotypic resistance ZCl, was assayed in vitro and in vivo in comparison to non-resistant *L. major* (MRHO/IR/75/ER).

Materials and Methods
Clinical samples, parasite isolation, and culture
This experimental study was performed by sampling ten non-healing CL patients who failed to recover despite receiving at least one full course of glucantime from health centers in Isfahan and its suburbs. They were positively diagnosed by microscopic observations for leishmaniasis. The parasites were isolated from the lesion by transferring the small amount of them in the Novy-Mac Neal-Nicolle) medium at 24 ± 1°C.

After isolation, parasites were transferred to the RPMI 1640 medium, supplemented with 10% inactivated fetal calf serum (FCS), 100 μg/mL streptomycin, and 100 IU/mL penicillin G, and finally cultured at 24±1 °C. *L. major* (MRHO/IR/75/ER) and *L. tropica* (MRHO/IR/02/Mash10), as controls, were also cultured through a similar process.

Species identification
The species were identified by the conserved region of kinetoplast DNA (kDNA) minicircles and the nested polymerase chain reaction (PCR) method. The total DNA of the isolates and controls was extracted with a commercial kit (GeNet Bio, Seoul, Korea) and according to the manufacturer's instructions. The first and second stages of nested-PCR were performed using CSB7XR: ATT TTT CGC GAT TTT CGC AGA ACG and CSB2XF: CGA GTA GCA GAA ACT CCC GTT CA GC primers in the first step, as well as 13Z: ACT GGG GGT TGG TGT AAA ATA G and LiR: TCG CAG AAC GCC CCT primers in the second one (13).
The PCR products were electrophoresed by applying 1.5% agarose gel, stained by ethidium bromide, and were observed under a transilluminator. *L. major* (MRHO/IR/75/ER) and *L. tropica* (MHOM/ IR/02/Mash10) were used as standards.

In vitro experiment
The J774 murine macrophage cell line was purchased from Pasteur Institute of Tehran, Iran and grown at 37 °C and 5% CO_2 in RPMI-1640 supplemented with 20% heat-inactivated FCS and 100 μg/mL streptomycin and 100 IU/mL penicillin. After mass production, a scraper was utilized to detach cells from the plates. Subsequent to Neubauer chamber cell counting, $2×10^6$ cells/wells were seeded to each 3.5 cm^2 well of six-well plates in the mentioned condition for 5-6 days. A well with a 22×22 coverslip on the bottom was considered for the control group.

The amastigote macrophage model was used to assay gene expression, and two wells were devoted for each sample. One of them was considered for pre-treatment and the other was supposed to treat by the half-maximal inhibitory concentration of glucantime (IC$_{50}$), namely, 7.2 μM (14).

Then, late stationary promastigotes of samples and the standard *L. major* strain (MRHO/IR/75/ER) were separately added to the grown, adhesive J774 macrophages at a 7:1 ratio of promastigotes/macrophage. The medium was removed 4-6 hours after incubation at 34 °C and 5% CO_2, and then the cells were washed with phosphate buffer saline, and fresh medium was added as well. The

plates were incubated at 37 °C in the presence of 5% CO_2 for an additional 24 hours. Next, the coverslip of the control plate was removed and stained with Giemsa, and the phagocytic act was investigated microscopically.

The total RNA of the phagocytic amastigotes was extracted with RNeasy Mini Kit (QIAGEN, Germany) and then reverse transcribed into cDNA with the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. The obtained cDNA was used to assess the profiles of RNA expression.

In vivo experiment

Four six-week-old inbred female Balb/c mice were obtained from Tehran Pasteur Institute (Tehran, Iran) and divided into eleven groups each containing 10 animals. For Leishman lesion, 10 groups were inoculated with 2×10^6 stationary promastigotes of 10 different *L. major* samples isolated from the patients, and one group was inoculated with the *L. major* (MRHO/IR/75/ER) at the base of the tails. *Leishmania* ulcer was created after 4-5 weeks. Direct sampling and microscopic examination were performed to ensure the existence of parasites in the lesions. For treatment, the mice in each group received glucantime intralesional (60 mg/kg/d) for two weeks (14). Sampling was performed before treating the mice, and then RNA was extracted using RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. The isolated RNA was reverse transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions, and *in vivo* examinations were continued to evaluate the profiles of RNA expression.

Quantitative-reverse transcriptase real-time PCR

Quantitative-reverse transcriptase real-time PCR (q-RT-PCR) was used to assess the profiles of RNA expression. The total reaction volume was 20 µL (contacting SYBR Master 1x: 10 µL; RNase-free water: 7.5 µL; Forward and reverse primers each: 0.5 µL; cDNA: 1.5 µL), and the followed conditions included 95°C/5 minutes for 1 cycle, and then 95°C/10 seconds and 60°C/30 seconds both for 40 cycles. As the template, four genes were analyzed, including PgpA, Gsh1, and Aqp1, and as internal control 18s rRNA. Forward and reverse primers were designed and blasted by the National Center for Biotechnology Information (Table 1).

Gene expression was quantified and analyzed by measuring threshold (CT) and applying the following formula:

$$\Delta\Delta CT = [(CT \text{ of treated sample gene} - CT \text{ of internal control gene}) - (CT \text{ of untreated sample gene} - CT \text{ of internal control gene})]$$

The results were analyzed by the comparative threshold cycle methods ($2^{-\Delta\Delta CT}$) and appropriate statistical analysis (15). Differences were considered significant at a level of $P \leq 0.05$.

Results

The molecular product of the nested-PCR on agarose gel 1% showed an amplified band of 560 bp, and in comparison with the standards, *L. major* (MRHO/IR/75/ER) (560 bp) and *L. tropica* (MHOM/IR/02/Mash 10, 750 bp), all samples were suggested to be *L. major* (Figure 1). Figure 2 displays the result of infecting the J774 cell line murine macrophages with promastigotes and the transformed intracellular amastigotes.

The gene expression of PgpA, Gsh1, and Aqp1 was studied for all isolates. Statistical analysis revealed that the expression of the studied genes in the isolates did not increase after exposure to glucantime (Table 2). The expression of these genes in isolates was also the same in comparison with *L. major* (MRHO/IR/75/ER), and after exposure to glucantime represented no increase in expression both in vitro and in vivo (Table 3 and Figure 3). The efficacy of the primers and the PCR was estimated to be 95%.

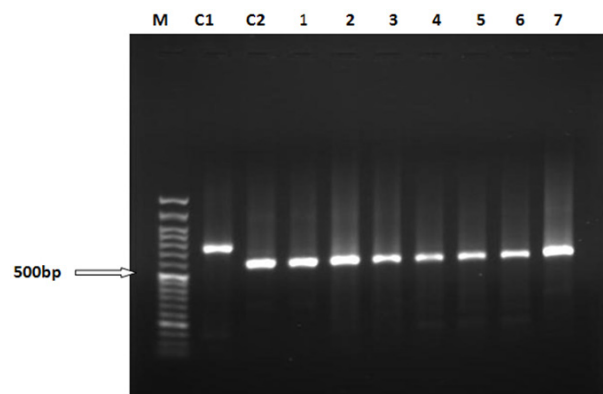**Figure 1.** Agarose gel (1%) electrophoresis of the second phase of nested-PCR product of genomic DNA from patients with resistance phenotype. *Note.* PCR: Polymerase chain reaction. Lane M is a 50 bp DNA ladder (Fermentas), C1: DNA from *L. tropica* (MHOM/IR/02/Mash10), C2: DNA from *L. major* (MRHO/IR/75/ER). Lanes 1-7 are samples from non-healing individuals.

Table 1. Forward and reverse primers

Gene	Accession Number	Forward Primer	Reverse Primer
Gsh1	XM-00168252	GCTCCACGATGACAC GGTTACC	GGATTCCACACGG CACAGTTG
PgpA	XM-00168329	GGTCTCATCTCGCTG TCACT	ACGCTCCACGCT GTTCAT
Aqp1	XM-001684934	TGCCACGATGCCGAG ACTC	GTGTTCGTCCTCCTCTTC CTCAT
18srRNA	Housekeeping	CCAAAGTGTG GAGATCGAAG	GGCCGGTAAAGG CCGAATAG

Note. PgpA: P-glycoprotein A; GSH: G-glutamyl cysteine synthetase; Aqp1: Aquaglyceroporin 1.

Table 2. The mean expression of PgpA, Gsh1, and Aqp1 genes (Δ CT) in isolated from patients and standard *Leishmania major* (MRHO/IR/75/ER) affected by glucantime

Gene	Standard *L. major* (MRHO/IR/75/ER)			Patients sp		
	Glu⁻	**Glu⁺**	***P* value**	**Glu⁻**	**Glu⁺**	***P* value**
	In vitro					
PgpA	1.12±0.01	1.49±0.01	0.157	1.34±0.29	1.69±0.76	0.125
GSH	1.4±0.01	1.42±0.01	0.655	1.35±0.2	1.48±0.25	0.065
AQP1	0.53±0.01	0.5±0.01	0.182	0.57±0.13	0.83±0.47	0.097
	In vivo					
PgpA	1.27±0.007	1.32±0.007	0.158	1.27±0.2	1.53±0.74	0.222
Gsh	1.21±0.01	1.28±0.01	0.157	1.19±0.11	1.37±0.38	0.113
Aqp1	0.53±0.01	0.59±0.01	0.156	0.55±0.1	0.63±0.07	0.611

Note. PgpA: P-glycoprotein A; Gsh: G-glutamyl cysteine synthetase; Aqp1: Aquaglyceroporin 1. *P* value was calculated by paired sample t-test.

Discussion

Pentavalent antimonial compounds (e.g., meglumine antimoniate or glucantime) are the first-line leishmaniasis treatment (1). However, there are some reports of non-healing cases with glucantime and the existence of drug resistance (5). Gene expression analysis was used to investigate the non-healing phenotypic cases that have been observed in the Trypanosomatidae family (16). Intracellular amastigotes are the ideal materials and are considered as the gold standard for the leishmaniasis study (16,17).

In this study, *L. major* was selected because it is endemic in our zone (Isfahan province at the center of Iran), and there are a few gene expression relevant reports about that (2).

Researchers have shown that the expression rate of some kinds of resistance genes, including ornithine decarboxylase, trypanothione reductase (TR), and Aqp1 in resistant and sensitive strains is different, and it was higher in resistant cases (10,18).

Genomic variation in sodium stibogluconate (Pentostam) resistance has been reported in some species

Figure 2. J774 Cell Line Murine Macrophages Containing Amastigotes (Magnification 1000X).

of *Leishmania*, including *L. tropica,* which is the cause of anthroponotic CL (19).

The first MDR homologous gene (PgpA) was an extra chromosomal circle (H-circle) amplified in a methotrexate, arsenite-resistant

Leishmania tarentolae promastigote cell line (19,20). A four-fold decrease was observed in the aggregation of antimony in PgpA transfected *L. major* versus the wild type (20).

In a research study, 97 samples were collected from the ulcers of leishmaniasis patients from some endemic areas of Iran and tested for mutation detection in MDR genes with conformation sensitive gel electrophoresis) and restriction fragment length polymorphism, 10 cases (11%) among them had a disorder in the sequence of drug-resistance genes (19).

Moreover, in Indian visceral leishmaniasis due to *Leishmania donovani*, the AQP1 gene demonstrated a higher copy number in antimony-resistant isolates when compared with sensitive isolates. The down-regulation of AQP1 RNA levels was not consistently found in the antimony-resistant isolates. This study indicated that the down-regulation of AQP1 may be one of the mechanisms of antimony resistance and suggested the important role of the AQP1 gene in the generation of antimony-resistant phenotypes although it is not the sole determinant of antimony resistance in the Indian field isolates (21).

The transfection of the PgpA gene and its overexpression led to antimony-resistance to *L. infantum* promastigote and amastigote (22). The amplification of PgpA and Gsh1 genes was observed in the SbIII- and arsenite-resistant mutants (23).

Torres et al clarified that the *L. guyanensis* isolates of non-healing patients have the overexpression of Gsh1 (24).

In this study, non-healing isolates and susceptible species were investigated, and no significant difference was found in the expression levels of tested genes both in vitro and in vivo. In spite of the achievements of previously mentioned studies, the results of this study indicated that there was no innate resistance against glucantime in *L. major* isolated from non-healing patients. The results
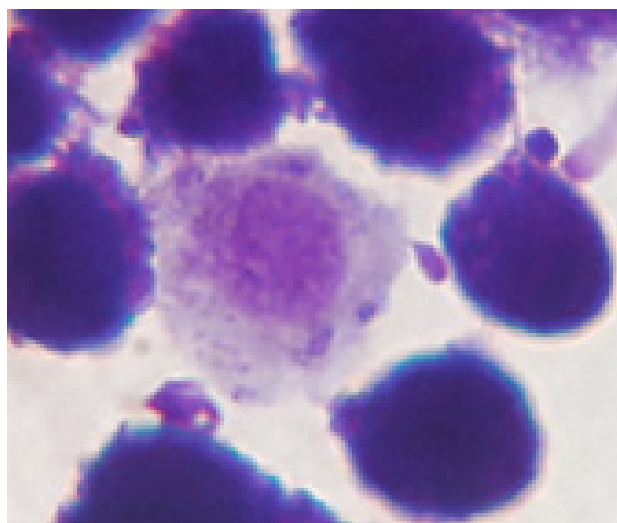
Table 3. Comparison of relative gene expression data ($2^{\Delta\Delta-CT}$) in standard *Leishmania major (MRHO/ER/75/IR)* and samples isolated from patients

Gene	$2^{\Delta\Delta-C_T}$ (In vitro)			$2^{\Delta\Delta-C_T}$ (In vivo)		
	L. major (MRHO/IR/75/ER)	Patients' isolates	*P* value	*L. major* (MRHO/IR/75/ER)	Patients' Isolates	*P* value
PgpA	0.77±0.1	0.78±0.59	0.371	0.83±0.58	0.96±0.49	0.573
Gsh	0.99±0.007	0.91±0.18	0.573	0.88±0.1	0.95±.153	0.161
Aqp1	1.02±0.007	0.83±0.3	0.278	0.95±0.006	0.96±.11	0.361

Note. L. major: Leishmania major; PgpA: P-glycoprotein A; GSH: G-glutamyl cysteine synthetase; AQP1: Aquaglyceroporin 1. *P*-value was computed by the Mann-Whitney test.
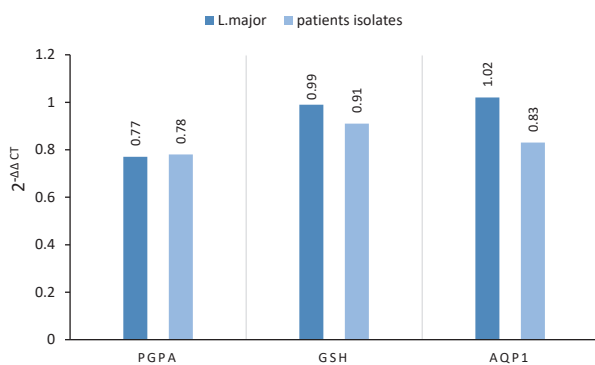
Figure 3. Comparison of $2^{\Delta\Delta-CT}$ in *Leishmania major* (MRHO/ER/75/IR) and patients' isolated samples (*in vitro* exam).

conform to the findings of a study undertaken in Brazil on *Leishmania braziliensis* and *L. guyanensis* samples from patients with different responses to antimonial drugs. In the mentioned study, the RNA expression of six genes, by playing a role in antimony metabolism (Aqp1, MRPA, Gsh1, Gsh2, TRYR, and TDR1) was assessed using the q-real-time PCR, and molecular data were compared with the clinical phenotypes. Among the isolates from patients with *L. braziliensis* with different treatment outcomes, no significant difference was found in the expression levels of the mentioned genes except for Gsh1 (24). Additionally, the findings of some studies in Nepal and Peru showed that there is no significant relationship between the sensitivity of parasites to antimony compounds *in vitro* and *in vivo*, indicating that resistance to glucantime is not inherent, and host body factors are highly important in the effectiveness of the drug (25,26).

In a study on the treatment of CL, patients represented significant variations in response to sodium stibogluconate (Pentostam) while no differences to this drug were observed in the sensitivity of *L. major* isolates from patients in the amastigote-macrophage model (27).

Therefore, our data support the suggestion that non-healing leishmaniasis frequently depends on incomplete irregular intermittent drug use, and this is the major reason for induced resistance.

The results of a research study on 312 patients demonstrated that they failed to recover despite receiving one or more courses of antimony. It was further found that only 26% of patients were treated according to the WHO guidelines while 72% of them received the drug fewer than 40 days, 42% took the drug irregularly, and 36% stopped the drug receiving arbitrarily (28).

However, treatment failure is a complex interplay between various factors related to either drug, parasite, or host, and thus is not necessarily exclusively linked to drug susceptibility. The quality of host's immunity and genetic, pharmacological factors, drug quality, intrinsic drug properties, treatment duration, and even differences in manufacturers, along with the biological sensitivity of the species of *Leishmania*, individually and collectively, influence the outcome of treatment. Therefore, due to the mentioned reasons, clinical response to antimony is in the complicated relationship with parasite sensitivity and other factors relevant to the patient or the drug (29).

Conclusion
Although in many cases, the lack of treatment has been shown to be the innate resistance in different species of *Leishmania*, no inherent resistance was observed in the examined *L. major* samples in this study. In addition, no correlation was found between the healing of lesions and the level of gene expression. Accordingly, further investigations are required to find more reasons for the resistance against antimonials. Nonetheless, it is important to mention that probably large-scale drug misuse induces and develops drug resistance, and it is recommended that patients be treated by experienced and skilled physicians according to approved protocols.

Authors' Contribution
SHH supervised and coordinated the project. SS, SS, and RA performed the experimental section. SS designed and was involved in the preparation of the manuscript.

Conflict of Interests
The authors declare that they have no conflict of interests.

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