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Original Article

Comparison of Proteome Profiling of Two Sensitive and Resistant Field Iranian Isolates of *Leishmania major* to Glucantime® by 2- Dimensional Electrophoresis

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Received 05 Jun 2014
Accepted 26 Nov 2014

Keywords:
Leishmania major,
Resistant,
Sensitive,
Meglumine Antimoniate (Glucantime®),
2- Dimensional Gel Electrophoresis,
Proteome map,
Iran

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Abstract

Background: In this study, two-dimensional gel electrophoresis (2-DE) method was applied to determine and compare the protein spots expressed in the two field isolates of *Leishmania major* and recovered from the patients who were clinically sensitive and resistant to Glucantime® treatment.

Methods: *Leishmania* parasites were isolated from the cutaneous lesions of two CL infected patients in Shiraz, south of Iran. The species of the two isolates were identified as *L. major* using Nested-PCR. Sensitivity (Sh-214S) and resistance (Sh-120R) of the two isolates to meglumine antimonite were checked by the standard in vitro assays. Both sensitive and resistant *L. major* isolates were harvested in RPMI 1640 medium. Protein extractions were performed using TCA/Acetone method and the protein spots were determined by a two-dimensional gel electrophoresis (2-DE). The gels were stained with silver nitrate and analyzed by Image Master 2D Melanie-6 software.

Results: About 2967 protein spots were detected. Overall, 89 protein spots represented considerable changes of expression in the resistant isolate of *L. major* compared to the sensitive isolate. Of these, 60 and 29 protein spots were up-and down regulated, respectively. In addition, 11 protein spots present in the resistant isolate were noticed to be absent in the sensitive isolate.

Conclusion: A number of proteins showed significant changes of expression in the drug-resistant *L. major*; moreover, the roles of these proteins probably enhanced the parasite resistance to the drug and increased parasite survival in the cells.

Introduction

Leishmaniasis is a vector-born disease disseminated throughout the world, especially in tropical and subtropical regions, causing serious public health problems in 77 countries with an incidence of 1-1.5 million cases annually (1). Cutaneous leishmaniasis (CL) is one of the most common forms of the disease reported to be endemic in different parts of Iran (2). In general CL presents in two main forms: anthroponotic CL (ACL) and zoonotic CL (ZCL), ACL and ZCL are caused by *L. tropica* and *L. major*, respectively. ZCL reaches out from northeast to the center and west of Iran as well as all the southern provinces (3-5). Approximately, 75% of CL cases belong to ZCL (6). Most of ACL are reported from northeast and central parts of Iran (3, 5).

Recently, cases of cutaneous leishmaniasis reported have increased in most parts of Iran (2, 5). Regarding the difficulty of vector control and absence of vaccine against this parasite, chemotherapy has an important role in controlling the disease (7). Meglumine antimoniate (Glucantime®) is the best option for the treatment of leishmaniasis, but the prevalence of parasites resistant to this drug in ACL and ZCL cases have enhanced. Antimonial treatment failure has been reported in 10-12% of ACL patients and 16% of ZCL patients in Iran because of drug resistance (6, 8). Within the first course of treatment, 40% of patients have not shown a suitable response to meglumine antimoniate (9).

Studies on gene amplification can be helpful, but since they are involved in different mechanisms and several genes are up- or down-regulated concurrently, this problem is hard to be conducted. Proteins play an important role in the regulation of cellular responses against the drugs (10, 11). Proteomic approaches have been employed to investigate protein expressions of protozoan parasites such as *Plasmodium falciparum* (12), *Trichomonas vaginalis* (13), and *Giardia lamblia* (14). Comparative proteome analyses have been successfully used in *Leishmania* spp. for an understanding of diverse outcomes of *Leishmania* against drug responses (15). Proteomic approaches to study *Leishmania* in general are at their early stages and partial proteome maps have only recently been reported based on the two-dimensional gel electrophoresis (2- DE) to separate proteins with regard to their isoelectric and molecular weight points (16,17).

The goal of our study was to perform a comparative analysis of 2-DE protein maps of the two clinical sensitive and resistant field isolates of *L.major* to Glucantime® for the study of over- or down-expression patterns of proteins with the final objective of employing proteomic technologies to search for specific proteins of resistant *Leishmania* to the drug. This is the first proteomic analysis of the two sensitive and resistant field isolates of *L. major* to Glucantime® in Iran.

Materials and Methods

Leishmania parasite isolates

Leishmania parasites were isolated from the cutaneous lesions of two patients infected with cutaneous leishmaniasis in Shiraz as the capital city of Fars Province, south of Iran. They revealed a significant level of unresponsiveness to Glucantime® among other patients with cutaneous leishmaniasis caused by *L. major* in Iran (18).

One of them was clinically resistant (Sh-120R) to Glucantime® and the other was sensitive (Sh- 214S) to the drug. The resistant case was treated during two full courses of intralesional Glucantime® administration (Rhone Poulenc Rorer, Paris, France) but no recovery was achieved and the patient still presented an active lesion (2). During parasitological examinations, infection was confirmed by the microscopic identification of *Leishmania* amastigotes in stained smears via a high magnification (1000×). A susceptible CL case after treatment with Glucantime® and the follow- up course led

to a complete recovery since no amastigotes were found during the following parasitological investigations. The patients reported no previous use of anti- leishmanial drugs, CL history or acute or chronic medical conditions.

This study was reviewed and approved by the Ethics Committees of Ahvaz Jundishapur University of Medical Sciences, Iran.

Species identification
DNA extraction and Nested PCR

DNA extraction was performed according to the protocol of commercial kit (Roche, Germany). To carry out the Nested PCR, we used the primers CSB1XR (ATTTTTCGCGATTTTCGCAGAACG) and CSB2XF (CGAGTA GCAGAAACTCCCGTTCA) as the forward and reverse primers, respectively, in the first step and in the second step, 13Z (ACTGGGGGTTGGTGTAAAATAG) and LiR (TCGCAGAACGCCCCT) were used to amplify the mini-circle variable kDNA (19, 20).

Amastigote drug (Glucantime®) susceptibility assay

The drug susceptibility of the two clinical isolates was implemented in the J774A.1 monocyte-macrophage mouse cell-line. Briefly, J774A.1 (5×10^4 cells/well) were grown in RPMI (Gibco/BRL) with a 15% FBS (Gibco/BRL) plated in 8 chamber LabTek tissue culture slides (Nunc, NY, USA) and incubated at 37˚C for 24 h to allow cell adherence. Then, the cells were infected with late logarithmic promastigotes at a parasite-to-macrophage ratio of 10:1. After 5 h of incubation at 37˚C, free promastigotes were removed with 3 times washing and the cells were re-incubated at the presence of serial dilution of Glucantime® for 72 h. Each 5-ml ampoule of Glucantime® (Sanofi-Aventis, Paris, France) contained 1.5 g meglumine antimoniate. The serial dilutions of Glucantime® used for the sensitive and resistant isolates were 2, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70 µg/ml (the doses were obtained based on the previous screening test). Fresh Glucantime® was added and slides were incubated for an additional 72 h and then stained with Giemsa. 3 slides were used for each isolate. The number of amastigotes was counted in 100 randomly chosen macrophages. The percentage of infected macrophages and the number of parasites per infected cell were evaluated through microscopic examinations. An inhibitory concentration of 50% (IC_{50}) is defined as the effective dose of Glucantime® that decreases the survival of *L. major* by 50% (21, 22).

Cell culture

L. major including the sensitive (Sh- 214S) and resistant Sh(- 120R) isolates was selected for mass production. Three replicates of promastigotes of each isolate were separately cultured. Promastigotes were sub- cultured in RPMI1640 medium (Gibco/ BRL) supplemented with 10% FBS (Gibco/BRL), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco/ BRL). Cultures were incubated at 25°C. Promastigotes were harvested after the last 5- day sub-culture at the late log phase and centrifuged at 3000 rpm and then washed 3 times with sterile PBS (pH 7.2- 7.4). Afterwards, 400×10^6/ ml of promastigotes were collected for the protein extraction.

Protein Extraction

The promastigotes were re-suspended in 5 mM Tris- HCl (Roche, Germany), pH= 7.8, containing 1mM of phenylmethylsulfonyl fluoride as a protease inhibitor (Roche, Germany), sonicated 3 times at 40 Hz for 10 seconds with the intervals of 50 seconds, and kept at 4°C for 4 hours. A 20% TCA with mM DTT (Merck, Germany) were used for the precipitation of proteins at -20°C for 1h. Samples were then centrifuged at 13000×g at 4°C for 15 minutes and washed with cold acetone containing 20 mM DTT and the residual acetone was removed by air-drying overnight. The samples were dissolved in 100 µl lysis buffer containing 7M urea, 2M thiourea (Merck, Germany), 1% ampholyte, pH=4- 6.5, 1% ampholyte, pH= 5- 7 (Bio-Rad), and 4%

CHAPS (Merck, Germany) detergent (23, 24). Protein concentration was determined by Bradford assay using BSA as the standard (25).

Two- dimensional gel Electrophoresis assay (2-DE)

For the first dimension, electrophoresis was applied onto IPG strips of 18 cm, pH=4-7 (Bio-Rad, Hercules, CA) rehydrated by loading the samples diluted with rehydration buffer (8M urea, 4% CHAPS, 2% ampholyte, 50 mM DTT, and traces of bromophenol blue) overnight. After the gel rehydration, IEF was performed at 300V/ 1h, 500V/ 1h, 1000V/ 2h, and 3500V/ 12h using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham, Piscataway, NJ) (26).

For the protein to be transferred from the first to the second dimension, IPG strips were incubated in an equilibration solution (50 mM Tris- HCl, pH= 8.8, 6M urea, 20% glycerol) (Merck, Germany), 2% SDS (Sigma), and 0.01% bromophenol blue (Merck, Germany) containing 2% DTT for 15 minutes and then re- incubated in the equilibration solution containing 2.5% iodoacetamide (Merck, Germany) for 15 minutes. Strips were placed on top of 10-15% gradient SDS-PAGE and sealed with agarose solution (Bio- Rad) (0.5% agarose plus a few grains of bromophenol blue).

The 2DE was carried out at 16 mA/gel and 24 mA/gel at 20 °C for 30 minutes until the dye front reached the bottom of the gel (24, 26, 27). Gels were silver- stained under the same conditions by freshly prepared silver reagents.

Image analysis

The gels were stained with silver nitrate scanned at a resolution of 300 dpi using the Bio- Rad GS- 800 densitometer and analyzed by Image Master 2D Melanie- 6 software. Each set of gel replicates for both the sensitive and resistant *L. major* isolates was combined into the average gels, which represented spots reproducibly present on both sets of the replicate gels. Spot filtering and editing were performed manually since the spots were neither detected by the software's automatic spot detection process nor separated correctly. Statistical analysis of protein variations was implemented by Student's *t*- test. It was concluded that protein spots are significantly up- or down- regulated when $P< 0.05$. The PI and molecular mass of proteins were assessed by standard markers (Amersham Pharmacia Biotech) (24, 27).

Statistical analysis

In vitro drug susceptibility assay was presented as IC50 (50% of inhibitory concentration), which was subsequently determined using linear regression analysis. All experiments were conducted at least 3 times and the results are expressed as the mean ± standard deviations (SDs). The significance of differences was designated by Student's *t*-test and the level of acceptable significance was 95% ($P < 0.05$).

Results

Nested PCR

The two sensitive and resistant *Leishmania* isolated produced amplified fragments of 560 bp for *L. major*. Amplification reactions in 1.5 % agarose gel electrophoresis using a 100 bp DNA ladder (Roche, Germany) are shown in Fig. 1.

Fig. 1: Gel electrophoresis of PCR products. **Lane 1)** DNA size marker 100bp, **lane 2)** Negative control, **lane3)** *L. tropica* (positive control standard = 750bp), **lane 4)** *L. major* (positive control = 560 bp), lanes **5, 6)** *L. major* isolates obtained from skin lesions of the patients sensitive and resistant isolates to Glucantime®

Drug susceptibility of L. major field isolates to Glucantime®

In vitro drug sensitivity of *L. major* to Glucantime® was determined in J774-1 cells. The results indicated IC_{50} value of Sb (V) for the resistant isolate was 25.6± 3.7 µg/ ml, whereas that of the sensitive isolate was 5.12± 1.3 µg/ ml. The IC_{50} of the resistant isolate was about 5- fold higher than that of the sensitive isolate.

Expression of protein spots in a two-dimensional gel electrophoresis

After the completion of automated and manual editing of the image for refining the artifacts, approximately 2967 protein spots were detected in each isolate. Overall, there was a significant homogeneity in the protein distribution and positioning between the two sensitive and resistant *Leishmania* isolates. Images resulted from the triplicate gels were analyzed. Representative 2DE maps of the sensitive and resistant *L. major* isolates are shown in Fig. 2.

Overall, 650 protein spots visually noticeable by Melanie- 6 software analysis were done while 115 protein spots displayed significant changes under drug resistance. Proteins were extracted from the two sensitive and resistant *L. major* isolates with a pH of 4- 7. PH range of the IPG strips of isoelectric focusing of the differentiated proteins was 4.16 to 6.52. Molecular weights of the differentiated proteins were between 14.1 and 83.2 kDa.

IF of 115 proteins from among 650 spots were detected with remarkable changes under drug resistance in the two isolates. Induction factor (IF), pH isoelectric focusing (*PI*), and molecular weights (MW) of the differentiated proteins between the two isolates are shown in Table 1.

These data were indicative of the differential expression of the resistant isolate to Glucantime® compared to that of the sensitive isolate in 115 protein spots from among which, 60 and 29 protein spots were significantly over-expressed and down-expressed, respectively. Additionally, 11 protein spots present in the resistant isolate were absent in the sensitive isolate. IF<0.5 and IF>2 were demonstrated for the down-and over-expressions of the protein spots. Close-up and 3D images revealed some protein spots between the two isolates are different in the expressed protein spots (Fig. 3, A and B).

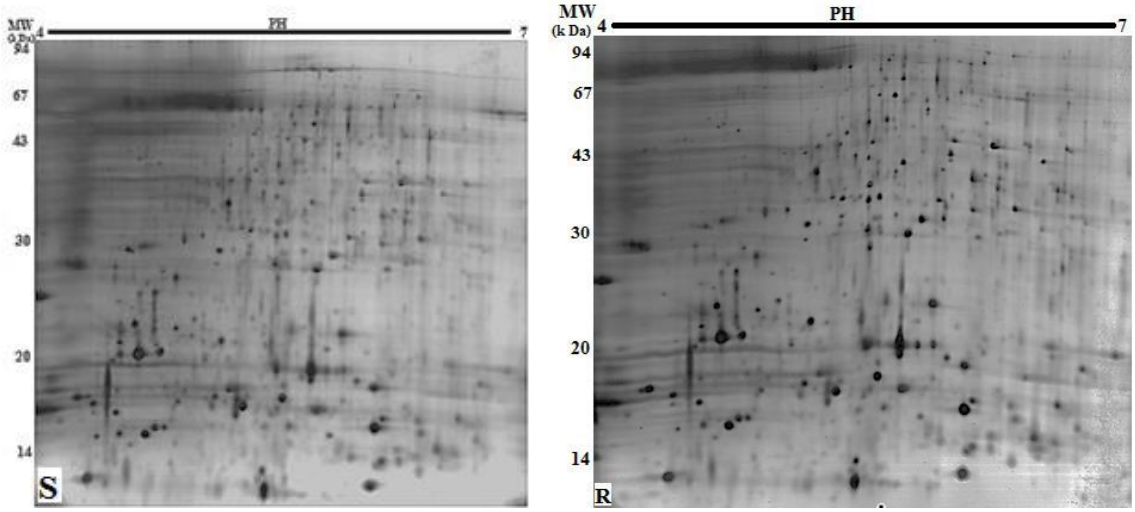

Fig. 2: 2DE gel image of Glucantime® sensitive (S) and resistance (R) isolates of *L. major* promastigotes that stained by silver nitrate on 18 cm IPG strips with pH range of 4- 7 and SDS-PAGE 12%

Table 1: Spots differently expressed between Glucantime® sensitive and resistant *L. major* isolates detected after 2D gels. **(A)** IF >2 showed the high expression **(B)** IF< 0.5 demonstrated the down expression **(C)** Absence of protein spots in resistant *L. major* isolate **(D)** Presence of protein spots in resistant *L. major* isolate

(A) High expression protein spots in resistant *L. major* isolate

No.	Spot Number	IF[a]	PI[b]	MW[c]	No.	Spot Number	IF	PI	MW
1	13	2.045456	4.16	14.32	**31**	220	2.662166	5.91	46.19
2	21	2.598937	5.42	14.92	**32**	235	2.298648	6.36	43.10
3	23	2.907549	5.20	14.28	**33**	258	4.254391	4.72	14.15
4	24	2.279519	5.34	15.18	**34**	259	3.81401	4.94	15.22
5	26	2.016594	5.30	15.17	**35**	271	3.772084	6.52	15.17
6	31	2.271391	5.18	16.13	**36**	281	4.730606	5.17	15.16
7	36	3.74182	6.11	15.17	**37**	284	2.255951	4.98	16.10
8	41	2.981907	6.30	15.20	**38**	286	2.633021	4.89	17.15
9	56	2.597753	5.72	21.32	**39**	289	2.759802	5.49	15.79
10	58	2.552658	5.52	18.35	**40**	294	2.074687	5.80	19.85
11	60	2.795147	5.22	20.18	**41**	306	2.039297	5.12	20.14
12	70	2.52878	5.50	20.37	**42**	317	3.270161	5.07	21.96
13	80	2.666548	5.11	29.11	**43**	318	2.730142	5.12	21.94
14	84	2.371862	5.05	29.9	**44**	319	2.667469	5.07	18.17
15	88	2.484961	5.31	26.9	**45**	353	2.594021	5.47	29.92
16	90	2.186684	5.21	27.14	**46**	360	4.36526	5.48	27.57
17	94	2.251584	5.53	30.13	**47**	362	2.337795	5.53	25.19
18	115	2.07755	5.92	29.8	**48**	364	3.287226	5.37	32.11
19	125	2.86259	6.13	25.8	**49**	385	2.775042	5.08	36.90
20	128	2.450641	6.23	26.6	**50**	392	2.259497	6.10	43.12
21	133	3.091465	5.01	26.9	**51**	403	2.428465	6.09	41.91
22	139	4.144915	5.27	36.11	**52**	411	4.610171	5.54	40.14
23	140	2.659075	5.72	33.6	**53**	416	2.828872	5.95	62.48
24	150	2.623845	6.16	18.18	**54**	428	3.150142	4.70	27.12
25	158	2.564301	4.94	20.85	**55**	430	7.84849	5.19	36.48
26	173	2.86451	5.86	40.52	**56**	431	3.243078	5.62	39.19
27	176	2.675983	5.55	45.78	**57**	433	9.256858	5.86	42.98
28	183	2.001409	5.78	38.72	**58**	435	6.068436	5.32	37.86
29	204	3.836405	5.67	57.69	**59**	454	10.4884	4.96	15.87
30	218	2.620985	5.75	55.10	**60**	461	33.86496	5.34	26.99

(B) Down expression protein spots in resistant *L. major* isolate

No.	Spot Number	IF	PI	MW	No.	Spot Number	IF	PI	MW
1	57	0.329713	5.85	22.7	**16**	266	0.23296	5.84	14.45
2	69	0.223998	5.45	21.7	**17**	273	0.354916	5.66	14.12
3	73	0.40258	5.67	26.11	**18**	384	0.379501	4.77	41.27
4	87	0.314991	5.25	26.4	**19**	404	0.282337	6.03	54.75
5	120	0.477984	6.27	33.11	**20**	405	0.147329	6.04	50.37
6	126	0.47344	6.31	26.15	**21**	412	0.46891	6.11	65.64
7	155	0.308663	5.41	14.10	**22**	417	0.309168	6.25	59.20
8	182	0.291121	5.58	36.43	**23**	422	0.464629	5.04	28.64
9	190	0.496649	5.43	46.25	**24**	425	0.191415	6.01	25.89
10	216	0.179107	5.57	68.11	**25**	445	0.342916	5.57	83.21
11	237	0.375255	5.94	78.14	**26**	449	0.363821	5.69	14.13
12	243	0.241714	6.50	34.68	**27**	462	0.111509	5.60	22.17
13	247	0.337436	6.27	39.55	**28**	463	0.316515	6.20	25.49
14	254	0.445859	4.45	14.68	**29**	480	0.309031	6.13	36.34
15	265	0.401693	5.57	14.19					

Table 1: Continued…

(C) Absence protein spot in resistant *L. major* isolate

No.	Spot Number	PI	MW
1	81	6.02	50.42
2	432	5.70	43.54
3	437	5.46	67.68
4	438	5.40	67.57
5	448	6.13	40.33
6	452	6.34	14.66
7	453	6.52	14.16
8	465	5.91	29.47
9	467	5.48	28.65
10	468	5.45	27.10
11	484	6.71	33.82
12	485	6.76	33.12
13	489	4.31	14.59
14	490	5.42	14.14
15	491	6.04	18.14

(D) Presence protein spot in resistant *L. major* isolate

No.	Spot Number	PI	MW
1	179	6.04	37.54
2	423	6.07	14.11
3	427	4.70	27.56
4	429	4.66	28.12
5	466	5.43	30.61
6	470	4.81	40.92
7	471	4.72	40.52
8	472	4.81	37.66
9	476	4.48	36.12
10	477	4.47	36.12
11	492	4.95	16.84

a. **IF**. Induction Factor. The induction factor calculated by dividing the percent volume of spots in gels. / **PI**. PH Isoelectric/**MW**. Molecular Weight

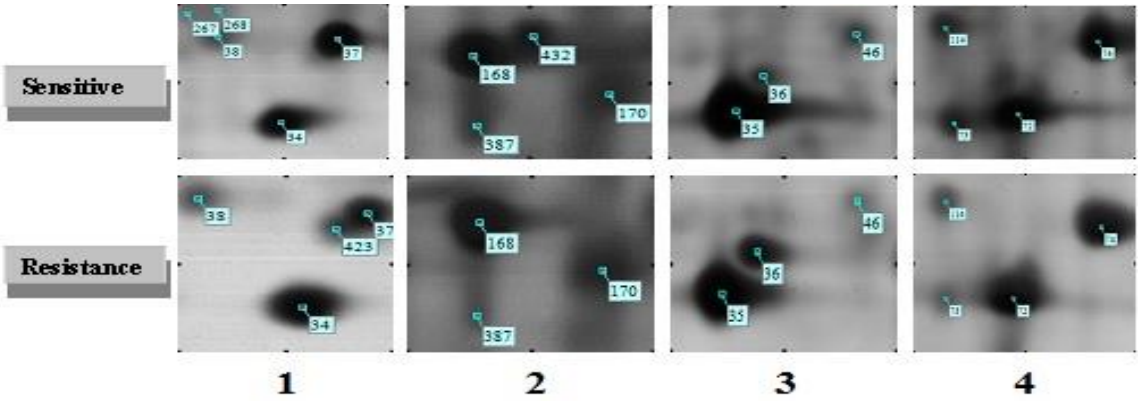

Fig. 3: A: Close- up views of differentially expressed protein spots in the promastigotes of *L.major* with significant vol. % changes in two Glucantime® sensitive and resistant isolates
1-Presence protein spot 423 in resistance isolate **2)** Absence protein spot 432 in resistance isolate **3)** Up regulate protein spot 36 in resistance isolate **4)** Down regulate protein spot 73 in resistance isolate

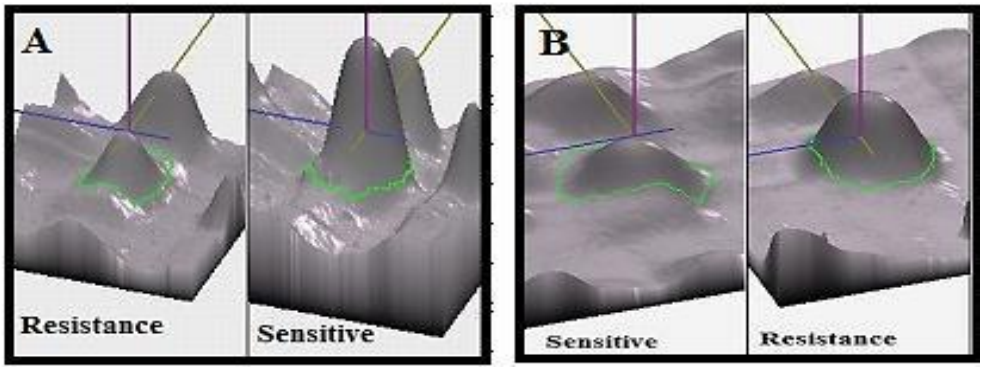

Fig. 3: B: 3D views of differentially expressed protein spots 73 **(A)** and 36 **(B)** in the promastigotes of *L.major* with significant vol. % changes in two Glucantime® sensitive and resistant isolates

Discussion

The first-line choice for the treatment of all forms of leishmaniasis in Iran is meglumine antimoniate (Glucantime®), which has been in use for more than 70 years in the world (28). Occurrence of the parasite resistance to this drug is increasing in some parts of the world, while the failure of antimonial treatment was especially reported in 16% of ZCL in Iran (6).

The intracellular model in a macrophage cell line is appropriate for evaluating the activity of anti-*Leishmania* drugs and identification of the resistant parasites can lead to the treatment failure. This approach was used to study the susceptibility of *L. major* isolates to Glucantime®.

Some genes whose expressions were significantly changed in Glucantime®-resistant *Leishmania* isolates might be implicated in the natural antimony resistance compared to the sensitive isolate genes (29, 30) such as overexpression of ubiquitin and AAP3 genes, upregulation of MRPA, PTP, and PGK genes, and down-regulation of AQP1, MAPK (21, 31), and calcineurin genes (22).

Proteins have the most important role against the drugs and regulation of cellular responses (10, 11, 32), while changes have been less remarkable in the protein profiles. One of the best techniques among the proteomic studies of protein profiling is 2- DE but it is not complete until the proteins could be identified by Mass spectrometry (24). Although a considerable amount of research on proteomic profiling exists for virulence factors in humans and bacteria, there are only a few reports for profiling parasite proteins (33). Yet, proteomes of many parasites stay unclear, but completion of genome sequencing and development of methods for protein separations will improve proteomic studies for these organisms. In this study, we used a 2- DE protein map to study the protein expression patterns of the sensitive and resistant *L.major* isolates to Glucantime® in the same region of the old world that allowed for the comparison of protein distribution and positioning between the resistant and sensitive *Leishmania major* isolates. Close matching of protein distribution and positioning for the two isolates was apparent. However, variability of some protein spots was observed.

After the analysis of each isolate, about 2967 protein spots were obtained, whereas approximately 3700, 2000, and 2236 protein spots in *L. major* (16), *L. donovani* (34), and *L. tropica* were discovered in other studies on a 2- DE gel map, respectively (24).

In this study, approximately 37% of 46% of *Leishmania* proteomic map found out by other researchers were covered in comparison with the prior studies carried out on *Leishmania* genome projects (16).

Comparison of the protein maps of the two isolates showed the molecular weights of all the proteins were 14.1 to 83.2 kDa with the *PI* of 4.16 to 6.52, while an enrichment of the protein spots within a pH of 4-7 has been also reported for other *Leishmania* species (29). Protein database analysis revealed most of these proteins have a role as the transferring proteins, heat shock proteins, skeletal proteins, and hypothetical proteins, while playing an important role in the protection of DNA structure and normal metabolism in the cells and enhancement of the parasite toleration during the drug resistance (30, 32).

Leishmania genome contains 8272 protein-coding genes, only 36% of which can be ascribed to a putative function (35). Therefore, protein identification for proteomic studies will be reliable in future when gel-to-gel reproducibility improves spot resolutions. Furthermore, for a complete study of proteomic map for the identification and determination of the functioning of this protein, other techniques such as mass spectrometry and western blot detection system can be very useful for knowing which proteins with what mechanisms have important roles in drug resistance (36).

Conclusion

2- DE is a strong tool to prove protein expression profiles between the two species of resistant and sensitive *Leishmania major* isolates. In addition, immunological assays can lead to the recognition of necessary parasite proteins and improvement of new *Leishmania* treatments and control strategies. Identification of more proteins related to the resistance mechanism requires further studies involving other technical methods like mass spectrometry.

Acknowledgments

The authors would like to thank Ms. N. Bavarsad- Ahmadpour from Department of Parasitology, Shiraz University of Medical Sciences, for their collaboration in the preparation of samples of *Leishmania*, Mrs. S. Charehdar, Dr. M. B. Khadem- Erfan, and Dr. E. Kazemi-Rad from Department of Medical Parasitology, School of Public Health, Tehran University of Medical Sciences, and Mr. H. Rezapour from Ministry of Health and Medical Education, Iran for their kindly cooperation. This work is a part of Ph. D thesis of Mehdi Zarean financially supported by a Grant (U- 91161) from Vice Chancellor of Research Development and Biotechnology and Molecular Biology Research Center, Ahvaz Jundishapur University of of Medical Sciences fully appreciated. The authors declare that there is no conflict of interests.

Index Descriptor and Abbreviations

ZCL, zoonotic cutaneous leishmaniasis; ACL, anthroponotic cutaneous leishmaniasis; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid performance liquid; 2DE, two dimensional gel electrophoresis; IEF, isoelectric focusing; DTT, dithiothreitol; FBS, fetal bovine serum; TCA, trichloroacetic acid in acetone; BSA, bovine serum albumin, IPG, immobilized pH gradient; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis, PI, pH isoelectric; MW, molecular weight; IF, induction factor.

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