Original Article

Protein Profiling on Meglumine Antimoniate (Glucantime®) Sensitive and Resistant *L. tropica* Isolates by 2- Dimentional Gel Electrophoresis: A Preliminary Study

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(Received 8 Sep 2008; accepted 17 Jan 2009)

Abstract

Background: Glucantime® is the first- line drug for the treatment of all forms of leishmaniasis. Unfortunately, the prevalence of parasites becoming resistant to Glucantime® is increasing in several parts of the world including Iran. As protein is the most important target for drugs in response to a variety of signals including drugs so, it seems expression protein patterns in sensitive and resistant *Leishmania* parasites could greatly help us about the mechanisms of responses to antileishmanial drugs. In this study, we used 2-dimentional gel electrophoresis (2-DE) method to determine protein expression profiles between drug (Glucantime®) sensitive and resistant *Leishmania tropica* isolated from Iranian anthroponotic cutaneous leishmaniasis (ACL) patients.
Methods: We used from the two confirmed genetically of Glucantime® sensitive (Mash-4) and resistant (Mash-927) field strains of *L. tropica,* isolated from ACL patients in north eastern Iran. The two *Leishmania* isolates were cultured, promastigotes were harvested followed by protein extraction using TCA/Aceton to study protein profiling, 2-DE was done and gels stained with silver nitrate.
Results: At least 2236 distinct protein spots were detected. Twelve spots out of them, showed significant changes in expression in resistant compared to sensitive isolates. Of these, 11 protein spots were up- and one was down-regulated.
Conclusions: This preliminary study has showed that a number of proteins differentially expressed in drug (Glucantime®) resistance *L. tropica* and probably the role of these proteins are increasing the parasite resistance against the drug and delay in cell death.

Keywords: *2 Dimentional gel Electrophoresis (2 DE), Protein profiling,* L. tropica*, Glucantime®, Resistant, Sensitive, Iran*

Introduction

Cutaneous leishmaniasis (CL) is endemic in several parts of the world and remains a serious public health problem in numerous countries including Iran (1, 2). In recent years the reported anthroponotic cutaneous leishmaniasis (ACL) cases have been increased in most parts of Iran so, is a matter of concern for health authorities (3, 4). In respect of hardships in vector control and the lack of an effective vaccine against this parasite, the control of ACL relies more on chemotherapy (5).The first line choice of treatment against leishmaniasis are meglumine antimoniate (Glucantime®) but prevalence of parasites becoming resistant to this drug is increasing in several parts of the world, including Iran (6-8). Proteins as the most important target for drugs are known to be modified in response to a variety of intracellular and extra cellular signals including drugs.

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Two-dimentional gel electrophoresis (2-DE) is the most popular method for determining the protein profiling. 2-DE method could separate proteins according to their isoelectric and molecular weight points. Despite 2-DE is a major step but, the proteomics is not completed until the proteins could be identified by mass spectrometry (9, 10).

The aim of this preliminary study was to determine the proteins differentially over or down expressed in two strains of *L. tropica* isolated from patients showed sensitivity and resistant to Glucantime® through protein profiling by 2-DE method. This is the first proteome analysis of *L. tropica* in cutaneous leishmaniasis patients in Iran.

Material and Methods

Study subject

The samples of this study were cultured promastigotes of two confirmed genetically related of Glucantime® sensitive (Mash-4) and resistant (Mash-927) field strains of *L. tropica,* which have been isolated from ACL patients in Mashhad, north eastern Iran (7). These two field isolates previously were identified by RAPD-PCR and pulsed field gel electrophoresis (PFGE) (4, 8), which have been kept in liquid nitrogen.

Cell culture

The *L. tropica* isolates including sensitive and resistant strains recovered from liquid nitrogen. For mass production promastigotes were sub cultured in RPMI1640 (Gibco) medium supplemented with 10% Fetal Bovine Serum (FBS-Gibco). Cultures were incubated at 25° C. Promastigotes from 5-day culture were harvested for protein analysis.

Protein Extraction

Promastigotes were grown to late log phase and about 400×10^6 were harvested by centrifugation at 3000 rpm and washed three times in PBS (pH 7.2-7.4). Protein extraction was performed by Lammeli assay (11) using cold acetone (12).To eliminate contaminants such as salts, nucleic acids, etc., the cells resuspended in 400µl cold acetone, 10% trichloroacetic acid (TCA) and 0.07% 2-mercapto ethanol (2-ME) and incubated in -20˚C for 45 min. Samples were centrifuged in 15000 rpm in 15 min, the pellets resuspended in above solution for another 45 min again in -20˚C. For protein extraction the pellets were resolubilized in 50µl of 2DE lysis buffer containing 7 M urea, 2 M thiourea, 2% NP-40, 5% 2-ME and 2% ampholine (13). The sample was centrifuged at 13000 rpm for 10 min at 4˚C, the supernatant was allowed as protein extracted for 2 dimentional gel electrophoresis. Protein concentration was determined by Bradford assay using bovine serum albomine (BSA) as standards (14).

2- Dimentional gel Electrophoresis assay

Isoelectric focusing (IEF) was performed on 18 cm immobilized pH gradient (IPG) strips (BioRad, USA) with pH range of 4-7. IPG strips were rehydrated in rehydration buffer (8M urea, 2% CHAPS, 20 mM dithiothreitol (DTT), and traces of bromophenol blue). For analytical and preparative gels, 114 µg protein was added to rehydration buffer. IEF was performed using Multiphor II. Gels were run at 300V/1h, 500V/1h, 1000V/2h, and 3500V/12h after focusing. IPG strips were equilibrated for 15 min in the equilibration solution (50 mM Tris–HCl pH 8.8, 6M urea, 20% glycerol, 2% SDS, and 0.01% bromophenol blue) containing 2% DTT. The second equilibration was as the first except that DTT was replaced with 2.5% iodoacetamide. The second dimention was developed with a 12% SDS-polyacrylamide gel. Protein spots were visualized by staining with silver nitrate. The entire of 2-DE assay was done in Agricultural Biotechnology Research Institute of Iran (ABRI) in Karaj-Tehran Iran (15).

Image analysis

Silver stained gels were scanned with a GS-800 Densitometer (BioRad, USA) at a resolution of 600 dots and analyzed using Melanie-3 software. Statistical analysis of protein variations was carried out using the student *t*-test (statistical significance was assumed for *P*-

values less than 0.05) on vol% of matched spots. The molecular mass of proteins in the gels was estimated by standard markers (Amersham pharmacia Biotech). Isoelectric phocusing (PI) was determined by measuring spot migration on 18 cm IPG (pH :4-7). Spots were concluded to be significantly up- or down regulated when $P< 0.05$ (ABRI) (16).

Results

After 2-DE gel electrophoresis of the proteins were extracted from late log phase of two sensitive and resistant *L. tropica* isolates in pH 4-7, the resulting images of triplicate gels were analyzed. About 2236 protein spots were reproducibly detected in triplicate of each strain (Fig. 1). From these 609 were visually reproducible and detectable by Melanie-3 software and future analysis have been done on. Of these, 12 proteins spots showed significant changes under drug (Glucantime®) stress. Molecular weight (MW) and pH Isoelectric focusing (PI) differentiated proteins between two mentioned strains were classified in Table 1. According to this table the molecular weight of 12 differentiated proteins was between 37.9 to 71.8 kDa.

According to the pH range [4-7] of the IPG strips the pH isoelectro phocusing of the differentiated proteins was 5.14 to 6.73 (Table 1). Induction factor (IF) of 12 proteins with significant changes under drug stress among 609 spots were reproducibly detected in three replicate of each Mash-4 and Mash-927 strains (Table 1). According to these data 12 protein spots had differential expression in resistant isolate in compare with sensitive isolate about the Glucantime drug. So, among the intended proteins 11 protein spots were up-regulated and high expressed with significantly changed (IF >2) in the stress drug strain (Spot No: 255, 260, 281, 285, 287, 329, 334, 456, 393, 552 and 603). Another protein, spot 219, that was down-expressed up to 2-fold in the susceptible strain and not significantly (IF < 0.5) changed in the resistant strain. Fig. 2 showed the comparison of a number differentially over or down expressed protein spots between two mentioned isolates, spots numbered and indicated by an arrow.

Table 1: MW (Molecular weight), PI (pH Isoelectric focusing) and IF (Induction factor) of differentiated proteins between Glucantime sensitive and resistant *L. tropica* strains after 2-DE gel electrophoresis. IF < 0.5 demonstrated the down –expression and IF >2 showed the high- expression of protein spots

No.	Spot Number	MW (kDa)	PI	IF
1	334	71.8	5.21	2.661448
2	329	64.5	5.16	2.265531
3	552	58.2	6.73	2.132971
4	260	59.9	6.15	2.065648
5	603	57.1	6.25	2.742148
6	393	57.2	6.50	2.433316
7	255	56.2	5.31	2.060316
8	287	58.1	5.67	2.626318
9	456	52.6	5.14	2.056550
10	285	49.8	6.17	2.211269
11	281	48.9	6.28	2.184886
12	219	37.9	5.39	0.481062

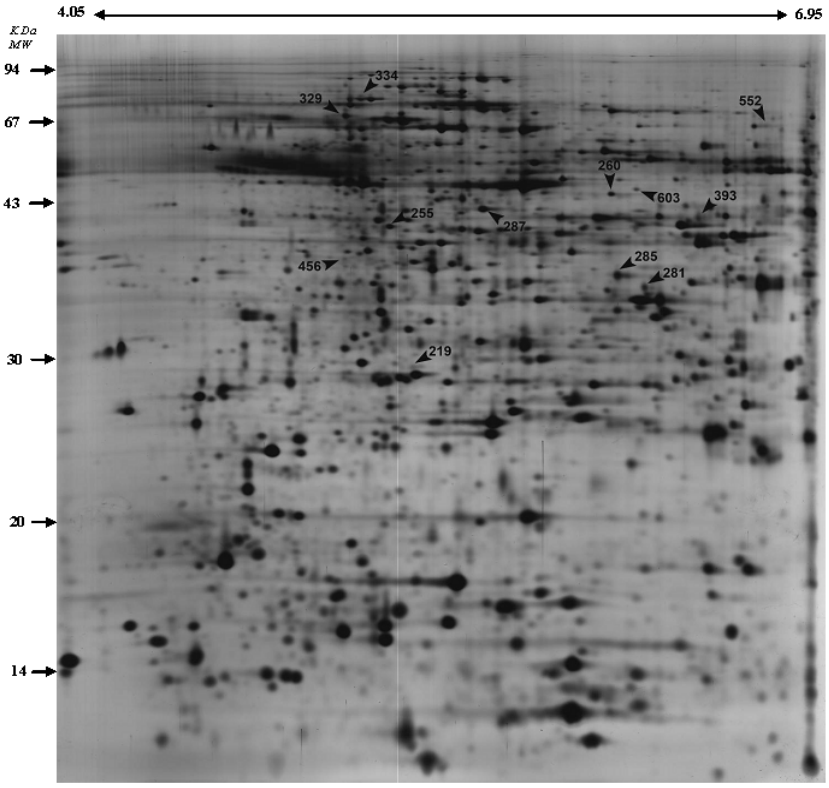

Fig. 1: 2-DE gel image of matched both Glucantime sensitive/resistant strains of *L. tropica* promastigotes. Marked differentiated protein spots numbered and indicated by an arrow, are listed in Table1. Isoelectric focusing was done on 18 cm IPG strips with pH range of 4-7 and SDS-PAGE was performed on 12% gradient slab gels. Gels were stained by silver nitrate

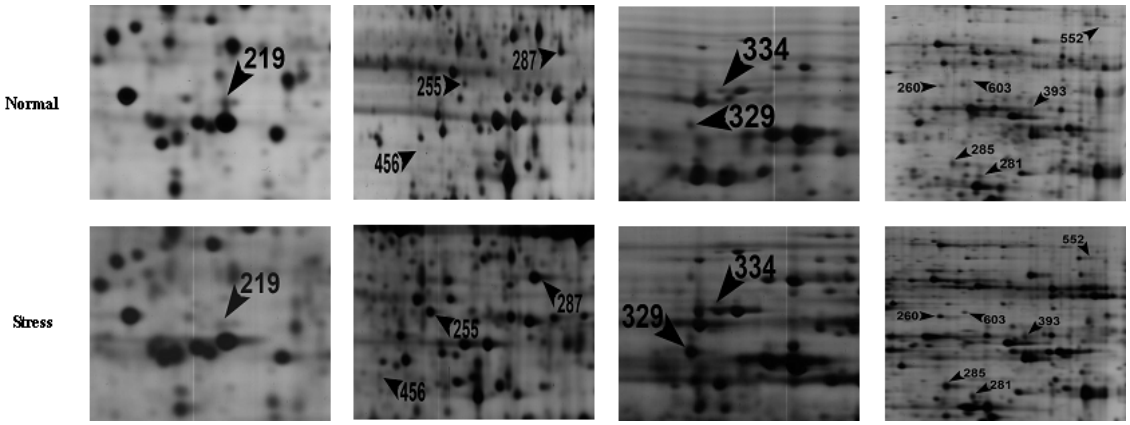

Fig.2: Comparison of differentiated spots with significant vol. % changes in two Glucantime sensitive (Normal) (up) and resistant (Stress) (down) *L. tropica* strains. The differentially expressed protein spots are numbered and indicated by an arrow

Discussion

Control of anthroponotic cutaneous leishmaniasis caused by *L. tropica* relies mostly on case-finding and chemotherapy. The first line drug for treatment of leishmaniasis are pentavalent antimony (Glucantime®) but the prevalence of resistant to this line drug is increasing in several endemic leishmaniasis region such as India, Europe, Middle East including Iran (8,17,18). The genomic studies showed several genes whose expression was significantly altered in Glucantime® resistant *Leishmania* isolates in compared to sensitive strain (18, 19). Proteins are the most important target for drugs which are known to be modified posttranslational in response to a variety of intra and extra cellular stress including drugs.

In the recent years, 2-DE has been used as one of the best methods for protein separating among a rapidly proteomics technologies. 2-DE method separates proteins according two points including isoelectric points, and to their molecular weight. Although the 2-DE was an important step in proteomics study, but just analysis of the protein map profiling is not complete until the proteins could be identified. Mass spectrometry is the most important assay for protein identification (20). In this study we used 2-DE protein arrays in pH: 4-7 to determine the possible differences in protein profiling of two genetically related pair of Glucantime® sensitive (Mash-4) and resistant (Mash- 927) *L. tropica* strains isolated from Iranian cutaneous leishmaniasis patients. The protein profile of promastigote forms of two strains were prepared in three replicate conditions and analyzed by Image master software for any expressional differences. Three replicates of promastigotes of each strain were separately cultured and proteins were extracted and mapped. Each map containing 2236 protein spots. Previous work revealed about 3700 proteins spots using *L. major* (21), 2000 protein spots using *L. donovani* (22), 719 spots in *L. guyanensis* (21). In other studies, showed 1000 protein spots in *Toxoplasma gondii* (23) and 1300 to 1400 protein spots in 2-DE gel map of *Plasmodium falciparum* were seen (24). In this study , our proteome map covers about 28% of the 8000 genes in the *Leishmania* genome in comparison with the previous study which showed 46% in *L. major* (21). After analysis of matched maps of two sensitive and resistance field isolates by software showed 12 proteins with significant alteration in normalized volume. Molecular analysis of resistant responsive proteins revealed that a large number of these proteins (11 spots) are up and only one was down regulated. The molecular weight of all up or down regulated proteins were 30 to 70 kDa with the PI 5.14 to 6.73.

Protein Data base analysis (25) according to the MW and PI of the proteins , revealed that most of these proteins showed homology with skeletal proteins, transfer proteins, oxidative protein stress , tolerance enzymes, heat shock proteins and hypothetical proteins (25-27). The function of drug resistant proteins were involved in the protection of DNA structure and damage of cell during drug stress, and up express regulation enzymes have been associated with both normal metabolism in cell as well as in the detoxification of oxidative and other stress responses for increasing the parasites toleration. As we mentioned above more work is needed to identify this landmark protein spots by precise method like mass spectrometry to know better proteins and the mechanisms of resistance/susceptibility *Leishmania* isolates to anti leishmanial treatment.

Acknowledgements

This work was supported financially by a grant (No: 132/5603) from Vice Chancellor for Research, Tehran University of Medical Sciences, Iran. The authors thank Dr. Ghasem Hosseini Salekdeh from Agricultural Biotechnology Research Institute of Iran in Karaj, for his kind cooperation and Dr. S. Shojaee for good suggestion and technical assistance. The authors declare that they have no conflict of interest.

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