Comparative proteomics study on meglumine antimoniate sensitive and resistant *Leishmania tropica* isolated from Iranian anthroponotic cutaneous leishmaniasis patients

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دراسة بروتيوميّة مقارنة حول الليشمانيات المدارية الحساسة لأنتيمونات الميغلومين وتلك المستعصية عليها من المستفرَدة مرضى تلك من الإيرانيين المرضى بداء الليشمانيات الجلدية البشرية

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الخلاصة: في سبيل تحديد تغيُّرات التعبير عن الروتينات، المتعلّقة باستعصاء الليشمانيات الجلدية على أنتيمونات الميغلومين، فقد أجرى الباحثون تحاليل بروتيومية مقارنة للذّراري المستعصية والذّراري الحساسة من الليشمانيات المدارية المستفرَدة من مرضى إيرانيين، باستخدام الرحلان الكهربي الثنائي الأبعاد للبروتينات الخلوية، ومن ثَمَّ تحديد البروتينات التي يُعَبَّر عنها تعبيراً مختلفاً باستخدام القياس الطيفي الكُتْلَويّ لزمن الارتشاف والتأيُّن بالليزر المُساعَد بالمصفوفة. وقد أدى تحليل الصور للخرائط الموافقة إلى التعرف على سبعة بروتينات مفرطة التعبير أو ناقصة التعبير؛ وهذه البروتينات هي: مستقبل بروتين الكيناز سي المفعَّل، والألفا توبولين، ومُخَلِّقة البروستا غلاندين إف 2 ألفا، ومصاوِغَة ثنائي سلفيد البروتين، وبروتين النقل الحويصلي، بالإضافة إلى بروتين افتراضي. وقد أظهرت الدراسة مدى فائدة دراسة البروتيوميات في التعرف على البروتينات التي يمكن أن تعبِّر عن الفوارق بين المستفرَدات الحساسة والمستفرَدات المستعصية من الليشمانية المدارية.

ABSTRACT In order to define the protein expressional changes related to the process of meglumine antimoniate resistance in anthroponotic cutaneous leishmaniasis (CL), we performed a comparative proteomics analysis on sensitive and resistant strains of *Leishmania tropica* isolated from Iranian CL patients. Cell proteins were analysed with 2-dimensional electrophoresis and differentially expressed proteins were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Image analysis of the matched maps identified 7 proteins that were either over- or down-expressed: activated protein kinase c receptor (LACK), alpha tubulin (×2), prostaglandin f2-alpha synthase, protein disulfide isomerase, vesicular transport protein and a hypothetical protein. The study shows the usefulness of proteomics in identifying proteins that may express differences between sensitive and resistant *L. tropica* isolates.

Étude protéomique comparative de souches *Leishmania tropica* sensibles ou résistantes à l'antimoniate de méglumine chez des patients iraniens atteints de leishmaniose cutanée anthroponotique

RÉSUMÉ Afin de déterminer les modifications de l'expression des protéines liées au processus de résistance à l'antimoniate de méglumine dans la leishmaniose cutanée anthroponotique, nous avons réalisé une analyse protéomique comparative de souches sensibles et de souches résistantes de *Leishmania tropica* isolées à partir d'échantillons prélevés chez des patients iraniens atteints de la maladie. Les protéines cellulaires ont été analysées par électrophorèse bidimensionnelle ; des protéines différentiellement exprimées ont été identifiées par spectrométrie de masse à temps de vol avec désorption-ionisation laser assistée par matrice. L'analyse d'image des cartes appariées a permis d'identifier sept protéines qui étaient soit surexprimées, soit sous-exprimées : le récepteur de la protéine kinase C activée, la tubuline alpha (×2), la prostaglandine F2-alpha synthase, la protéine disulfide isomérase, une protéine du transport vésiculaire et une protéine hypothétique. L'étude souligne l'utilité de la protéomique dans l'identification des protéines pouvant être différentiellement exprimées selon le caractère sensible ou résistant des souches *L. tropica*.

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Introduction

With an annual rate of 1.5 to 2 million new cases throughout the world cutaneous leishmaniasis (CL) remains a serious public health problem in numerous countries [1,2]. From 13% to 25% of CL cases in the Islamic Republic of Iran are anthroponotic cutaneous leishmaniasis (ACL) caused by *Leishmania tropica* [3] and in recent years the reported number of ACL cases has increased in most parts of the country [4]. Since there is no effective vaccine against leishmaniasis, early diagnosis and appropriate treatment of patients are the best disease control measures in ACL [5]. Although the pentavalent antimonial (SbV)-based drug Glucantime® is the first choice for treatment and control of ACL, the incidence of SbV-resistant *Leishmania* spp. is increasing in several parts of the world [6,7], including the Islamic Republic of Iran [8]. Current data based on epidemiology and transmission studies of ACL foci of *L. tropica* in the north and north-east of the country have documented rates of acquired antimony drug resistance reaching 40% [3,8].

In spite of many studies, the mechanism of *Leishmania* antimonial resistance is still unclear [9–17]. Molecular methods, based on DNA, RNA and protein assays, have been used successfully *in vivo* and *in vitro* [9] to study resistance to anti-*Leishmania* drugs in different *Leishmania* spp [10]. Gene amplification studies have implicated various mechanisms and several genes that are over- or down-expressed. Changes in proteins profiles have been less well studied. Recently, however, comparative proteomics analysis based on 2-dimensional (2D) gel electrophoresis and mass spectrometry has been shown to be a powerful approach for determining the differences in protein patterns of subjects with leishmaniasis [17].

Our preliminary study using 2D gel electrophoresis in drug sensitive/resistant strains of *L. tropica* showed that some proteins were differentially expressed [18]. In the present study, we used a comparative approach with matrix-assisted laser desorption/ionization time of flight (MALDI-Tof-Tof) mass spectrometry to identify more of the proteins in clinical field isolates of promastigote forms of genetically-confirmed drug-sensitive and drug-resistant *L. tropica* parasites [8].

Methods

Study design

This comparative proteomics study used Sb(V)-sensitive and -resistant *L. tropica* strains with the aim of identifying any proteins with differential expression in drug-resistant isolates compared with drug-sensitive isolates. Three biological replicates were prepared by 3 distinct culture batches of the promastigotes in both isolates. Independent 2D electrophoresis gel samples were run and analysed using the ImageMaster software package. Differences greater than 2-fold were identified as valid protein spots by the software and these spots were excised from the gels and sent for identification by MALDI-Tof-Tof mass spectrometry.

Parasites

The 2 *L. tropica* strains used had been genetically confirmed previously by standard *in vivo* and *in vitro* genetic assays as either resistant (Msh-R878) or sensitive (Msh-S2) to meglumine antimoniate [8]. The isolates were recovered from Iranian patients from Mashhad city, in north-eastern Islamic Republic of Iran.

Cell culture

The sensitive and resistant *L. tropica* isolates were recovered from liquid nitrogen, and sub-cultured in RPMI1640 medium (Gibco/BRL) supplemented with 10% fetal bovine serum (Gibco/BRL). Cultures were incubated at 25 °C. Promastigotes at late log phase (about 400×10^6/mL) were harvested by centrifugation at 2500× g and washed 3 times with sterile phosphate-buffered saline (pH 7.2–7.4).

Sample preparation (protein extraction)

The cells were thawed and resuspended in 5 mM Tris–HCl, pH 7.8, containing 1 mM phenylmethylsulfonyl fluoride as a protease inhibitor. The samples were sonicated at 40 Hz 3 times for 10 s with 50 s intervals on ice bath. The homogenate was kept at 4 °C for 4 h. Proteins were precipitated by 20% trichloroacetic acid in acetone with 20 mM dithiothreitol (DTT) for 1 h at –20 °C. The samples were then centrifuged at 13 000× g for 15 minutes at 4 °C and the pellets were washed with cold acetone containing 20 mM DTT. Residual acetone was removed by airdrying. The pellets were resolubilized in 50 µL of sample lysis buffer containing 7M urea, 2M thiourea, 1% ampholyte pH 4–6.5, 1% ampholyte pH 5–7 and 4% CHAPS detergent. Protein concentration was determined by Bradford assay using bovine serum albumin as standards [19].

Two-dimensional gel electrophoresis

Isoelectric focusing was performed on 17 cm immobilized pH gradient (IPG) strips (BioRad) with pH range of 4–7. IPG strips were rehydrated overnight by loading the samples diluted with rehydration buffer containing 8 M urea, 4% CHAPS, 2%

ampholyte, 50 mM DTT, and traces of bromophenol blue. Isoelectric focusing was carried out using the Protean IEF cell (BioRad) beginning with a linear increase from 0 to 250 V for 20 min, followed by linear increase to 10 000 V, and remaining at 10 000 V to achieve total 60 000 V/h. After focusing, IPG strips were equilibrated for 15 min. in the equilibration solution (50 mM tris–HCl pH 8.8, 6 M urea, 20% glycerol, 2% sodium dodecyl sulphate (SDS), and 0.01% bromophenol blue) containing 2% DTT, and then reacted for a further 15 min. in the equilibration solution containing 2.5% iodoacetamide. The equilibrated strips were placed on top of 10%–15% gradient SDS polyacrylamide gel electrophoresis (SDS-PAGE) slab gels and sealed with 1% agarose. The second dimension electrophoresis was performed at 16 mA/gel for 30 min. and 24 mA/gel for 5 h at 20 °C. The 2D gels were stained with mass-spectrometry-compatible silver nitrate staining [20].

Image analysis

Gel images were scanned at a resolution of 300 dpi using the BioRad GS-800 densitometer. Spot detection and matching were performed using the ImageMaster 2D Platinum software (GE Healthcare) on silver-stained analytical gels. Statistical analysis of protein variations was carried out using the Student *t*-test with a confidence level of 95% on relative volume of matched spots.

Mass spectrometry analysis

The protein spots were manually cut from 2D gels and destained by 50% acetonitrile/100 mM ammonium bicarbonate. Trypsin (2 mg/mL in 25 mM ammonium bicarbonate) digestion was performed at 37 °C overnight. Extracted peptides were spotted onto a 192-well MALDI-TOF target plate for the proteomics analyser (Applied Biosystems 4700), performing peptide mass fingerprinting (PMF) and subsequent tandem (MS/MS) analysis on up to 10 precursor peptides. The PMF and MS/MS results were automatically compared with the *L. major* genome database using the Mascot search engine (Matrix Science). Mass tolerance settings of 1.2 Da for the parent ion and 0.5 Da for fragment ions were applied. Search settings allowed 1 missed cleavage with trypsin and 2 modifications (carboxamidomethylation of cysteine and oxidation of methionine). Statistical confidence limits of 95% were applied for protein identification.

Results

The protein spots in the range of pH 4–7 were analysed and are shown in Figure 1. There was an obvious similarity in protein spot distribution between the 2 isolates. A number of protein spots of interest in addition to the differentially expressed proteins were identified by mass spectrometry. Some of these proteins were structural proteins including alpha- and beta-tubulin, stress proteins such as heat shock protein (HSP) 70, oxidoreductase, peroxidase such as tryparedoxine peroxidase and other proteins with unknown functions such as hypothetical proteins (Table 1).

The next step of the study was to identify a number of these proteins to serve as differentials. Image analysis of the matched maps showed 7 differentially expressed proteins with significant alteration ($t > 2.57$) in normalized volume (Figure 1). These were 5 protein spots which were down-expressed and 2 which were over-expressed in drug-resistant isolates (Table 2, Figure 2).

The proteins, which were highly expressed under stress conditions or drug resistance in *L. tropica* isolates, were identified as activated protein kinase C receptor (LACK) and a hypothetical protein. The down-expressed proteins included prostaglandin f2-alpha synthase (PGFS), protein disulfide isomerase-2 (PDI-2), alphatubulin and vesicular transport proteins (CDC48 homologue).

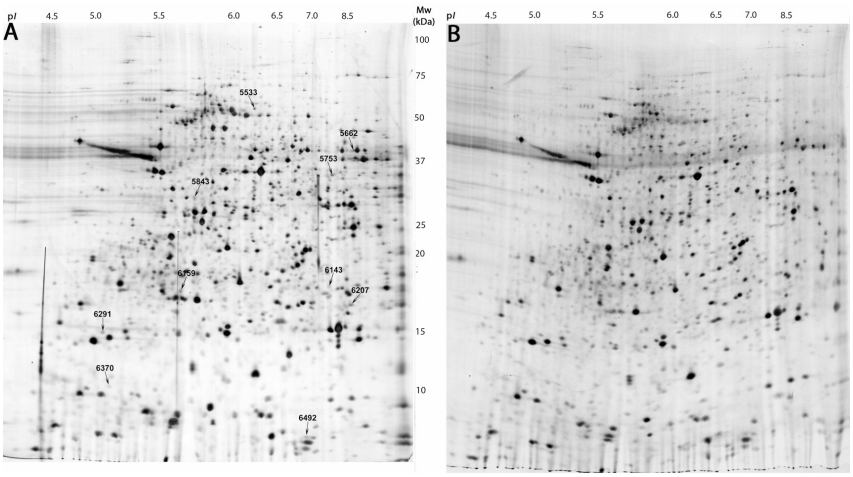

Figure 1 Two-dimensional map of the comparative proteome of antimonial-sensitive (A) and -resistant (B) *Leishmania tropica* isolates using an extracted protein. Numbered spots were identified and are listed in Table 2

Table 1 Identification of several protein spots derived from *Leishmania tropica* proteom using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis with mascot score above 52

Spot ID	Protein name	Accession no.	MW (Da)	pI	% coverage	Protein score	Biological process
6289	Beta-tubulin	LmjF33.0798	49 740	4.71	44.0	457	Tubulin is the major constituent of microtubules
6162	Enolase	LmjF14.1160	46 092	5.60	52.6	425	Magnesium ion binding
6362	Heat shock 70-related protein 1, mitochondrial, putative	LmjF30.2490	71 621	5.68	34.2	428	Stress response
6231	Heat shock 70-related protein 1, mitochondrial, putative	LmjF30.2550	70 609	5.67	47.5	398	Stress response
5840	Pyruvate dehydrogenase E1 beta subunit, putative	LmjF25.1710	37 898	5.64	48.2	339	Pyruvate dehydrogenase (acetyl-transferring) activity
6147	Cytochrome c oxidase subunit V, putative	LmjF26.1710	22 291	6.11	57.6	339	Electron transport respiratory chain
5990	Heat shock 70-related protein 1, mitochondrial, putative	LmjF30.2490	71 621	5.68	39.5	286	Stress response
5805	Reiske iron-sulfur protein, putative	LmjF35.1540	33 742	5.93	51.8	254	Oxidoreductase
6125	Cytochrome c oxidase subunit IV	LmjF12.0670	39 434	5.63	44.8	250	Electron transport respiratory chain
8061	Peroxidoxin (tryparedoxin peroxidase)	LmjF23.0040	25 342	6.43	47.7	235	Oxidoreductase
6132	Eukaryotic initiation factor 5a, putative	LmjF25.0720	17 812	4.83	75.3	228	Translation initiation factor activity
8151	Heat shock 70-related protein 1, mitochondrial, putative	LmjF30.2490	71 621	5.68	32.4	196	Stress response
6492	Protein disulfide isomerase	LmjF36.6940	52 377	5.22	19.9	195	Protein disulfide isomerase activity
5693	Putative uncharacterized protein	LmjF13.0450	13 330	5.27	29.7	194	Unknown function
6324	Hypothetical protein	LmjF13.0450	13.92	5.27	20.2	>52	Unknown function
8030	Cytochrome c oxidase subunit IV	LmjF12.0670	39 434	5.63	47.5	191	Electron transport respiratory chain
6231	Aldose 1-epimerase, putative	LmjF35.0970	41 428	5.95	27.7	181	Isomerase
6315	Tryparedoxin peroxidase	LmjF15.1140	21 173	6.43	35.7	168	Oxidoreductase
6151	Carboxypeptidase, putative	LmjF33.2540	57 050	5.55	34.6	166	Carboxypeptidase
6383	Ribonucleoprotein p18, mitochondrial, putative	LmjF15.0280	21 300	6.74	75.4	160	Ribonucleoprotein
5893	Putative uncharacterized protein	LmjF36.6760	28 999	5.53	41.8	157	Unknown function
6264	Putative uncharacterized protein	LmjF36.0480	26 102	4.92	25.7	143	Unknown function
5691	Calpain-like cysteine peptidase, putative	LmjF20.1310	12 936	5.27	36.5	111	Cysteine peptidase, clan CA, family C2, putative
5753	Protein disulfide isomerase	LmjF36.6940	52 377	5.22	11.5	96	Protein disulfide isomerase activity

Table 1 Identification of several protein spots derived from *Leishmania tropica* proteom using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis with mascot score above 52 (*concluded*)

Spot ID	Protein name	Accession no.	MW (Da)	pI	% coverage	Protein score	Biological process
5972	Phosphomannomutase, putative	LmjF36.1960	28 101	5.18	36.0	91	Hydrolase
5660	Cofilin-like protein	LmjF29.0510	15 715	5.56	26.6	82	Actin binding
6181	Tryparedoxin peroxidase	LmjF15.1080	22 128	6.32	17.5	82	Oxidoreductase activity
5605	Calpain-like cysteine peptidase, putative	LmjF14.0850	12 936	4.70	61.7	78	(see above)
5802	Putative uncharacterized protein	LmjF25.2010	30 292	5.80	30.4	73	Carbon–carbon lyase activity
6278	Tryparedoxin	LmjF29.1160	16 544	5.06	8.2	70	Cell redox homeostasis
6315	Tryparedoxin peroxidase	LmjF15.1080	22 128	6.32	17.5	68	Oxidoreductase
6152	Enolase	LmjF14.1160	46 092	5.60	31.7	61	Magnesium ion binding
6427	40S ribosomal protein S19 protein, putative	LmjF34.2780	19 376	10.82	59.8	59	Ribonucleoprotein
8097	Eukaryotic initiation factor 5a, putative	LmjF25.0720	17 812	4.83	53.0	58	Translation initiation factor activity
6143	Prostaglandin f2-alpha synthase	LmjF31.2150	31 850	5.85	23.9	58	Oxidoreductase activity
5533	Activated protein kinase c receptor	LmjF28.2740	34 400	6.05	22.4	56	Kinase activity
5753	Alpha tubulin	LmjF13.0360	49 759	4.89	14.6	53	Tubulin is the major constituent of microtubules

MW = molecular weight; pI = isoelectric point.

Discussion

The first choice chemotherapy for different leishmaniases is pentavalent antimony [10]. Recent progress in the completion of *Leishmania* spp. genomic sequences and access to these data permit scientists to co-study the expression of genes and proteins by powerful proteomics approach. Studying the proteome helps us to understand the differences in protein expression between *Leishmania* parasites reactive to and resistant to drugs [21].

The protein map of promastigote forms of the 2 strains was prepared and analysed for differences in gene expression. Each map contained about 1000 protein spots. Previous studies revealed about 2000 protein spots in *L. tropica* [18], 3700 in *L. major* [22], 2000 in *L. donovani* [23] and 719 in *L. guyanensis* [24]. The image analysis of matched maps of 2 sensitive/resistant field isolates showed 7 proteins with significant alteration in normalized expression in promastigotes. These landmark proteins were identified by mass spectrometry as LACK, alpha-tubulin, PGFS, PDI-2, vesicular transport protein and some proteins identified as hypothetical proteins.

LACK was one of the over-expressed proteins in resistant *L. tropica* isolates. Studies showed that production of LACK is the immune response in susceptible BALB/c mice against *Leishmania* and remains a good candidate for a vaccine for human leishmaniasis [25]. This protein is also required for parasite viability and represents a potential drug target for the treatment of leishmaniasis [26].

Other landmark proteins found were structural proteins including alpha-tubulin; both alpha- and gamma-tubulin are important for cell shape, growth and differentiation and for inhibition of cellular proteolysis [27]. Decreased expression levels of these proteins are probably related to reduced protection against antimonial drugs in resistant isolates.

Vesicular transport proteins identified as putative proteins were other proteins down-expressed in our resistant *L. tropica* strains. These proteins are located close to the flagellar pocket. Down-expression of these transport proteins decreases the transport and therefore the concentration of antimonial drugs within the cell [28].

We also report here identification of a 54 kDa protein which was shown to be PDI. Expression of PDI

Table 2 Summary of protein identification using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis

Match no.	Protein name	Accession no.	Theoretical MW(kDA)/pI	Observed MW(kDA)/pI	Sequence coverage (%)	Protein score	Student t-test	Expression in drug-resistant isolate
6492	Protein disulfide isomerase (PDI-2)	LmjF36.6940	52.7/5.22	10/6.8	22.2	195	2.10	Down-expressed
5843	Alpha-tubulin	LmjF13.0360	50.5/4.89	36/5.6	8.0	61	3.50	Down-expressed
6143	Prostaglandin f2-alpha synthase (PGFS)	LmjF31.2150	32.0/5.85	20/8.4	23.9	58	3.55	Down-expressed
5533	Activate protein kinase C receptor (LACK)	LmjF28.2740	34.8/6.05	51/5.9	22.4	56	2.50	Over-expressed
5753	Alpha tubulin	LmjF13.0360	50.5/4.89	40/8.2	14.4	53	2.85	Down-expressed
6207	Vesicular transport protein (CDC48 homologue)	LmjF27.1050	73.2/6.13	17/8.6	1.4	41	5.40	Down-expressed
6291	Hypothetical protein, conserved	LmjF35.3650	91.2/9.65	15/5.2	4.3	35	2.91	Over-expressed

MW = molecular weight; pI = isoelectric point.

has been shown to result in attenuation of cell viability in response to hypoxia and to protect cells from apoptotic cell death in response to drug resistance in *in vivo* conditions [29]. Down-expression of this protein may be associated with parasite survival.

PGFS with 32 kDa molecular weight was another differentiation protein in field isolates of sensitive/resistant *L. tropica*. The PGFS protein exhibits 99.3% identity in cytosol of promastigotes in Old World *Leishmania* spp. in *L. donovani* and *L. tropica* [30].

A number of proteins that were over-expressed in resistant isolates were identified as hypothetical proteins, but their function remains unknown. The current sequencing data of protozoan parasites suggest that about 60% of the putative gene products are hypothetical and have no known homologues [21].

Other proteomic studies of drug resistance on promastigotes of *L. donovani* field Indian isolates against Sb(V) reported differential expression of at least 2 proteins. The HSP83 and a small kinetoplastid calpain-related protein were shown to be increased through modification of the programmed cell death in the resistant parasite [17]. Another study showed that in methotrexate-resistant *Leishmania* isolates some proteins, such as pteridine reductase (PTR1) and trypanothione reductase, were post-translationally modified and over-expressed. These proteins could allow the *Leishmania* parasite to quickly adjust its response to the oxidative pathway. However, the role of PTR1 in drug-resistant *Leishmania* spp. is not clear [31].

Conclusion

In conclusion, proteomics seem to be a potential method to differentiate protein expression in Sb(V)-resistant *L. tropica* strains compared with sensitive isolates. Over-expression of LACK and some hypothetical proteins as well as down-regulation of PGFS, PDI-2 and vesicular transport protein in drug-resistant strains were identified. These altered proteins seem to play a role in the drug stress response, perhaps allowing the *Leishmania* parasite to quickly adjust its response to the drug and delay cell death. Identification of more proteins related to the resistance mechanism requires further studies involving other technical methods.

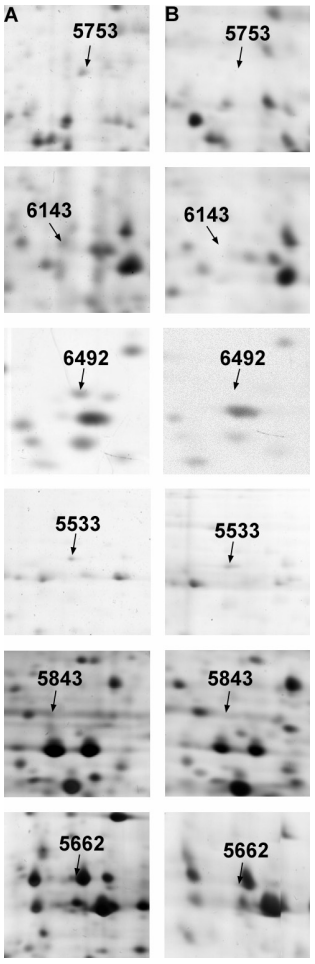

Figure 2 Comparison of 2-dimensonal electrophoresis gel analysis shows an increase or decrease in normalized volume of a number of protein spots shown in Table 2. In antimonial-sensitive (column A) and -resistant (column B) *Leishmania tropica* isolates

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