Original Article

Downregulation of Calcineurin Gene Is Associated with Glucantime® Resistance in *Leishmania infantum*

Mohammad Bagher KHADEM ERFAN 1, * Mehdi MOHEBALI 1,2 Elham KAZEMI-RAD 1, Homa HAJJARAN 1, Gholamhossein EDRISSIAN 1, Setareh MAMISHI 1, Mojtaba SAFFARI 4, Reza RAOOFIAN 4, * Mansour HEIDARI 4, 5

1. Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
2. Center for Research of Endemic Parasites of Iran (CREPI), Tehran University of Medical Sciences, Tehran, Iran
3. Pediatric Infectious Diseases Research Center, Tehran University of Medical Sciences, Tehran, Iran
4. Department of Medical Genetics, Tehran University of Medical Sciences, Tehran, Iran
5. Stem Cell Preparation Unit, Farabi Eye Hospital, Tehran University of Medical Sciences, Tehran, Iran

Received 20 Mar 2013
Accepted 11 July 2013

Abstract

**Background:** Pentavalent antimonials are the first line drugs for the treatment of leishmaniasis. Unresponsiveness of *Leishmania* spp. to antimonial drugs is a serious problem in some endemic areas. Investigations on molecular mechanisms involved in drug resistance are essential for monitoring and managing of the disease. Calcineurin is an essential protein phosphatase for number of signal transduction pathways in eukaryotic cells and it has a mediated role in apoptosis. This study aimed to determine of biomarker(s) in Glucantime® resistance strain of *L. infantum*.

**Methods:** We used cDNA amplified fragment length polymorphism (cDNA-AFLP) and real time-RT PCR assays to compare gene expression profiles at the mRNA levels in resistant and susceptible *L. infantum* field isolates.

**Results:** The cDNA-AFLP results showed downregulation of calcineurin in resistant isolate in comparison with susceptible one. Significant downregulation of calcineurin (0.42 fold) (*P*<0.05) was found in resistant isolate compared to susceptible one by real time-RT PCR.

**Conclusion:** This is the first report of calcineurin implication in Glucantime® drug resistance of field (natural) isolate of *L. infantum*. Downregulation of calcineurin could protect parasites from antimonial-induced apoptosis.

Keywords

*Leishmania infantum*, Calcineurin gene, Antimonial resistance, cDNA-AFLP, Real-time RT-PCR
Introduction

Several species of Leishmania are causative agents of a wide spectrum leishmaniasis which ranging from self-healing skin lesions to dangerous visceral forms (1). Global prevalence of leishmaniasis accounts for 12-15 million people and incidence rate of the disease is estimated 2 million new cases annually (2). Despite importance of the disease and serious attempts of researchers, no desirable vaccine is yet available against the disease. Rapid detection and appropriate treatment in most clinical forms of leishmania-sis have essential role in control of the disease (3-5).

Pentavalent antimonials sbV, such as Glucantime® have been used clinically as the first line drug against leishmaniasis since seven decades (6). Although these drugs have been administrated long time worldwide, some biochemical aspects of sbV metabolism in Leishmania are not uncovered (3). The sbV is a prodrug and kills Leishmania by sbIII which is an activated form of sbV. The exact site of this activation and involved mechanisms in this process are still unknown (7).

Unfortunately, in recent years the efficacy of antimony therapy has been challenged by occurrence of drug resistance. The major problem in treatment of leishmaniasis in some regions such as India is emerging of sbV-resistant parasites (8). Unresponsiveness to Glucantime® has been also reported from Iran (9).

Various mechanisms are involved in drug resistance of Leishmania such as drug entry, drug metabolism, drug transport, programmed cell death (10). For instance, Aquaglyceroporine (AQP1) is a plasma membrane protein of Leishmania and involves in entry of activated form of drug sbIII to parasite (11). Transfection and knock out experiments of AQP1 have shown that decrease of AQP1 led to resistance (11, 12). TDR1 and lmACR2 of parasite have been detected which involved in sbV reduction (13, 14). It is well documented that MRPA is one of the most important gene in drug transport and sequestration (12, 15). In addition, some studies illustrated the involvement of Heat Shock Proteins (HSPs) in resistance by modulating some phase of apoptosis pathway (10). Furthermore, it has been suggested that the expression of wild-type calcineurin could be a key element in the processes of apoptosis. In the eukaryotic cells such as cardiac and nerve cells the biological function of this protein is dependent to calcium/calmodulin (16, 17).

In spite of the fact that several techniques such as RT-PCR, proteomics and microarray are currently employed for identification of molecular mechanism that are involved in antimonial resistance of Leishmania, some aspects of this issue require further investigations by other approaches (18, 19). cdNA-AFLP as a method allows researchers to monitor transcriptional changes associated to development and modification in cellular functions. In contrast to some techniques such as GeneChip, microarray which are relied on specific probes, cdNA-AFLP does not need any prior knowledge of gene sequences (20, 21).

In this study we aimed to utilize cdNA-AFLP for identification of potential biomarkers in antimonial drug resistance in Leishmania.

Materials and Methods

Isolation of parasites

Two natural Leishmania spp. isolated from visceral leishmaniasis patients by bone marrow aspirations. One of them was clinically resistant to Glucantime® but another one was sensitive to the drug. Resistant case has been treated three periods with systemic administration of Glucantime® but did not lead to cure. Susceptible case after bone marrow aspiration has been treated with Glucantime® and led to cure. These isolates were confirmed as L. infantum by ITS1PCR-RFLP method (22). Parasites were cultured in RPMI 1640 medium (Gibco/BRL) supplemented with 10% fetal bovine and incubated at 25°C.
Amastigote drug susceptibility assay

Drug susceptibility of amastigotes of *L. infantum* to Glucantime® was determined by cultivation of the parasites in the J774A.1 macrophage-mouse line. Briefly, macrophages (5 X 10⁴ macrophages/well) were cultured in RPMI-1640 with 10% FBS in eight-chamber LabTek tissue-culture slides and incubated at 37 °C for 24 h. In order to infect macrophages by stationary phase promastigotes, 5 X 10⁵ promastigotes/well were added to macrophages then incubated at 37 °C for 4 h. Subsequently, cells were incubated for 72 h with serial dilutions of Glucantime®. Pentavalent antimony concentrations for sensitive isolate used 2, 4, 6, 8, 10 and 12 µg/ml and for resistant isolate were 35, 40, 45, 50, 55, 60, 65 and 70 µg/ml (the doses were used based on our previous screening test). Fresh drug was added to slides for an additional 72 h. After staining by Giemsa, based on counting the amastigotes in 100 randomly chosen macrophages, the IC₅₀ is defined as the effective dose of Glucantime® that decreases the survival of *L. infantum* by 50%. Experience was performed triplicate and IC₅₀ values were determined by linear regression analysis.

RNA extraction

Total RNA was extracted using Tripure kit according to the manufacturer’s protocol (Roch, Mannheim, Germany) with minimal modifications. Briefly, 1 x 10⁸ promastigotes were packed by centrifugation. Subsequently, the pellets were lysed in 1 ml Tripure reagent and followed by adding 200 µl chloroform. After centrifugation, the aqueous phase was collected and precipitated by adding of 500 µl isopropanol. Then, pellets were treated with 75% ethanol and air-dried. Precipitated RNA was dissolved in RNase free water. The quantity and quality of RNA was examined using nanodrop (ND-1000, Thermo-Scientific Fisher, US) and gel electrophoresis, respectively. The extracted RNA was treated with DNasel to eliminate any genomic contamination (Qiagen, Germany).

cDNA AFLP

cDNA AFLP was conducted as described previously (23). Briefly, single strand cDNA (sscDNA) was synthesized with 10 µg of total RNA, 10 mM of dNTP (Fermentas, Burlington, Canada), 20 pmol/µl OligodT (Fermentas, Burlington, Canada), 20 pmol/µl random hexamer (Fermentas, Burlington, Canada) and incubated at 65°C for 10 min following by addition of 200 U RevertAid premium Reverse Transcriptase (Fermentas, Burlington, Canada) 20 U Ribolock RNase inhibitor (Fermentas, Burlington, Canada) and 4 µL of 5X reverse transcriptase buffer containing Tris-HCl (PH 8.3) (Fermentas, Burlington, Canada) then incubated at 25°C for 10 min following by 50°C for 60 min. The integrity of cDNA was checked with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene primers as housekeeping (Table 1). The PCR condition was: (5 min at 94°C; 30 cycles of 30s at 94°C; 30s at 55°C; 45s at 72°C and 7 min at 72°C). Double strand cDNA (dscDNA) was synthesized with 1.5 U Ribonuclease H (Roche, Mannheim, Germany), 40 U DNA Polymerase I (Fermentas, Burlington, Canada), 10 mM of each dNTP and incubation at 16 °C for 2 h, followed by purification with High Pure PCR Cleanup Micro Kit (Roche, Mannheim, Germany). Double restriction digestion was performed with 5 U *Mbo* I and 5 U *EcoR I* (Fermentas, Burlington, Canada) on 5 µg purified dscDNA at 37°C for 2 h.

Digested dscDNA fragments were ligated by AFLP adaptors (Table 1) 8 µg AD *EcoR I*, AD *Mbo I* and 4 µg ad *EcoR I*, ad *Mbo I*. Ligation was conducted in a final volume of 60 µl; it was done in following conditions: 1 min at 55 °C, decreasing to 10 °C over 45 min

Available at: [http://ijpa.tums.ac.ir](http://ijpa.tums.ac.ir)
Table 1: Primer and adaptors sequences used in cDNA-AFLP and Real-Time RT-PCR

| NAME              | SEQUENCES                                           | PRIMERS          |
|-------------------|-----------------------------------------------------|------------------|
| AD ECOR1          | 5’-ACCGACGTGACTATCCATGAAG-3’                        | Adaptor          |
| AD ECOR1          | 5’-AATTCTTCAGG-3’                                   |                  |
| AD MBOI           | 5’-GATCTGCGGTA-3’                                   |                  |
| PR ECOR1          | 5’-ACCGACGTGACTATCCATGAAGAATTTC-3’                  | Pre-amplification|
| PRMBOI            | 5’-CCTATCCAGACTCTCACCGCAGATC-3’                     |                  |
| S1 ECOR1          | 5’-ACCGACGTGACTATCCATGAAGAATTTC-3’                  | Sensitive        |
| S2 ECOR1          | 5’-ACCGACGTGACTATCCATGAAGAATTTC-3’                  |                  |
| S3 ECOR1          | 5’-ACCGACGTGACTATCCATGAAGAATTTC-3’                  |                  |
| S4 ECOR1          | 5’-ACCGACGTGACTATCCATGAAGAATTTC-3’                  |                  |
| S1 MBOI           | 5’-CCTATCCAGACTCTCACCGCAGATC-3’                     |                  |
| S2 MBOI           | 5’-CCTATCCAGACTCTCACCGCAGATC-3’                     |                  |
| S3 MBOI           | 5’-CCTATCCAGACTCTCACCGCAGATC-3’                     |                  |
| S4 MBOI           | 5’-CCTATCCAGACTCTCACCGCAGATC-3’                     |                  |
| G6PDH F           | 5’-ATCAACGACGCACTGCTTG-3’                           | Housekeeping     |
| G6PDH R           | 5’-TTCATCCGCTTCCTTACG-3’                            |                  |
| Calcineurin F     | 5’-GTTCTTCAATGGACCCGAGAG-3’                         | Target Gene      |
| Calcineurin R     | 5’-TGAAACTGTGCATCACCTTGAA-3’                        |                  |

(i.e. 1°C per 1 min) then 6U T4 DNA Ligase (Roche, Mannheim, Germany) was added to reaction and incubated at 4°C overnight. Purification was carried out by High Pure PCR Clean up Micro Kit (Roche, Mannheim, Germany) then Pre-amplification was performed with pre-amp primers (Table 1) according to this program: 30 cycles of 94°C, 30 s, 64°C, 30 s, and 72°C, 45 s and final extension at 72°C for 7 min.

Pre-amplified products were subjected to PCR using sensitive primers which containing adaptor sequences plus one nucleotide 3’ (Table 1). Products resulting from sensitive amplification were separated on 10% non-denaturing polyacrylamide gel electrophoresis (PAGE) and stained with silver nitrate.

Isolation, cloning and sequencing

Selected bands were extracted from the PAGE. The eluted DNA was re-amplified by selective (sensitive) primers (Table 1) in 30 cycles with the appropriate selective primers (Table 1) from resultant profile. PCR products were checked on 1.5% agarose gel then cloned in to a pGEM-T Vector System I (Promega, Fitchburg, USA). Recombinant plasmids were sequenced for Identification of cloned transcribed-derived factors (TDFs), using universal primers (T7 promotor and SP6) (Bioneer, Seoul, South Korea). Homology searches were done in non-redundant nucleic and protein databases BLAST. (http://www.ncbi.nlm.nih.gov/BLAST/).

Real Time PCR analysis

Real-Time (RT-PCR) was conducted to investigate differences in expression patterns of identified gene(s) between resistant and susceptible isolates. Specific primers were designed by primer 3 version 0.4.0, (http://frodo.wi.mit.edu/) according to identified target gene (Table 1). cDNA was generated from 1µg of total RNA using QuantiTect® Reverse Transcription (Qiagen, Germany) according to manufacturer’s instruction.

This experiment was done in triplicate with 20µl volumes using IQ SYBR green Super mix (Takara, Japan), in an RT-PCR machine (Cobett, RG-3000, Australia). The PCR condition was as follows: activation at 95°C for 30s, amplification at 95°C for 5 s, 60°C for 34 s for 48

Available at: [http://ijpa.tums.ac.ir](http://ijpa.tums.ac.ir)
cycles. Comparing the cycle thresholds (CTs) of the identified genes with housekeeping gene (GAPDH) the relative value of the expression level of each target gene was analyzed by using the $2^{-\Delta\Delta CT}$ method.

**Results**

*Susceptibility of Leishmania infantum amastigotes to Glucantime®*

The IC50 values of sensitive isolate was $5.33 \pm 1.15$ µg/ml while this value for resistance isolate was $43.33 \pm 5.77$ µg/ml.

**cDNA AFLP**

cDNA AFLP approach was conducted to identify differential transcriptomics expression in resistant and susceptible isolates to Glucantime®. Total RNA was extracted from samples in logarithmic phase (data not shown). The single strand cDNA (sscDNA) was synthesized as mentioned in materials and methods. Using specific primers of housekeeping gene (GAPDH), the integrity of cDNA was checked and confirmed (Fig. 1).

![Fig. 1: Detection of housekeeping gene, GAPDH, (165bp) by reverse transcriptase-PCR (RT-PCR) on an ethidium bromide stained agarose gel (1.5%). M: 50 bp (base pair) molecular weight marker. S: Susceptive, R: resistant, NTC: No template control](image)

The cDNA AFLP products from different primer combinations represented on 8% non-denaturating PAGE (Fig. 2).

To detect involved gene(s) in drug resistance mechanisms, different bands containing TDFs were excised from PAGEs and cloned into TA-cloning vector. The recombinant plasmids containing cDNA AFLP products were sequenced. Sequencing results were searched in non-redundant nucleic and protein databases.

![Fig. 2: Pattern of TDFs which extracted from cDNA- AFLP PAGE. Sensitive amplification of cDNA AFLP on a PAGE from three different primer combinations: S3 MboI /S3 EcoRI, S2 MboI /S4 EcoRI and S1 EcoRI /S2 EcoRI. The arrows indicate extracted TDFs with cod number: 1, 2, 3 (related genes mentioned in table 2).M; (50 bp) molecular weight marker. S:sensitive. R: Resistant](image)

In this study, three genes including calcineurin and two unknown genes were identified with different mRNA expression levels in resistant *L. infantum* isolate compared to the sensitive isolate (Table 2).

**Validation of cDNA AFLP results using real time RT-PCR**

In order to confirm the cDNA AFLP results, we explored expression of detected gene expressions in samples. Real time RT-PCR discovered the downregulation of calcineurin gene (Fig. 3). Figure 3 shows significant downregulation of calcineurin (0.42 folds) ($P<0.05$) in resistant isolate compare to susceptible.
Table 2: Differentially expressed transcription-derived fragments identified by cDNA-AFLP

| Code No | Length (bp) | Accession No          | Annotation           | E-value |
|---------|-------------|-----------------------|----------------------|---------|
| 1       | 120         | XM-001468586.1        | Calcineurin          | 2e-31   |
| 2       | 180         | XM_001470402.1        | hypothetical proteins| 1e-12   |
| 3       | 260         | XM_001470402.1        | hypothetical proteins| 3e-13   |

Fig. 3: Relative expression pattern of calcineurin gene by real time RT-PCR which was measured in L. infantum antimony sensitive compared to resistance. The expression of GAPDH was used to normalize the data. The values are the mean ± SD of at least three different experiments (P<0.05)

Discussion

Since development of effective vaccines against leishmaniasis has not been successful, chemotherapy is the only choice to manage the disease (3). Emerging of large-scale increase in antimony drugs resistance has been appeared as a serious problem in some endemic regions. To address this problem, several laboratories have undertaken different strategies (18, 19). In the present study, we utilized cDNA-AFLP as a valuable method to discovery gene(s) potentially involved in drug resistance of L. infantum. We counted about 1500 TDFs which were differentially expressed in the studied samples.

We found downregulation of calcineurin in Glucantim-resistant isolate compared with susceptible. Calcineurin is a calcium and calmodulin-dependent protein phosphatase and the only protein phosphatase regulated by Ca^{2+} and involved in diverse cellular activities including, cell survival and apoptosis (24-26).

Several studies reported the roles of this protein in adaptations under different environmental factors, in terms of salt levels and temperature changes (25, 26). For instance, in yeast, adaptation to high salt environment occurs by induction of Pmr2p expression via calcineurin activation (24). Adaptation to environmental temperature changes by calcineurin has been well described in Cryptococcus neoformans and Arabadopsis thaliana (25, 27, 28). Odom et al. showed its critical role in surviving of C. neoformans in serum (25). Calcineurin by interaction with other biomolecules such as heat shock proteins provides suitable thermotolerance and virulence in Leishmania major (29).

In spite of the fact that calcineurin directly or indirectly is implicated in surviving, a line of evidence suggested that under different condition it could play adverse function. For example, calcineurin could trigger apoptosis in different organisms by particular concentration of cytosolic ROS (16, 30). Due to high similarities between mammalian and Leishmania calcineurin in terms of function and structure(16), we suggest that downregulation of calcineurin might have a negative effect on apoptosis in Leishmania spp. Consistent with our findings for first time a study by Dhein et al. reported that calcineurin mediates apoptosis in lymphocytes (31). The activation of calcineurin is depended upon cytoplasmic calcium ion concentrations (16). In this regard, a study using cardiac cells revealed that the increasing of intracellular Ca^{2+} levels led to cellular apoptosis by activation of calcineurin and some transcriptional factors (32). Furthermore, solid evidence supported the implication of elevated intracellular Ca^{2+} concentrations in Leishmania parasite cell death (33-35).
Apoptosis has been also observed and characterized by externalization of phosphatidylserine and nuclear DNA fragmentation in amastigotes of *L. donovani* treatment with antimony compounds (33). Antimony drugs induced the productions of some oxidative agents such as nitric oxide (NO) or hydrogen peroxide (H$_2$O$_2$) which play a role in leishmanicidal effects of these drugs (36). It is quite understandable that oxidative stress is responsible for elevation of intracellular Ca$^{2+}$ in *Leishmania* and due to this event, apoptosis could be occurred by calcineurin activation.

**Conclusion**

For the first time, we identified calcineurin as a drug resistance target gene in *L. infantum*. We suggest that downregulation of this gene might be involved in survival rate of *Leishmania* by inhibiting the apoptosis. However, further studies are needed to define precise biological function of calcineurin in the process of *Leishmania* drug resistance.

**Acknowledgements**

The authors would like to express their appreciation to Mrs. Soroor Charedar, Mrs. Orkideh Saydi Dinehkabodi, and Mrs. Shirin Farahyay, Dr. khoshzaban and Dr. r jabarvand for their kind cooperations. This project was financially supported by the Vice-Chancellor for Research, Tehran University of Medical Sciences, Iran (Project No: 88-04-27-9682). The authors declare that there is no conflict of interests.

**References**

1. Choi CM, Lerner EA. Leishmaniasis: recognition and management with a focus on the immunocompromised patient. Am J Clin Dermatol. 2002; 3(2):91-105.
2. Varela M RE, Lorenzo Muñoz D, Robledo SM, Kolli BK, Dutta S, Chang KP, et al. *Leishmania* (*V. panamensis*): An in vitro assay using the expression of GFP for screening of antileishmanial drug. Exp Parasitol. 2009; 122(2):134-9.
3. Maltezou HC. Drug resistance in visceral leishmaniasis. J Biomed Biotechnol. 2009; 2010(7).
4. Romero GAS, Boelaert M. Control of visceral leishmaniasis in Latin America—a systematic review. PLoS Negl Trop Dis. 2010; 4(1):e584.
5. Kheirandish F, Bandehpour M, Haghhighi A, Mahboudi F, Mohebali M, Mosaffa N, et al. Molecular cloning and expression of Iranian *Leishmania major* pteridine reductase 1. Iranian J Parasitol. 2008; 3(2):1-9.
6. Decuyper S, Vanaerschot M, Brunkert K, Imamura H, Müller S, Geary TG. Molecular Mechanisms of Drug Resistance in Natural *Leishmania* Populations. PLoS Negl Trop Dis. 2012; 6(e):5154.
7. Kothari H, Kumar P, Sundar S, Singh N. Possibility of membrane modification as a mechanism of antimony resistance in *Leishmania donovani*. Parasitol Int. 2007; 56(1):77.
8. Sundar S, More DK, Singh MK, Singh VP, Sharma S, Makharia A, et al. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. Clin Infect Dis. 2000; 31(4):1104-7.
9. Hadghi 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(5):e162.
10. Jeddi F, Piarroux R, Mary C. Antimony Resistance in *Leishmania*, Focusing on Experimental Research. J Trop Med. 2011; 2011.
11. Gourbal B, Sonuc N, Bhattacharjee H, Legare D, Sundar S, Ouellette M, et al. Drug uptake and modulation of drug resistance in *Leishmania* by an aquaglyceroporin. J Biol Chem. 2004; 279(30):31010-7.
12. Marquis N, Gourbal B, Rosen BP, Mukhopadhyay R, Ouellette M. Modulation in aquaglyceroporin AQP1 gene transcript levels in drug-resistant *Leishmania*. Mol Microbiol. 2005; 57(6):1690-9.
13. Denton H, McGregor JC, Coombs GH. Reduction of anti-leishmanial pentavalent antimonials drugs by a parasite-specific thiol-dependent reductase, TDR1. Biochem J. 2004; 381(Pt 2):405.
14. Zhou Y, Messier N, Ouellette M, Rosen BP, Mukhopadhyay R. *Leishmania major* LmAcr2 is

Available at: [http://ijpa.tums.ac.ir](http://ijpa.tums.ac.ir)
a pentavalent antimony reductase that confers sensitivity to the drug pentostam. J Biol Chem. 2004; 279(36):37445-51.

15. Hansen C, Hansen EW, Hansen HR, Gammelgaard B, Stürup S. Reduction of Sb (V) in a human macrophage cell line measured by HPLC-ICP-MS. Biol Trace Elem Res. 2011; 144(1):234-43.

16. Rusnak F, Mertz P. Calcineurin: form and function. Phycol Res. 2000; 80(4):1483-521.

17. Hata R, Masumura M, Akatsu H, Li F, Fujita H, Nagai Y, et al. Up-regulation of calcineurin Abeta mRNA in the Alzheimer's disease brain: assessment by cDNA microarray. Biochem Biophys Res Commun. 2001; 284(2):310-6.

18. Guimond C, Trudel N, Brochu C, Marquis N, El Fadili A, Peytavi R, et al. Modulation of gene expression in *Leishmania* drug resistant mutants as determined by targeted DNA microarrays. Nucleic Acids Res. 2003; 31(20):5886-96.

19. t'Kindt R, Scheltema RA, Jankevics A, Brunker K, Rijal S, Dinehkabodi OS, Ghaffari SH, Saffari M, Dinehkabodi OS, Ghaffari SH, et al. Overexpression of aldo-keto-reductase in azole-resistant clinical isolates of *Candida glabrata* determined by cDNA-AFLP. DARU. 2013; 21(1).

20. Decorosi F, Viti C, Mengoni A, Bazzicalupo M, Giovannetti L. Improvement of the cDNA-AFLP method using fluorescent primers for transcription analysis in bacteria. J Microbiol Meth. 2005; 63(2):211-5.

21. Farahyar S, Zaini F, Kordbacheh P, Rezaei S, Safara M, Raoofian R, et al. Overexpression of ald-o-keto-reductase in azole-resistant clinical isolates of *Candida glabrata* determined by cDNA-AFLP. DARU. 2013; 21(1).

22. Kazemi-Rad E, Mohebali M, Hajjaran H, Rezaei S, Marnishi S. Diagnosis and characterization of *Leishmania* species in Giemsa-stained slides by PCR-RFLP. Iran J Public Health. 2008; 37(1):1.

23. Saffari M, Dinehkabodi OS, Ghaffari SH, Moarresi MH, Mansouri F, Heidari M. Identification of novel p53 target genes by cDNA AFLP in glioblastoma cells. Cancer Lett. 2009; 273(2):316-22.

24. Klee CB, Ren H, Wang X. Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. J Biol Chem. 1998; 273(22):13367-70.

25. Odom A, Muir S, Lim E, Toffaletti DL, Perfect J, Heitman J. Calcineurin is required for virulence of *Cryptococcus neoformans*. The EMBO Journal. 1997; 16(10):2576-89.

26. Blankenship JR, Wormley FL, Boyce MK, Schell WA, Filler SG, Perfect JR, et al. Calcineurin is essential for *Candida albicans* survival in serum and virulence. Eukaryot Cell. 2003; 2(3):422-30.

27. Kudla J, Xu Q, Harter K, Gruissem W, Luan S. Genes for calcineurin B-like proteins in *Arabidopsis* are differentially regulated by stress signals. Proc Natl Acad Sci U S A. 1999; 96(8):4718-23.

28. Yoshida T, Toda T, Yanagida M. A calcineurin-like gene ppb1+ in fission yeast: mutant defects in cytokinesis, cell polarity, mating and spindle pole body positioning. J Cell Sci. 1994; 107(7):1725-35.

29. Niederer T, Dandash O, McConville MJ. Calcineurin is required for *Leishmania major* stress response pathways and for virulence in the mammalian host. Mol Microbiol. 2011; 80(2):471-80.

30. Aramburu J, Heitman J, Crabtree GR. Calcineurin: a central controller of signalling in eukaryotes. EMBO reports. 2004; 5(4):343.

31. Dhein J, Walczak H, Bäumler C, Debatin K, Krammer PH. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). Nature. 1995; 373(6513):438-41.

32. Bishopric NH, Andreka P, Skepak T, Webster KA. Molecular mechanisms of apoptosis in the cardiac myocyte. Curr Opin Pharmacol. 2001; 1(2):141-50.

33. Shaha C. Apoptosis in *Leishmania* species & its relevance to disease pathogenesis. Indian J Med Res. 2006; 123:233-44.

34. Lindoso JAL, Cotrim PC, Goto H. Apoptosis of *Leishmania* species in hamsters infected with visceral leishmaniasis. Int J Parasitol. 2004; 34(1):1-4.

35. Sudhandiran G, Shaha C. Antimonial-induced increase in intracellular Ca2+ through non-selective cation channels in the host and the parasite is responsible for apoptosis of intracellular *Leishmania donovani* amastigotes. J Biol Chem. 2003; 278(27):25120-32.

36. Sereno D, Holzmuller P, Mangot I, Cuny G, Ouaissi A, Lemesre JL. Antimicrobial-Mediated DNA Fragmentation in *Leishmania infantum* Amastigotes. Antimicrob Agents Chemother. 2001; 45(7):2064-9.

Available at: [http://ijpa.tums.ac.ir](http://ijpa.tums.ac.ir)