# Identification of immunoreactive proteins in secretions of *Leishmania infantum* promastigotes: an immunoproteomic approach

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## Abstract

**Background:** In the Mediterranean region, *Leishmania infantum* is the main cause of visceral leishmaniasis. Dogs with canine visceral leishmaniasis are an important reservoir of visceral leishmaniasis. Control of canine visceral leishmaniasis could disrupt transmission of visceral leishmaniasis to humans. The secreted antigens of *Leishmania* promastigotes are potential stimuli of the host immune system. Proteomic techniques facilitate the identification of new protein markers.

**Aims:** This study aimed to identify immunoreactive proteins in the secretions of *L. infantum* promastigotes which could be possible targets for the diagnosis and treatment of canine visceral leishmaniasis and the development of vaccines against the disease.

**Methods:** Secretions of *L. infantum* promastigotes were obtained from the cultivation of $6 \times 10^9$ promastigotes in serum-free RPMI-1640 medium during a period of 72 h. After deionization and lyophilization, two-dimensional gel electrophoresis was used for protein separation followed by Western blotting. Thirteen common and repeatable immunoreactive spots were analysed by mass spectrometry.

**Results:** Nine proteins were identified by spectrometry. Most of these proteins were involved in metabolism pathways, survival and pathogenicity of *Leishmania* parasites. Phospholipase C, immune inhibitor A, chitin-binding protein and a single peptide match to chain A crystal structure of selenomethionine were observed in the secretions of *L. infantum* promastigotes.

**Conclusions:** The proteins identified in metabolism pathways, survival and pathogenicity of *Leishmania* parasites are possible targets that could be used for the diagnosis and treatment of canine visceral leishmaniasis and the development of vaccines against the disease in the future.

Keywords: visceral leishmaniasis, *Leishmania infantum*, dogs, proteomics, mass spectrometry

Citation: Rashidi S; Nguewa P; Mojtahedi Z; Shahriari B; Kalantar K; Hatam G. Identification of immunoreactive proteins in secretions of *Leishmania infantum* promastigotes: an immunoproteomic approach. East Mediterr Health J. 2020;26(12):1548–1555. https://doi.org/10.26719/emhj.20.114

Received: 24/09/19; accepted: 09/03/20



## Introduction

Visceral leishmaniasis (VL) is the severe form of a group of diseases caused by *Leishmania donovani* complex. In VL, the reticuloendothelial system is invaded by these parasites. *L. infantum* is the main cause of VL in the Mediterranean region. The prevalence of VL is high in endemic areas such as the Indian subcontinent and south-west Asia *(1,2)*. Dogs infected with canine VL (CVL) are an important reservoir for VL *(2)* and by controlling canine VL, the transmission cycle of VL to humans may be disrupted.

The available anti-leishmanial drugs have important side-effects and there is growing evidence of drug resistance in leishmaniasis *(3,4)*. In the absence of suitable drugs for VL, using proteomic techniques to identify new markers for diagnosis, treatment and vaccination is an appropriate strategy for the control of leishmaniasis in humans and animal reservoirs *(5)*.

Proteomic techniques can show the proteome profile of cells and biological fluids and can also provide more information on protein functions and post-translational modifications of proteins *(5)*. The application of mass spectrometry and proteomic techniques in the laboratory and in the identification of therapeutic targets has been reported in the past decade *(6–9)*. Furthermore, proteomic techniques have received more attention in parasitology for the indication of possible new targets in the diagnosis and treatment of and vaccination against protozoan parasites such as *Leishmania (5,10)*.

The importance of proteome identification of protozoa secretions has been highlighted *(11,12)*. Studies have shown that the antigens from *Leishmania* parasites that are able to stimulate Th1 cells are appropriate candidates for designing vaccines against leishmaniasis *(13,14)*. Secretions of *Leishmania* promastigotes as activators of the host immune response have been suggested to be suitable sources of antigens for the design of vaccines and diagnostic tests in leishmaniasis *(15,16)*.

Given these issues and the ability of proteomic technology to provide information on secreted proteins from *Leishmania* parasites, our study aimed to identify immunoreactive proteins in the secretions of *L. infantum* promastigotes using sera of dogs infected with CVL. Identification of these proteins might open a new path for the effective diagnosis and treatment of and vaccination against CVL in the future.

## Methods

### Obtaining secretions

*L. infantum* (MCAN/IR/07/Moheb-gh strain: from CVL-infected dogs) promastigotes were mass cultivated at 25 °C in Schneider insect culture medium (Sigma Chemical Co., United States of America) supplemented with 20% (v/v) bovine serum (heat-inactivated at 56 °C for 50 min), 100 U/mL penicillin and 100 µg/mL streptomycin. The promastigotes were collected in the exponential growth phase (on the third day) by centrifugation at 2000 × g for 10 min at 4 °C and then washed three times with serum-free RPMI-1640 medium (Shelmax Co., China).

Washed promastigotes ($6 \times 10^9$ promastigotes) were transferred to 10 mL serum-free RPMI-1640 to obtain secretions. After checking the viability of the promastigotes at different times using flow cytometry and propidium iodide, the secretions were collected after 72 h. The secretions obtained were centrifuged at 9000 × g for 30 min at 4 °C and the supernatants were collected *(17)*.

### Deionization and lyophilization of secretions

Contamination of the secretions with the ions (salts) stops the isoelectric focusing stage. To remove these ions, 50 mL of supernatant were aliquoted in dialyzed bags containing a ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche, Germany) at 1 × final concentration. The mixture was dialyzed overnight at 4 °C using a membrane (with a 14 kDa molecular mass cut-off) in 10 L of 1 mM ammonium bicarbonate buffer (Sigma) with four solution changes *(18)*. The dialyzed secretions were frozen and lyophilized to dryness. As a negative control, this procedure was repeated with the RPMI-1640 without promastigote secretions. Lyophilized proteins were resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS and 2% immobilized pH gradient buffer (pH 3–10); GE Healthcare, Sweden), aliquoted and stored at −70 °C. The protein concentration was determined using Bradford assay and bovine serum albumin (Sigma, Germany) as standard.

### Gel electrophoresis and Western blotting

About 40 µg of the secreted proteins recovered were electrophoresed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4% stacking gels over 12% separating gels (Roche Applied Science, Germany). Sample lysates were boiled at 100 °C for 5 min in SDS gel loading buffer (6X) consisting of 375 mM Tris-HCl (pH 6.8), 12% SDS, 60% glycerol, 30% 2-mercaptoethanol and 0.6% bromophenol blue. Then, gels were run using an electrophoresis system (Bio-Rad, United States of America).

In the next step, the sample lanes were transferred (Voltage 20, 1 h) from the gel to a polyvinylidene difluoride membrane (Bio-Rad, USA). After the transfer, 3% bovine serum albumin in phosphate buffered saline was used for blocking the non-specific binding sites on the polyvinylidene difluoride membrane (12 h at 4 °C). Pooled sera from five CVL-infected dogs (asymptomatic and symptomatic) were used to probe the membranes *(19)*. All CVL-infected and non-infected sera had been previously checked for CVL using the direct agglutination test. Pooled sera from non-infected dogs were used as negative control. Antibody binding proceeding was done with 1:1000 dilutions of primary anti-sera in skim milk (2 h at room temperature). After three washes (15 min each) with phosphate buffered saline containing 0.5% Tween-20, the rabbit anti-dog IgG (1:4000 dilutions; Abcam, USA) was then allowed to react (1.5 h at room temperature). Finally, the immunoreactive bands were detected using diaminobenzidine tetrahydrochloride substrate (Sigma, Germany). The ChemiDoc MP Imaging System (Bio-Rad, USA) was used to scan the bands obtained. To confirm the results, the experiment was repeated three times using sera from different CVL-infected and non-infected dogs.

### Western blotting

The protean isoelectric focusing cell system (Bio-Rad, USA) was used for isoelectric focusing. About 200 µg of the protein sample was added for each immobilized pH gradient strip (pH 3–10 nonlinear, 18 cm) by active rehydration (50 V, 20 °C, 14 h) in rehydration buffer (8 M urea, 2 M thiourea (Merck, Germany), 0.3% w/v dithiothreitol, 2% w/v CHAPS, 2% vol/vol immobilized pH gradient buffer (pH 3–10) and bromophenol blue) followed by isoelectric focusing for a total of 62 000 Vh.

After isoelectric focusing, each strip was equilibrated in 10 mL equilibration buffer (6 M urea, 50 mM Tris, pH 8.8, 2% w/v SDS, 30% w/v glycerol) containing 65 mM dithiothreitol (in the first step) and 135 mM iodoacetamide (in the second step) at room temperature for 15 min. Two-dimensional gel electrophoresis followed by Western blotting was done using the same procedures described earlier. To confirm the results, each experiment was repeated three times using pooled sera from different CVL-infected and non-infected dogs.

After the immunodetection step, GS-800 calibrated densitometer (Bio-Rad) and Prodigy SameSpots software (version 1.0) (Nonlinear Dynamics, United Kingdom of Great Britain and Northern Ireland) were used to scan the spots and analyse the digital images, respectively. The reference image was an image with the greatest number of spots. Each spot was analysed in a semi-automated way and background intensity was subtracted from each image.

### Isolation of immunoreactive spots

After mapping the immunoreactive spots on the membrane, two-dimensional gel electrophoresis was conducted again with the secreted proteins lysate. Coomassie brilliant blue staining (containing 8% ammonium sulfate (Sigma, USA), 1.6% orthophosphoric acid (Sigma, USA), 20% methanol (Merck, Germany), and 0.12% Coomassie blue G 250 (Merck, Germany)) was used for visualization of spots (overnight incubation) *(20)*. After staining, gels were destained with distilled water. Finally, the immunoreactive spots identified on the membrane were punched from the gel using a pipette.

### In-gel digestion of protein samples and mass spectrometry

In-gel digestion of protein samples and mass spectrometry analysis were done according to the protocol of the metabolomics and proteomics laboratory technology facility, Department of Biology, University of York, United Kingdom *(20)*. According to the company procedures, MASCOT scores of more than 62 are significant ($P$ < 0.05).

### Database search

The mass spectra generated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF/TOF MS) were searched against peptide masses in the UniProt database (555 594 sequences, 199 016 217 residues) (Table 1) and also the NCBI database (132 460 369 sequences; 48 620 496 129 residues) (Table 2).

### Ethical considerations

Experiments with animals were done following guidelines of the Institutional Animal Care and Committee on Ethics of Animal Experimentation of the Shiraz University of Medical Sciences.

## Results

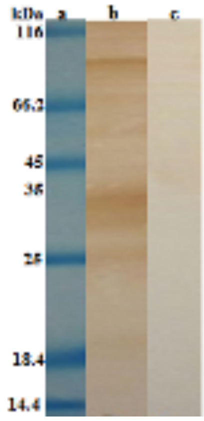
### Viability of promastigotes

When we obtained secretions, to decrease the release of secretory proteins from dead promastigotes to the secretions, we carried out propidium iodide staining to check the viability of *L. infantum* promastigotes. The propidium iodide results showed that the best time for collecting the secretions with a high concentration was 72 h after cultivating in serum-free RPMI-1640 medium. The rate of promastigotes viability at this time was 83% *(17)*.

### SDS-PAGE and Western blotting

Before doing the two-dimensional gel electrophoresis following Western blotting, we determined the molecular weights of the immunoreactive protein bands in the secretions. As shown in Figure 1, we detected several sharp bands between 25 and 35 kDa and 66.2 and 116 kDa. We also saw a weak band between 45 and 66.2 kDa. Performing Western blotting before 2-DE-Western blotting facilitates the identification of immunoreactive spots on the polyvinylidene difluoride membrane and gel.

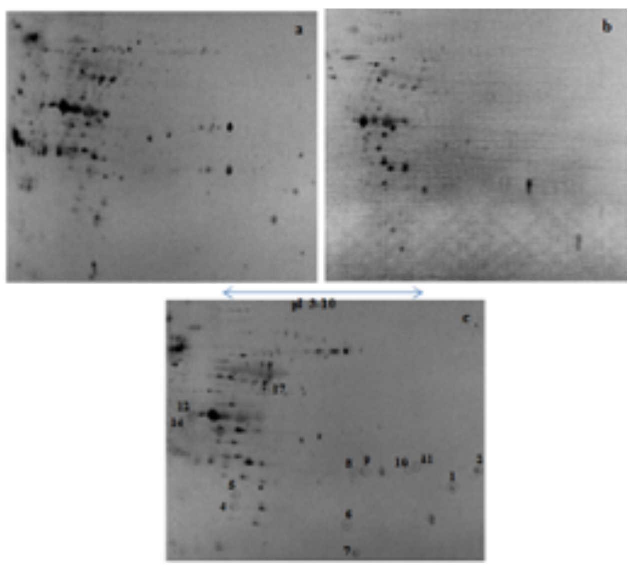
**Figure 1 Western blotting of the secretions on the polyvinylidene difluoride membrane: a) ladder, b) probed membrane with sera of dogs infected with canine visceral leishmaniasis, c) probed membrane with sera of dogs without canine visceral leishmaniasis**




### Western blotting and identification of immunoreactive proteins by mass spectrometry

We extracted and lysed secretions of *L. infantum* promastigotes. Since the aim of our study was to identify immunoreactive proteins in the secretions of *L. infantum* promastigotes, we separated proteins in lysates by using isoelectric focusing over a pH range from 3 to 10 and then two-dimensional gel electrophoresis. Three gels were run for each test. We detected more than 1000 spots and about 100 immunoreactive spots on each of the gels and polyvinylidene difluoride membranes, respectively. We isolated 13 repeatable and intense immunoreactive spots from the

**Figure 2 Immunoproteome of the secretions on the polyvinylidene difluoride membrane: a) probed membrane with sera of dogs infected with canine visceral leishmaniasis, b) probed membrane with sera of dogs without canine visceral leishmaniasis, c) selected spots of gel for mass spectrometry analysis**



gel for mass spectrometry analysis (Figure 2). Of these spots, nine immunoreactive proteins were characterized by MALDI TOF/TOF MS. Due to contamination with keratin, three spots showed spectra dominated with keratin. The identified proteins were chitin-binding protein, immune inhibitor A (immune In A), a peptide matched to bacillolysin (δ-endotoxin), a single peptide match to hs1vu complex proteolytic subunit-like, a single peptide match to chain A crystal structure of selenomethionine, iron superoxide dismutase, phospholipase C, and some proteins matched to enterotoxins.

We used the *L. infantum* genome project database (www.genedb.org) and published literature for the identification of multiple functions of the detected proteins. The scores of all the proteins obtained were significant (> 62). According to the mass spectrometry data, spot number 17 (SEC 13) was identified as "immune In A" and "selenomethionine" in the Uniprot and NCBI databases, respectively.

## Discussion

We applied an immunoproteomic approach on secretions of *L. infantum* promastigotes, using pooled sera from CVL-infected dogs (asymptomatic and symptomatic), to find immunoreactive proteins that could be possible new targets for diagnosis and treatment of, and vaccination against CVL. The use of pooled sera might reduce the effect of individual animal immune response variations on *L. infantum* antigens.

Chitin-binding protein as an immunoreactive protein in the secretions of *L. infantum* promastigotes has been previously reported in *Toxoplasma gondii (21)*. Chitin-binding protein is part of chitinase enzyme and synergistic effects of chitin-binding proteins on chitinases have been described in bacteria *(22)*. Although the activity of chitinase is two-to-four- fold higher in amastigotes than in promastigotes in vitro *(23)*, in a previous study on the secretions of *L. donovani*, chitinase and chitin-binding proteins were not detected in mass spectrometry results *(24)*. More investigations on the elucidation of the potential action of chitin-binding proteins in *Leishmania* and gene sequencing and cloning of chitin-binding proteins *(23)* might be useful to understand the role of chitin-binding proteins in the biology of *Leishmania* parasites.

The function of immune In A in *Leishmania* parasites is still not known; however, this protein has been described as a neutral metalloprotease in bacteria *(25)*. Based on the presence of metalloproteases in *Leishmania* parasites and their involvement in parasite virulence, the discovery of immune In A as a possible metalloprotease in *Leishmania* parasites suggests that this protein may be of clinical interest for leishmaniasis prognosis and the prediction of treatment efficacy *(26)*. Since IgA protease is the only homologous protein of immune In A, the detection of anti-immune In A in CVL- and VL-infected sera might suggest new approaches using tools based on immune In A for the diagnosis of leishmaniasis in the future *(27)*.

We identified a peptide that matched bacillolysin (δ-endotoxin). Bacillolysin is a virulence factor and lethal toxin in bacteria *(28)*. Interestingly, the role of the disulfide bond A as a homologue of the protein disulfide isomerase is associated with the production of toxins in bacteria *(29)*. Because protein disulfide isomerases are important proteins in the pathogenicity of *Leishmania* parasites, the possible expression of such endotoxins in these parasites could be related to protein disulfide isomerases *(29,30)*. Due to the scarce data on endotoxins in *Leishmania* parasites, molecular techniques including gene expression and quantitative real-time polymerase chain reaction could be used to confirm the production of endotoxins in these parasites.

The survival, virulence and proteolytic functions of hs1vu in *Leishmania* parasites have been described in previous studies *(31–33)*. In addition, the relationship between hs1vu and 3M3I protein (a hypothetical protein in *L. major* likely involved in nucleotide metabolism) may be helpful to elucidate the metabolic function of hs1vu in *Leishmania* parasites *(34)*. The potential and critical functions of hs1vu, and the overexpression of this protein

**Table 1 Identification of immunoreactive proteins in the secretions of *Leishmania infantum* promastigotes (UniProt)**

| Spot name | No. of spots on gel | Protein name | No. of matched peptides | Mr (theoretical) | pI (theoretical) | Score | Protein sequence coverage (%) | Accession no. |
|---|---|---|---|---|---|---|---|---|
| SEC 5 | 10 | PLC | 4 | 32.3 | 7.14 | 101 | 7 | P09598 |
| SEC 6 | 6 | A single peptide match to Enterotoxin (fragment) | 1 | 47.8 | 4.23 | 90 | 35 | P80567 |
| SEC 9 | 8 | PLC | 2 | 32.3 | 7.14 | 101 | 7 | P09598 |
| SEC 12 | 9 | PLC | 4 | 32.3 | 7.14 | 275 | 14 | P09598 |
| SEC 13 | 17 | A single peptide match to Immune In A | 1 | 74.9 | 5.13 | 54 | 1 | P23382 |

*Mr = molecular weight; pI = isoelectric point; PLC = phospholipase C.*

Table 2 **Identification of immunoreactive proteins in the secretions of *Leishmania infantum* promastigotes (NCBI)**

| Spot name | No. of spots on gel | Protein name | No. of matched peptides | Mr (theoretical) | pI (theoretical) | Score | Protein sequence coverage (%) | Accession no. |
|---|---|---|---|---|---|---|---|---|
| SEC 1 | 4 | A single peptide match to hs1vu | 1 | 24.2 | 6.07 | 73 | 5 | XP_001469616.1 |
| SEC 2 | 7 | A single peptide match to chain A, crystal structure of selenomethionine | 1 | 83.3 | 5.75 | 73 | 1 | 4YU5_A |
| SEC 4 | 14 | Chitin-binding protein | 3 | 49.9 | 6.18 | 295 | 9 | WP_001035088.1 |
| SEC 6 | 6 | Enterotoxin 40 kDa subunit | 2 | 43.09 | 6.85 | 171 | 6 | CRG01534.1 |
| SEC 8 | 2 | A single peptide match to antigen | 1 | 36.3 | 6.01 | 85 | 4 | EOP01994.1 |
| SEC 10 | 12 | A single peptide match to bacillolysin (insecticidal δ-endotoxin) | 1 | 62.6 | 5.78 | 77 | 2 | EEM05310.1 |
| SEC 11 | 1 | Iron superoxide dismutase | 2 | 26.5 | 8.46 | 185 | 12 | XP_001463371.1 |
| SEC 12 | 9 | Phospholipase C | 3 | 32.2 | 6.5 | 212 | 11 | WP_002010569.1 |
| SEC 13 | 17 | A single peptide match to chain A, crystal structure of selenomethionine | 1 | 83.3 | 5.75 | 82 | 1 | 4YU5_A |

*Mr = molecular weight; pI = isoelectric point.*

in the viscerotropic form of *L. tropica (31)* suggest a role of this protein as a valuable marker in the treatment and diagnosis of CVL and VL in a clinical setting.

Selenoproteins are involved in the regulation of oxidative stress in the cells. Isolation of leishmanial-selenomethionine derivatives from *L. donovani* proteins, and the sequencing of the gene encoding this protein in Kinetoplastida support our detection of selenomethionine through mass spectrometry assays *(35,36)*.

We also identified Fe-SOD which protects *Leishmania* parasites against radical superoxide anions. The expression of SODs as conserved molecules with a high degree of homology in *Leishmania* species *(24,37,38)* highlights the possible use of these proteins in the development of candidate drug and vaccine targets in leishmaniasis. In recent years, nanovaccines against leishmaniasis have been evaluated by producing the recombinant form of leishmanial-SODB1 and loading on the chitosan nanoparticles *(39)*.

The report of phospholipase C-orthologue genes and phospholipase C-related signalling pathways in protozoa in previous studies confirms our results on the expression of phospholipase C in secretions of *Leishmania* parasites *(40,41)*. It has been suggested that the escape of protozoan parasites from parasitophorous vacuoles could be mediated by phospholipase C *(42)*. The expression of inositol phosphosphingolipid phospholipase C-like protein, the cleavage of the glycophosphatidyl-inositol anchor through phospholipase C and the induction of GP63-shedding via phospholipase C in *Leishmania* parasites are related to the virulence and pathogenicity functions of phospholipase C in these parasites *(43–45)*. Nevertheless, the exact role of phospholipase C is unknown in *Leishmania* parasites so far. Given the aforementioned functions of phospholipase C in the pathogenicity of *Leishmania* parasites and also the role of phospholipase C in hydrolysis of miltefosine, the use of phospholipase C-inhibitors can be evaluated as a therapeutic target in CVL and VL in the future *(46)*.

## Conclusion

The isolation of high protein concentrations in proteomic studies on secretions provides reliable data. Since secreted antigens of *Leishmania* promastigotes are potential stimulants of the host immune system, the identified immunoreactive proteins in our study might be valuable proteins for developing diagnostic candidates and vaccine targets in the future. In addition, according to the main roles of such molecules in metabolism pathways, survival and pathogenicity of *Leishmania* parasites, they could be possible therapeutic targets for CVL in the future. Validation of our results – the proteins we found expressed in *Leishmania* parasites – through further laboratory techniques including gene cloning, Western blotting, enzyme-linked immunosorbent assay (ELISA) and quantitative real-time polymerase chain reaction is warranted.

**Funding:** National Institute for Medical Research Development (NIMAD), Tehran, Islamic Republic of Iran (grant number 971405).

**Competing interests:** None declared.

## Identification de protéines immunoréactives dans les sécrétions des promastigotes de *Leishmania infantum* : une approche immunoprotéomique


### Résumé

**Contexte :** Dans la Région méditerranéenne, *Leishmania infantum* est la principale cause de leishmaniose viscérale. Les chiens atteints de leishmaniose viscérale canine constituent un important réservoir de leishmaniose viscérale. La lutte contre la leishmaniose viscérale canine pourrait empêcher la transmission de cette maladie à l'homme. Les antigènes sécrétés par les promastigotes de *Leishmania* sont des stimuli potentiels du système immunitaire de l'hôte. Les techniques protéomiques facilitent l'identification de nouveaux marqueurs protéiques.

**Objectifs :** La présente étude visait à identifier des protéines immunoréactives dans les sécrétions de promastigotes *L. infantum* qui pourraient être utilisées pour le diagnostic et le traitement de la leishmaniose viscérale canine et la mise au point de vaccins contre cette maladie.

**Méthodes :** Les sécrétions de promastigotes *L. infantum* ont été obtenues à partir de la culture de $6 \times 10^9$ promastigotes dans un milieu sans sérum RPMI-1640 pendant une période de 72 h. Après déionisation et lyophilisation, on a utilisé une électrophorèse bidimensionnelle en gel pour séparer les protéines, suivie d'un transfert de type Western. Treize points immunoréactifs communs et répétables ont été analysés par spectrométrie de masse.

**Résultats :** Neuf protéines ont été identifiées par spectrométrie. La plupart de ces protéines étaient impliquées dans les voies métaboliques, la survie et la pathogénicité des parasites *Leishmania*. La phospholipase C, l'inhibiteur immunitaire A, la protéine de liaison à la chitine et un peptide unique correspondant à la structure cristalline de la chaîne A de la sélénométhionine ont été observés dans les sécrétions de promastigotes *de L. infantum*.

**Conclusions :** Les protéines identifiées dans les voies métaboliques, la survie et la pathogénicité des parasites *Leishmania* sont des cibles possibles qui pourraient être utilisées pour le diagnostic et le traitement de la leishmaniose viscérale canine et la mise au point de vaccins contre la maladie à l'avenir.


## تحديد البروتينات المناعية التفاعلية في إفرازات مُشَيِّقَة "الليشمانيا الطفلية": نهج البروتيوميات المناعية

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### الخلاصة

**الخلفية:** في إقليم شرق المتوسط، تُعتبر "الليشمانيا الطفلية" السبب الرئيسي لداء الليشمانيات الحشوي. ويُعتبر الكلاب المصابون بداء الليشمانيات الحشوي مستودعاً مهماً له. ويمكن أن تؤدّي مكافحة داء الليشمانيات الحشوي في الكلاب إلى وقف انتقاله إلى البشر. وقد تكون مستضدات مُشَيِّقَة "الليشمانيا" المُفرزة هي محفزات محتملة للنظام المناعي في جسم المضيف. ومن شأن تقنيات البروتيوميات أن تُسهل من إمكانية تحديد الواسمات البروتينية الجديدة.

**الأهداف:** هدفت هذه الدراسة إلى تحديد البروتينات المناعية التفاعلية في إفرازات مُشَيِّقَة "الليشمانيا الطفلية"، والتي من الممكن أن تكون مستهدفات محتملة لتشخيص وعلاج داء الليشمانيات الحشوي في الكلاب وتطوير لقاح مضاد لهذا المرض.

**طرق البحث:** حُصل على إفرازات مُشَيِّقَة "الليشمانيا الطفلية" من مزرعة مُشَيِّقَة 6×910 في وسط RPMI–1640 خال من الأمصال خلال فترة 72 ساعةً. وبعد إزالة التأيين والتجفيد بالتجميد، استُخدم اثنان من أجهزة الرحلان الكهربي الهلامي ثنائي الأبعاد لفصل البروتينات، وأعقَب ذلك إجراء لطخة ويسترن. واستُخدم قياس طيف الكتلة في تحليل ثلاثة عشر موضعاً مناعياً تفاعلياً مشتركاً ومتكرراً.

**النتائج:** حدد قياس طيف الكتلة تسعة بروتينات. وكان أغلبها موجوداً في مسارات التمثيل الغذائي، والبقاء على قيد الحياة، وإمراضية طفيليات "الليشمانيا". وقد لوحظ في إفرازات مُشَيِّقَة "الليشمانيا الطفلية" وجود فوسفوليباز C، والمثبط المناعي A، والبروتين الذي يربط الكيتين، ومطابقة البِبْتيد الواحد لسلسلة التركيب البلوري للسيلينوميثيونين A.

**الاستنتاجات:** تُعتبر البروتينات المُحددة في مسارات التمثيل الغذائي، والبقاء على قيد الحياة، وإمراضية طفيليات "الليشمانيا" مُستهدفات محتملة يمكن استخدامها في تشخيص داء الليشمانيات الحشوي في الكلاب ومعالجته وتطوير لقاحات مضادة لهذا المرض في المستقبل.

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