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REVIEW ARTICLE

Using proteomics as a powerful tool to develop a vaccine against Mediterranean visceral leishmaniasis

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Abstract Visceral leishmaniasis (VL) is a tropical infectious disease, which is called Mediterranean visceral leishmaniasis (MVL) in the Mediterranean area. In spite of many attempts, no effective commercial vaccine exists for MVL. To find new targets for developing antileishmanial vaccines, knowing parasite antigens that provoke the immune system are on demand. Nowadays, proteomics methods are defined as approaches for analysis of protein profiling of different cells. Within this framework, detection of new antigens is becoming more facilitated. In this review, we aimed to introduce possible targets using proteomics so; they could be used as candidates for developing vaccines against MVL. It can shed new light in the near future on the development of promising vaccines for MVL.

Keywords Mediterranean visceral leishmaniasis · Proteomics · Vaccine target

Introduction

Visceral leishmaniasis (VL) is caused by *Leishmania donovani* and *L. infantum* in the old world, while it is due to *L. chagasi* in the new world. In Mediterranean regions, *L. infantum* is the main cause of Mediterranean visceral leishmaniasis (MVL) (Azizi et al. 2006; Barati et al. 2015; Fakhar et al. 2006; Gharekhani et al. 2016; Ghatee et al. 2013; Hatam et al. 2010). Approximately every year 500,000 new cases of VL occur in the world (Barati et al. 2015; Ghatee et al. 2013). In the zoonotic pattern, reservoirs are dogs and rodents (Postigo 2010; Reithinger et al. 2007; Sabzevari et al. 2013). Evidence has shown that dogs can act as a natural host for MVL, and human is its accidental host (Fig. 1). In VL, parasites tend to infect macrophages throughout the viscera. Manifestations of VL in human are different and vary and sometimes it could appear in a life-threatening progressive visceral form of the disease. For instance, the confection of HIV with VL is a serious problem for health care conditions (Alvar et al. 2008).

With respect to the diagnostic of VL, the methods are different. They include parasitology, biochemical, serological and molecular approaches (Mohammadi-Ghalehbin et al. 2011; Oryan et al. 2013; Rassi et al. 2007). Chemotherapy is the first choice for VL treatment but unfortunately, using the available drugs is accompanied by the side effects (Murray et al. 2005). Side effects and resistance to available drugs prompt the investigators to invent new drugs. Doubtless, an effective vaccine would be an appropriate treatment.

Nowadays, new techniques such as proteomics give us a promising approach for discovering new targets to develop a protective vaccine for infectious diseases like leishmaniasis. The aim of this review was to provide a view about those markers characterized by proteomics technique in previous articles.

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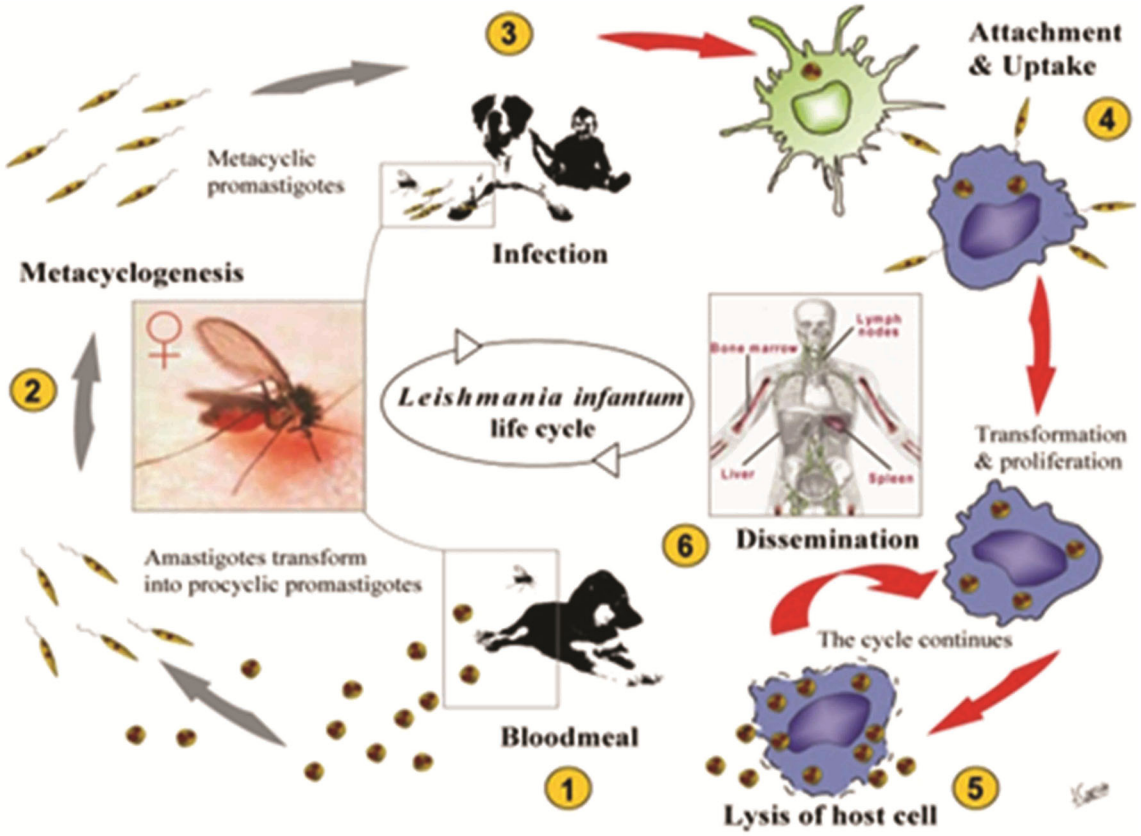

Fig. 1 Life-cycle of *L. infantum: Leishmania* has a digenetic life-cycle consisting of an extracellular flagellated stage (promastigote) in the sandfly vectors, and an aflagellated intracellular stage (amastigote) in the animal reservoirs and human. Reproduced with permission from Nieto et al. (2011)

Vaccines for leishmaniasis

Th1 is responsible for the immunity against the *Leishmania*. This subset of T- cells by producing of IFN-γ can activate the macrophages to kill the parasite (Mansueto et al. 2007; Nylén and Gautam 2010). In the treated patients, it has been shown that recovery and resistance to reinfection are related to the development of antigen-specific Th1 cell responses (Nylen and Gautam 2010; Roberts 2005; Tripathi et al. 2007). Taken together, a good strategy for developing a vaccine against leishmaniasis, such as MVL, should focus on eliciting the Th1 cells against the *Leishmania* parasites.

Some research groups characterized a number of *Leishmania* proteins which produced variable protection against *Leishmania* in animal models (Coler et al. 2007; Foroughi-Parvar et al. 2015; Foroughi-Parvar and Hatam 2014; Kushawaha et al. 2011; Singh et al. 2012). Also, using killed *Leishmania* vaccine has shown different results in different studies (Kedzierski 2010; Srivastava et al. 2016). On the other hand, live attenuated ones have shown an effective protection in some studies (Bhattacharya et al. 2015; Gannavaram et al. 2014; Ismail et al. 2017). The use of attenuated *Leishmania* parasites as a vaccine is very interesting because they are somehow mimicking the natural infection. In addition, it leads to similar immune responses. By means of molecular techniques, the recombinant proteins are becoming another new approach for vaccines development (Coler et al. 2007; Dias et al. 2017). In contrast to the substantial effort to develop a vaccine, there is no approved vaccine against human leishmaniasis, while there are approved vaccines for dogs (Reithinger et al. 2007).

Nowadays, by completion of genome sequencing of *Leishmania*, it seems that invention of a vaccine is becoming an easier task than before (Cantacessi et al. 2015; Llanes et al. 2015). Recently, researchers have attempted to use proteomics techniques for introducing immunodominant antigens in *Leishmania* parasites for designing new effective vaccines.

Proteomics

Proteome refers to the set of proteins encoded by the genome and defined as an analysis of proteins in order to determine their unique identity, quantity, function, and

interaction (Herosimczyk et al. 2006). Evidence has been shown that this method also practically suitable for analysis of the proteome of *Leishmania* genus (Murray et al. 2005; Reithinger et al. 2007). As most of the genes are conserved among the *Leishmania* species, there is a poor correlation between the transcripts and the proteins expressed by the *Leishmania* parasite (Iantorno et al. 2017) in *Leishmania* spp. Functional genomic analysis of mRNA also does not show the whole pattern of protein expression. A widespread technique, like western blotting, just looks at the change in one protein, but if we intend to check the changes in a large number of proteins in various biological materials and microorganism at the same time, proteomics facilitates this task (Lewandowicz et al. 2009) and helps us to discover biomarkers which can be applied to drug, vaccine, and diagnostic targets (Thongboonkerd 2004).

Methods and steps involved in proteomics analysis

Combination of proteomics, bioinformatics and mass spectrometry (MS) could give us a good quality of proteome maps. 2-dimensional gel electrophoresis (2DE) is a method which can separate the proteins mixtures based on the net charge by isoelectric focusing (IEF) (1 dimension) and based on to the molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (2 dimensions). After staining the gels, protein spots were digested by trypsin; then we extracted peptides sequence using tandem mass spectrometry (MS/MS) (Fig. 2) which is linked to a genome sequence database.

For reducing the complexity of proteins, fractionation by ammonium sulfate and digitonin prefractionation apply for detecting the markers with low molecular mass (McNicoll et al. 2006). For enhancing the visualization of targets with low molecular masson the gels, liquid phase IEF should be combined with 2DE (Brobey and Soong 2007).

Recently, proteomics methods have been improved by using multi-dimensional liquid chromatography (LC) and applied for proteomics of pathogenic organisms (Zhang et al. 2010).

Proposed targets for MVL vaccines using proteomics in promastigotes and amastigotes

To find new targets for developing anti-leishmanial vaccines, knowing the biology of the parasite and exact mechanism of host-parasite interactions are two important issues that are worth noticing. Within this framework, different studies have found out proteins which are involved in parasite's vitality, infectivity, and invasiveness

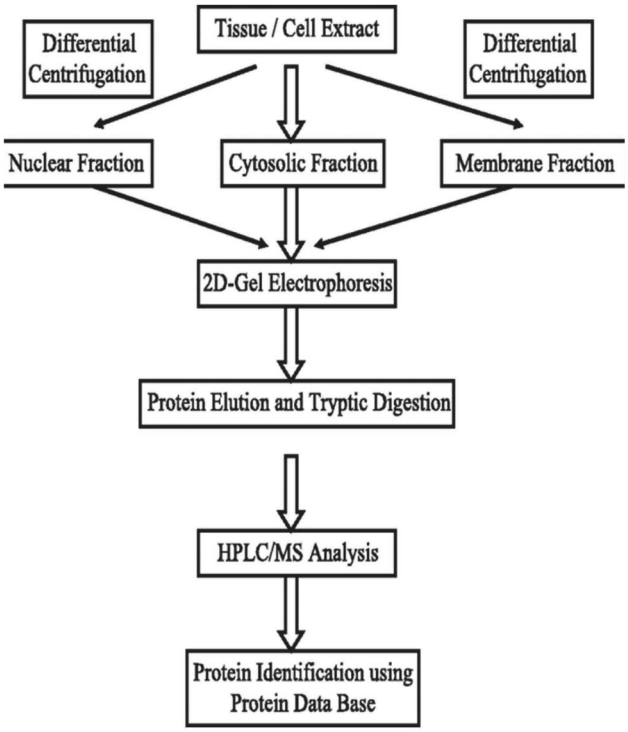

Fig. 2 Overview of steps involved in proteomics analysis: whole cell lysate or a special cell fraction can be analyzed by 2DE and mass spectrometry. Reproduced with permission from Smita et al. (2015)

(da Fonseca et al. 2014; Dea-Ayuela et al. 2006). Expressed proteins responsible for survival and infectivity are not directly related to the vaccine potential targets except the proteins which are immunodominant that can be applied for a vaccine against MVL in future. Recognition of immunodominant proteins by immunoproteomics method seems to be on demand for designing a practical vaccine in MVL.

The results of a study indicated that the immunodominant proteins in *L. infantum* promastigote membranes were located in molecular weight of 30–36 kDa range (Kamoun-Essghaier et al. 2005). The spots sequences showed LACK (*Leishmania* homolog of receptors for activated C-kinase) and a possible member of the aldehyde reductase family provokes humoral immunity in MVL (Gómez-Arreaza et al. 2011). These proteins belong to the conserved proteins which are permanently expressed in the eukaryotic cells.

Another study, using multiplex 2D on attenuated *L. infantum* promastigotes (Aravind et al. 2003), showed observable changes in the thiol-redox control system and made it less virulent (Daneshvar et al. 2012). This metabolic system is vital and protects the *Leishmania* against the oxidative burst of macrophages (Acestor et al. 2006). Also, the metabolism of hydrogen peroxide and trypanothione reductase activity in the attenuated type was

declined in comparison to the wild-type. Consequently, using immunodominant targets of attenuated promastigotes forms is probably helpful in improving vaccines.

A recent study on *L. infantum* promastigote in logarithmic phase introduced some immunodominant proteins such as ATPase beta subunit (chaperone function); propionyl carboxylase (in fatty acid metabolism); transketolase (in carbohydrate metabolism); succinyl-diaminopimelate desuccinylase (in amino acid metabolism and synthesis); proteasome subunit (in protein synthesis and catabolism); full size heat shock protein 70 (in stress response/chaperone) and adrenodoxin reductase, a non-antigenic protein which is related to the ergosterol biosynthesis pathway (Dea-Ayuela et al. 2006). Most of these proteins are immunodominant and have been proved associated with crucial physiological and virulence functions of *Leishmania* parasite. Thus, these results help us to identify new targets for developing vaccines for VL.

The result of a 2D immunoblotting study on the late-logarithmic phase of *L. infantum* promastigotes (Agallou et al. 2016) identified immunodominant proteins mostly include in stress responses and metabolic systems. These data also showed that eukaryotic initiation factor 4a (elF-4a) and LACK are known for their immunostimulatory potential and provoke antigen-presenting cells to produce IL-12 and TNFα.

Also, in this study, an in silico investigation proposed that the chaperonin HSP60, enolase, cyclophilin 2, dihydrolipoamide dehydrogenase, and cyclophilin 40 are restricted to MHC-I and/or MHC-II. The identification of proteins which are restricted to MHC-I and MHC-II is very crucial to have a long-lasting response of CD8+ and CD4+ respectively against the parasites. Based on the finding of this study, apparently, the combination of bioinformatics and immunoproteomics will probably guide us to predict vaccine targets.

The results of an immunoproteomics study on *L. infantum* promastigote stage (Coelho et al. 2012) show that the phosphoglycan beta-1, 3-galactosyltransferase, and flagellum transition zone component, which is linked to LPG synthesis, are more highly expressed. In addition, elongation factors, heat shock proteins such as HSP70, HSP83 and other chaperones, as well as tubulin and other housekeeping proteins, were observed. It seems that HSPs have important roles in immunity. Also, there are targets for the responses of the immunity system to a wide difference of pathogens including fungi, bacteria, helminths, and protozoa (Tsan and Gao 2009). These data are important for designing vaccines and biology of *L. infantum.*

In *Leishmania* parasites, over-expression of some genes is directly or indirectly related to the infectivity (Alcolea et al. 2009, 2010). In 2011 (Alcolea et al. 2011), an investigation by 2DE and MS processed the proteins of *L. infantum* promastigote in early logarithmic to stationary phase in the replicate axenic cultures. The observation indicated that the level of protein and mRNA in eukaryotic elongation factor 1a (eEF1a) subunit and the electron transfer flavoprotein (ETF), had the equal expression level and in the stationary phase it was decreased. In promastigotes in dividing logarithmic stage, a 51 kDa subunit of replication factor A showed an up-regulation. All of the proteins ascribed here display the same differential regulation values with the identical mRNA levels. Therefore, during the life cycle of *L. infantum*, the 40S ribosomal protein S12, the eEF1a subunit, the ETF, a-tubulin and the T-complex protein 1 subunit γ are regulated in different patterns both in proteome and transcriptome. It seems that they get close to the target for a vaccine for each stage.

A recent study on *L. infantum* promastigote in logarithmic phase (Dea-Ayuela et al. 2006) introduced some structural immunodominant targets, such as paraflagellar rod protein 3 (PAR3), which can be effective for vaccine targets in MVL. It has been demonstrated that some structural proteins in *Leishmania* are functional and several of them are crucial for developmental stages and parasite life cycle. Thus, these results support us to identify new targets for developing vaccines.

The results of a study (Kamoun-Essghaier et al. 2005) illustrated that elongation factor 1a is a mitochondrial protein which could stimulate the antibody in visceral leishmaniasis. Also, elongation factor 2 and elongation factor 1a are able to induce cellular immunity in leishmaniasis patients (Jaiswal et al. 2014; Kushawaha et al. 2011). All the mentioned proteins in the current investigation belong to the conserved proteins which permanently expressed in eukaryotic cells and could be a possible target for diagnosis and vaccine against MVL.

The results of another study by free flow electrophoresis (FFE) and IEF separation technique (Brotherton et al. 2012; Nissum and Foucher 2008) showed that glycolytic enzymes and flagella proteins upregulated in the *L. infantum* promastigotes. In amastigote, enzymes included in fatty acid β oxidation and gluconeogenesis was overexposed. In accordance with this data, post-translational modification occurs in each stage. Consequently, these observations could open a new horizon for developing a new vaccine.

In another research by labeling the cysteine-reactive light and heavy ICAT (Isotope Coded Affinity Tag) reagents on *L. infantum*,(Leifso et al. 2007), the authors displayed the paraflagellar rod proteins (PFR) (Portman and Gull 2010) and cell surface protease leishmanolysin (GP63) (Isnard et al. 2012; Lieke et al. 2008). The PFR is a multifunctional complex of the cytoskeleton structure and the motility of *Leishmania* parasite is dependent on it

(Carrilloa et al. 2008). GP63, which is a surface protease in *Leishmania,* is very important in the virulence and infectivity. Organisms that express gp63 can utilize the opsonic effects of complement while avoiding its lytic effects (Gupta et al. 2013). Thus, we can use these specific proteins to design effective vaccines in future.

The membranous proteins are crucial in the regulatory pathways of *Leishmania* parasites and host–pathogen interactions. A quantitative mass spectrometry study (Lynn et al. 2013) identified that approximately 20–40% of proteins in the amastigote and promastigote of *L. infantum* were different in expression. Drawing a map for proteins such as leishmanolysin (GP63), eEF-1α and amastin based on their functions in the metabolism, infectivity, and virulence could help us to detect proteins which can potentially be candid for inventing the MVL vaccines.

The analysis of proteins in developmental stages of *L. infantum* showed that 6.1% of proteins were related to the promastigote, and 12.4% belonged to the amastigotes (McNicoll et al. 2006). The correlation between amastigote specific protein isoforms and its mRNA was almost 53%, while in respect to the promastigote specific spots, no correlation was observed. A lot of proteins were indicated in multiple spots; consequently, that post-translational modification is too much in this parasite. In some cases, different isoforms seem to be proprietary to different life stages. These data proposed that post-transcriptional controls at translational and post-translational levels could perform remark function in the differentiation of *Leishmania*. Consequently, this data indicates the necessity of attention to post-translational modification of protein for development of a vaccine for leishmaniasis.

In a proteomics study (Leifso et al. 2007), histone glycolytic proteins and enzyme enolase (Gupta et al. 2014) were introduced in protein profiling of *L. infantum.* In addition to apoptosis roles, glycolytic proteins are essential for transcriptional control of histone gene expression (Sirover 2005). Based on the essential role of these proteins, they can be probable targets in VL vaccines in future.

In a proteomics study, the authors checked the virulence factors in the amastigote forms extracted from the macrophages (in vitro) and hamster (in vitro) tissues (da Fonseca et al. 2014). The results illustrated that over-expression of KMP-11, phosphomannomutase, metallopeptidase, EF-2, Rieske iron–sulfur protein precursor, and *S*-adenosylhomocysteine is associated to the virulence and degradation of host cell protective proteins. Also, tryparedoxin peroxidase and peroxiredoxin keep the *L. infantum* safe against the stress conditions (Iyer et al. 2008). On the other hand, EF-1 α inhibits the activation of macrophage and chaperones, and endoribonuclease L-PSP has a role in the prolongation of host cell lifetime and parasite survival. In conclusion, these proteins are indispensable for the parasite survival and pathogenesis; thus, targeting of these significant proteins should be considered for focusing on MVL vaccines.

In an immunoproteomics study on antigenic extracts of *L. infantum* axenic amastigotes (Coelho et al. 2012), ATP-dependent RNA helicase (Barhoumi et al. 2006) and amastin (Nasereddin et al. 2010) were presented. These proteins are comparable to the tissue amastigotes. In addition, elongation factors, heat shock proteins such as HSP70, HSP83, and other chaperones, as well as tubulin and other housekeeping proteins, have been observed. These data are in the same line with those of other studies (Moreira and Murta 2016; Ramírez et al. 2013). In addition, to use these markers in the improvement of vaccines for MVL, by focusing on these proteins, we can reach more information about the biology of *L. infantum*. Thus, further studies are recommended to be done in this regard.

An investigation in Canada (El Fakhry et al. 2002) used comparative 2DE and MS to introduce proteins that are differentially presented in the amastigote of *L. infantum.* They identified two proteins, which belong to the isocitrate dehydrogenase, energetic metabolism pathways, and the glycolytic enzyme triosephosphate isomerase. Isocitrate dehydrogenase exists in the tricarboxylic acid cycle, a metabolic pathway in which acetate is oxidized to produce ATP. Triosephosphate isomerase is the other protein indicating a high presentation in amastigotes, which is a high current enzyme that roles as an essential duty in glycolysis (Kushawaha et al. 2012). The kinetic analysis illustrates that their activity and amount are more in amastigote in comparison to promastigote. For instance, Triosephosphate isomerase (TIM) activity in *L. infantum* amastigotes was twofold higher in comparison to *L. infantum* promastigotes, probably due to amastigotes need to high levels of TIM activity to make ATP via glycolysis within host cells (Bente et al. 2003). Generally, these enzymes have a crucial role in metabolism, survival, and pathogenicity of the amastigote in *L. infantum* so can be effective in designing vaccines for MVL.

Proposed targets for MVL vaccines using proteomics in the excretory-secretory antigens (ES) of *L. infantum* promastigotes

Nowadays, scientists believe that excretory-secretory antigens (ES) are appropriate sources for immune system stimulating and vaccines in MVL (Joshi et al. 2014; Lemesre et al. 2005; Petitdidier et al. 2016). Shotgun proteomics approaches revealed that the proteins in secretions of *L. infantum* promastigote are associated with the nucleotide metabolism, carbohydrate metabolism, antioxidant activity, protein degradation, heat-shock response, and

other processes. In this survey, the ES immunodominant proteins were checked with the sera of animals with clinical manifestation for canine visceral leishmaniasis (CVL). In addition to the new research avenues in MVL probable vaccines (Braga et al. 2014), these isolated proteins are important to check the immune responses and the pathology of *L. infantum.*

The results of another immunoproteomics study on soluble exogenous antigens (SEAgs) of *L. donovani* (Kumar et al. 2015) indicated that enolase, carboxypeptidase and activated protein kinase C receptor homolog deviate the immune responses to the Th1 cells; consequently, they are suitable targets for vaccine development. Also, heat-shock 70-related protein1, glucose-regulated protein 78 and heat-shock 70-kDa proteins have been introduced as the inducer of Th1 immune responses. The finding of these new targets may be our dream in case of VL vaccines comes true. Since the *L. donovani* and *L. infantum* are two agents of VL in different areas, we are convinced to focus on the results of this research to propose these targets as a vaccine for MVL in the future.

Proteomics and drug resistance and the targets that can be proposed for MVL vaccine

In a study, the mitochondrion of *L. infantum* was exposed to Antimony (SbIII) and Miltefosine (MIL) (Vincent et al. 2015). Although these drugs induce the cell death through the changes on the *Leishmania* mitochondrion, the parasite can be adapted and withstand these drugs by changes in the proteins' pattern. Generally, mitochondrial DNA adapts the parasite to the different nutritional condition and also helps the parasite to grow in different hosts. That is why; the results of these studies can guide us to target the special proteins in the mitochondrion. Thus, by evaluation of their immunogenicity, we can reach a new source of antigens for designing a vaccine against *Leishmania* spp. Also, Hide et al. (2008) studied the mitochondrial protein content of *L. infantum,* using proteomics. They present mitochondrial fraction with lowest levels of cytosolic contamination by performing fractionation with the focus and enrichment of mitochondrial proteins.

A comparative proteomics study on resistance and sensitive strains of *L. infantum* to the Amphotericin B (AmB) (Brotherton et al. 2014) showed that in the resistance mutant strain, up-regulation was seen in the proteins which belonged to the glycolysis and tricarboxylic acid cycle. Interestingly, up-regulation was observed in reactive oxygen species (ROS) scavenging and heat shock proteins in the resistant mutant. Knowing the protein profiles of sensitive and resistant strains of *L. infantum* can improve our information about the selection of appropriate targets for MVL vaccines.

Conclusion

As reviewed above, proteomics technique gives us a facility for identification of protein profiling of *Leishmania* parasites. After identification of protein targets, we should identify immunodominant proteins which provoke the immune responses against *L. infantum*. These introduced targets can be evaluated in Elisa, western blotting and in vivo to prove their immunostimulatory property for designing a vaccine.

It seems that most of the proteins that reviewed the candidates for VL vaccines are located in the metabolism pathway of *Leishmania* parasites. Additionally, some of these proteins are located in the other part of the parasite such as flagella. It proposes that for obtaining more appropriate and effective vaccine targets, 2DE and mass spectrometry should be performed on the special fraction of the parasite, not on the whole parasite lysate.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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