

# Estimation of vector competence of Moroccan Sand fly Populations Using a Proteomic Approach

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## Research

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# Abstract

**Background** Phlebotomine vector born disease are distributed throughout the world, and different pathogens are associated with varying degrees of disease severity. In Morocco, sand fly populations are incriminated in the transmission of several pathogens, such *Leishmania spp.* and phlebovirus, in particular the most relevant sand fly species as the case of *Phlebotomus papatasi*. This species is the main vector of *Leishmania major* in Morocco, and incriminated in phlebovirus transmission. Not to pass over without investigating its possible role on the transmission of entomopathogenic parasite already reported in literature.

**Methodology and finding** The present study focused on proteomic analysis in *Phlebotomus papatasi* from four localities in Morocco. Proteomic analysis in female *P. papatasi* was performed with Liquid chromatography-tandem mass spectrometry (LC-MS/MS). A total of 239 identified proteins for *Leishmania spp.*, 26 identified proteins for phlebovirus and 1132 identified proteins for nematode were detected in the 20 pools of *P. papatasi* from investigated localities. The microscopic examination of 1752 sandflies collected showed, the infection of two specimens of female's *P. sergenti* with Tetranematid *Didilia spp.* and potential Microfilariae in Imintanout locality (Z2).

**Conclusion** This study presents the first report of nematodes in sand flies of Africa. Also, the first use of proteomic tools for estimation of vector competence of *P. papatasi* which could be a specific tool to a better understanding of the vectorial capacity of Moroccan sandflies population.

## Introduction

Phlebotomine sandflies (*Diptera: Psychodidae, Phlebotominae*) are small, fragile, blood-sucking insect with a wide range of hosts [1], allowing the pathogen transmission to humans and other animals [2]. These are considered as vectors of various diseases notably canine and human leishmaniasis, bartonellosis and several arboviruses [3]. Morocco is one of the countries where leishmaniasis constitutes a major public health concern and sand fly species exist with a significant geographical spread [4]. Moroccan populations of sandflies, as the other Mediterranean region's populations, are not only vectors of protozoa, but also viruses [5].

*Phlebotomus papatasi* is widely distributed around the Mediterranean basin [6]. This species is very common and has a significant ecological plasticity [7-9]. It is incriminated in the transmission of cutaneous leishmaniasis (CL) and of sand fly fever that has been established in the Mediterranean regions [10]. Beside this, *P. papatasi* is the main vector of zoonotic leishmaniasis due to *L. major* in Morocco, the most abundant form in terms of number of cases [11].

The wide distribution range of *P. papatasi* extends across North Africa, through Eurasia, and into India, it appears to be highly dependent upon environmental conditions [12]. *Phlebotomus papatasi* associated with all CL to *L. major* endemic foci across the country in arid areas of Morocco [8]. *P. papatasi* is most

active in the hot, dry season and it was the most abundant species when ambient temperature is in the 32–36 °C range [13]. *P. papatasi* was found well adapted to arid climate conditions [14, 15].

Techniques as liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) become a powerful and effective approach to better understand complex and dynamic host-pathogen interactions at the protein level [16]. Evidently proteins play an important role in different molecular networks and perform many biochemical functions of organisms. Label-free liquid chromatography-mass spectrometry (LC-MS/MS) routinely quantifies and identifies thousands of proteins across multiple samples in a single run, providing an unprecedented opportunity to examine changes in the proteomics profile of a biological fraction or organism [17]. In the present study, we analyse four Moroccan populations of *P. papatasi* females in order to intend and improve the information available about this species in Morocco by the estimation of its vector competence in the transmission of several pathogens.

## Materials And Methods

### Sand fly collection and specimen treatment

Sandflies were collected using miniature CDC light traps (John W. Hook Co., Gainesville, FL, U.S.A.) between June 2018 and June 2019 from four localities in Morocco; three of them are endemic foci of leishmaniasis (Z1: Errachidia (31°56'52.6"N 4°25'47.7"W), Z2: Imintanout (31°10'18.1"N 8°51'02.4"W), Z3: Zagora (30°20'52.5"N 5°50'13.1"W)); and one non-endemic foci; NE: Marrakech (31°39'11.6"N 8°01'30.2"W)[18].

We used specimens trapped by CDC technique because it gives better specimens for the accurate proteomic analysis as sticky traps technique for collection could have affected the specimens and their proteomic analysis accuracy. [19].

A total of 1752 sandflies were collected and all specimens stored in 70% ethanol at – 20 °C in the Microbial Biotechnologies, Agrosiences and Environment Laboratory in Marrakech, Morocco until use.

Thereafter, analysis by LC MS/MS was carried out at the proteomic facility of the Research Institute of the McGill University Health Centre (RI-MUHC; Montréal, Canada). Fifteen specimens of females *Phlebotomus papatasi* (5 per pool) from each locality were used for proteomic analysis to detect proteins of medically important pathogens (*Leishmania spp.*, phlebovirus) and entomopathogenic parasite (Nematode) in sandflies.

The rest of collected sandflies were examined using a binocular microscope for the presence of potential nematodes species. The infected specimens were dissected to remove the nematode from the sand fly body and identified morphologically according to Moroccan sand fly Key [20]. Sandflies collected were conserved in 70% ethanol and transported to the RI-MUHC.

### Protein digestion with trypsin

The head and genitalia of the sand flies were mounted on slide and species identification were made using identification key [20]. A standard TCA protein precipitation was first performed to remove detergents from *P. papatasi* specimens. Protein extracts were then re-solubilized in 10  $\mu$ L of a 6M urea buffer. Proteins were reduced by adding 2.5  $\mu$ L of the reduction buffer (45 mM DTT, 100 mM ammonium bicarbonate) for 30 min at 37°C, and then alkylated by adding 2.5  $\mu$ L of the alkylation buffer (100 mM iodoacetamide, 100 mM ammonium bicarbonate) for 20 min at 24°C in dark. Prior to trypsin digestion, 20  $\mu$ L of de-ionized distilled water was added to reduce the urea concentration to 2M. Ten  $\mu$ L of the trypsin solution (5 ng/ $\mu$ L of trypsin sequencing grade from Promega, 50 mM ammonium bicarbonate) was added to each sample. Protein digestion was performed at 37°C for 18 h and stopped with 5  $\mu$ L of 5% formic acid. Protein digests were dried down in vacuum centrifuge and stored at -20 °C until LC-MS/MS analysis.

## LC-MS/MS

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed at the RI-MUHC proteomic facility as described by Atayde et al. 2019 [16]. Sample proteins were precipitated with 15% trichloroacetic acid (TCA)/acetone and digested with trypsin at a final concentration of 2 ng/ml. After an 18-hr incubation at 37C, the reactions were quenched by the addition of formic acid to a final concentration of 1% prior to the LC-MS/MS analysis. The LC column was a PicoFrit fused silica capillary column (New Objective) self-packed with C-18 reverse-phase material (Phenomenex). This column was installed on the Easy-nLC II system (Proxeon Biosystems) and coupled to the Q Exactive mass spectrometer (Thermo Fisher Scientific) equipped with a Proxeon nanoelectrospray Flex ion source. The buffers used for chromatography were 0.2% formic acid (buffer A) and 100% acetonitrile/0.2% formic acid (buffer B). Peptides were loaded on column at a flow rate of 600 nl/min and eluted with a two-slope gradient at a flow rate of 250 nl/min. Solvent B first increased from 2% to 40% in 85 min and then from 40% to 80% in 25 min. LC-MS/MS data were acquired using a data-dependent top 15 method and standard values were used for all the parameters of the mass spectrometer.

## Protein identification

The peak list files were generated with Proteome Discoverer (version 2.1) using the following parameters: minimum mass set to 500 Da, maximum mass set to 6000 Da, no grouping of MS/MS spectra, precursor charge set to auto, and minimum number of fragment ions set to 5. Protein database searching was performed with Mascot 2.6 (Matrix Science) against the Moroccan *Leishmania* species (*L. major*, *L. infantum*, *L. tropica*), Phleboviruses, Nematode protein databases. The mass tolerances for precursor and fragment ions were set to 10 ppm and 0.1 Da, respectively. Trypsin was used as the enzyme allowing for up to 1 missed cleavage. Cysteine carbamidomethylation was specified as a fixed modification and methionine oxidation as variable modifications. Data analysis was performed using Scaffold (version 4.10.0). Only proteins with minimum 3 peptides and peptide score higher than 20 were considered.

## Results

The study was conducted using 3 pools of 5 specimens of *P. papatasi* representing each four studied localities. We performed mass spectrometry (LC-MS/MS) to analyze the content of the purified Moroccan sand fly *P. papatasi* proteins. Pools were analyzed for the presence of proteins specific of *Leishmania spp.*, phlebovirus and nematode proteins. Besides, this analysis allows us to make differentiation of biomarkers peptides in *P. papatasi* populations between different localities. We compared the list of the identified proteins in different localities and found that the majority of the protein hits have been observed for all localities. As mentioned earlier, only proteins with high peptide and spectral counts have been chosen for the comparison (Table 1). Detailed spectrum and peptide report and sample of each locality are available in Additional file (S1). We identified different level of abundance for *P. papatasi* proteins, showing spot number, UniProt identifier, protein name, function annotated from BLAST similarity search (S1). All identified proteins of significance are involved with housekeeping functions, structural proteins of pathogen phlebotomine borne diseases, and entomophogenic proteins.

Our initial analysis has permitted to determine that nematode proteins detected by LC-MS/MS analysis were the most abundant with 1132 proteins identified, followed by *Leishmania spp.* and phlebovirus with 239 and 26 identified proteins respectively (Table 1 & Fig. 1).

**Table 1** Number of proteins identified and spectral count detected in Moroccan *P. papatasi*

Pathogen	Number of proteins identified	Number of spectral count
<i>L. infantum</i>	117	2520
<i>L. major</i>	108	2336
<i>L. tropica</i>	14	434
<i>Phlebovirus</i>	26	151
<i>Nematode</i>	1132	25061

### Identification of Specific *Leishmania spp.* Biomarker Proteins determined by Proteomics

*Leishmania spp.* peptides showed presence in different locality even in the non-endemic locality (NE) (Fig. 1). Comparing spectral counts for *Leishmania spp.* peptides from different localities studied, our results show a great significant difference for *L. tropica* proteins between different localities which is not for others *Leishmania spp.* By peptide group-based spectral count differentiation, the difference is significant ( $p$  value < 0.05) (figure 1). We describe *Leishmania* species determination on entomological samples based on partial sequencing of *Leishmania spp.* proteins such as the heat-shock protein 70 gene (hsp70), Alpha tubulin protein, Ubiquitin (Ubi) (Fig .2, Fig .3, and Fig. 4). Those proteins involved in a variety of cellular functions for *Leishmania spp.* We observed presence of *Leishmania spp.* biomarker proteins in *P. papatasi* in the different study sites even in the non-endemic area (Z4) (Fig .2, Fig .3, and Fig. 4). Some of interesting protein was detected is viscerotropic leishmaniasis antigen protein for *L. tropica*. This entity responsible for anthroponotic cutaneous leishmaniasis in Morocco [18].

## Identification of Specific Phlebovirus Biomarker Proteins determined by Proteomics

In regard to phlebovirus, the number of specific proteins according to locality is lower; we did not get many hits for them. We recorded some phlebovirus proteins such Sand fly fever virus (SFSV), Severe fever with Syndrome Virus (SFTSV) and Uukuniemi phlebovirus (UUKV).

## Identification of Specific Nematode Biomarker Proteins determined by Proteomics

Of interest, in all localities investigated during our study, we observed that nematodes and Onchoceridae proteins are the most abundant specific biomarker proteins in the sandflies, having high unique peptides identified and spectral count (Table 1) ( Fig.6, Fig. 7). However, direct observations by light microscopy of 1752 sandflies specimens collected from all localities revealed infection of only 2 specimens with entomopathogenic nematodes and potential filaria in Imintanout locality central area of Morocco (Fig. 8a & Fig. 8b). From morphological characteristic of those specimens, they are two females of *P. sergenti*. According to the body feature (body length was 3400  $\mu\text{m}$  and the body width 200  $\mu\text{m}$ ), size and egg diameter ( $26.4 \pm 2.2 \mu\text{m}$ ), the nematodes were identified as Tetradonematidae, *Didilia* species (Fig.9).

## Discussion

To estimate and study the vector competence of *Phlebotomus papatasi* in Morocco, we performed a proteomic analysis for Moroccan entities of *Leishmania spp.*, phleboviruses, as well as to check for the presence of entomopathogenic parasite in the different localities where the study was conducted. Proteomics methods have rarely been applied to compare vector competence of its natural populations of sandflies. Out of the 22 sandflies species, five species of the genus *Phlebotomus* are vectors of the three nosogeographic entities of leishmaniasis in Morocco: *Phlebotomus papatasi* proven vector of *L. major* [21], *P. ariasi*, *P. longicuspis* and *P. perniciosus* vectors of *L. infantum* [22], and *P. sergenti* proved vector of *L. tropica* [23]. Proteomics may improve our understanding of parasite biology and pathogenesis. On the other hand proteomic has also paved the way for the screening of pathogenic and entomopathogenic proteins of *Phlebotomus papatasi*. The analysis of the peptide digests of *P. papatasi* by LC-MS/MS resulted in a huge amount of information, and identified peptides with their corresponding proteins. Because peptide counts are not a reliable quantitative measure for sample comparison, we analyzed our proteomic data using the spectral count to provide a more efficient analysis [24]. Our results revealed that some specific biomarker proteins such Hsp-70,  $\alpha$ -tubulin and Ubiquitin of *L. infantum* and *L. tropica* proteins were present in *P. papatasi*. Those proteins have been investigated to permit the discrimination of medically important *Leishmania* species worldwide without the need for parasite isolation [25-28]. Among them, some proteins are highly conserved along the eukaryotic evolutionary tree and available on the genome sequences for several *Leishmania spp.* and important in genome organization of *Leishmania* species [29, 30]. The presence of all Moroccan *Leishmania* nosogeographic species proteins in *P. papatasi* could be explained by the overlapping of three *Leishmania spp.* in Morocco, which renders Moroccan leishmaniasis epidemiological profile not accurate. Effectively, *L. tropica* has been recorded in endemic *L. major* foci [31, 32]. Moreover, visceral (VL) leishmaniasis cases

have been found in established foci of zoonotic cutaneous leishmaniasis ZCL in Morocco [33]. Presence of viscerotropic *L. tropica* antigen protein could stem from the presence of zymodeme (*L. tropica*-279) responsible of canine VL in Morocco [34].

Moroccan populations of sandflies are not only vectors of protozoa, but also viruses. Es-Sette et al. (2015) identified the Toscana virus and its distribution in our country [5]. In central Morocco, Antibodies against the Naples virus and Sicilian virus have been observed in human populations living in this part of Morocco. Interestingly, antibodies of these two viruses were detected in areas where *P. papatasi* is present and abundant [35], raising the question of the potential incrimination of *P. papatasi* in the transmission of those viruses. Seemingly, our proteomic analysis for phlebovirus detection revealed a low number of identified peptides; for instance the polymerase SFSV protein recorded in Zagora (Z3) and Marrakech (NE). Also some emerging tick-borne virus [36-39] for instance; SFTSV glycoprotein detected in Imintanout (Z2), Zagora (Z3) and Marrakech (NE) and Errachidia (Z1), Zagora (Z3) and Marrakech (NE) for UUKV nucleocapsid protein were identified. Lower number of identified peptides could be due to RNA degradation while *P. papatasi* morphological identification was performed prior to proteomics analysis [40].

Unlike to what we found in our phlebovirus proteomic screening, a huge number of identified proteins were recorded for nematodes and *Onchocercidae* with a significant importance (Fig.1). Therefore, this finding lead us to microscopically monitor *P. papatasi* sandflies and other species of the different localities studied. In the past, entomoparasites of phlebotomine sandflies have been reported in the literature. In fact, Tetradonematid parasite nematodes were described to be found in the body cavity of phlebotomine sandflies in Pakistan, Saudi Arabia, Afghanistan and Portugal [41, 42]. However, they never been observed in Africa until now. This nematode was reported to infect *Luzomyia longipalpis* in a laboratory colony maintained at the National institute of Health in Bogota, Colombia [43]. AS well as to be detected in *P. sergenti* and *P. ariasi* in Portugal [44, 45]. Interestingly, *Onchocercidae* parasite has never been reported in sandflies. In our study the examination of 1752 sand fly specimens for nematodes and *Onchocercidae* presence from the fourth localities studied showed two observations of *P. sergenti* specimens infected by nematodes in Imintanout locality, which is known to be an endemic foci of *L. tropica*-mediated CL in Morocco [34]. From a morphological point of view the first nematode is a *Didilia spp* and the second is a potential microfilaria. This is the first observation of Tetranematid *Didilia spp.* in Africa and the first report of Microfilaria in sandflies.

Our results highlight two findings, included proteomics results *P. papatasi* and microscopic examination of sandflies. Firstly, the vector competences of Moroccan population of sandflies are lack estimated, as we see in ours study for *P. papatasi* main vector of *L. major* in Morocco [11]. Secondly, we found a high abundance of nematodes proteins in *P. papatasi* and we observed the first naturally infection by *Didilia spp.* and Microfilaria in female *P. sergenti* in Morocco. These observations open questions about the role of sandflies in the transmission cycle of these parasites in Morocco.

## Conclusions

In conclusion, the present study identified proteins for Moroccan nosogeographic entities of *Leishmania*, *phlebovirus* and *nematode* detected in wild caught of *P. papatasi*. *Microfilaria* and Tetranematid *Didilia spp.*, were identified in the same area where leishmaniasis occur. These results represent the first use of LC-MS/MS proteomics tool for estimation of vector competence of sand flies and the first detection of nematodes in African sand fly population.

Thus, more investigations on study of vector competence of Moroccan populations of sandflies are needed.

## Abbreviations

**L.:** *Leishmania*

**VL:** Visceral leishmaniasis

**CL:** Cutaneous leishmaniasis

**P:** *Phlebotomus*

**spp.:** Species

**LC-MS/MS:** Label-free liquid chromatography-mass spectrometry

**CDC:** Centers for Disease Control and Prevention

**TCA:** Trichloroacetic acid

**SFSV:** Sand fly fever virus

**SFTSV:** Severe fever with Syndrome Virus

**UUKV:** Uukuniemi phlebovirus

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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## Author's contribution

MO, AB, SB and MD were conceived the project, MD, GD, CM and SB were performing the experiment. MD, GD, CM, SB, HM, MO and AB were involved in data analysis writing and reviewing of the manuscript. All authors read and approved the final manuscript.

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## Figures

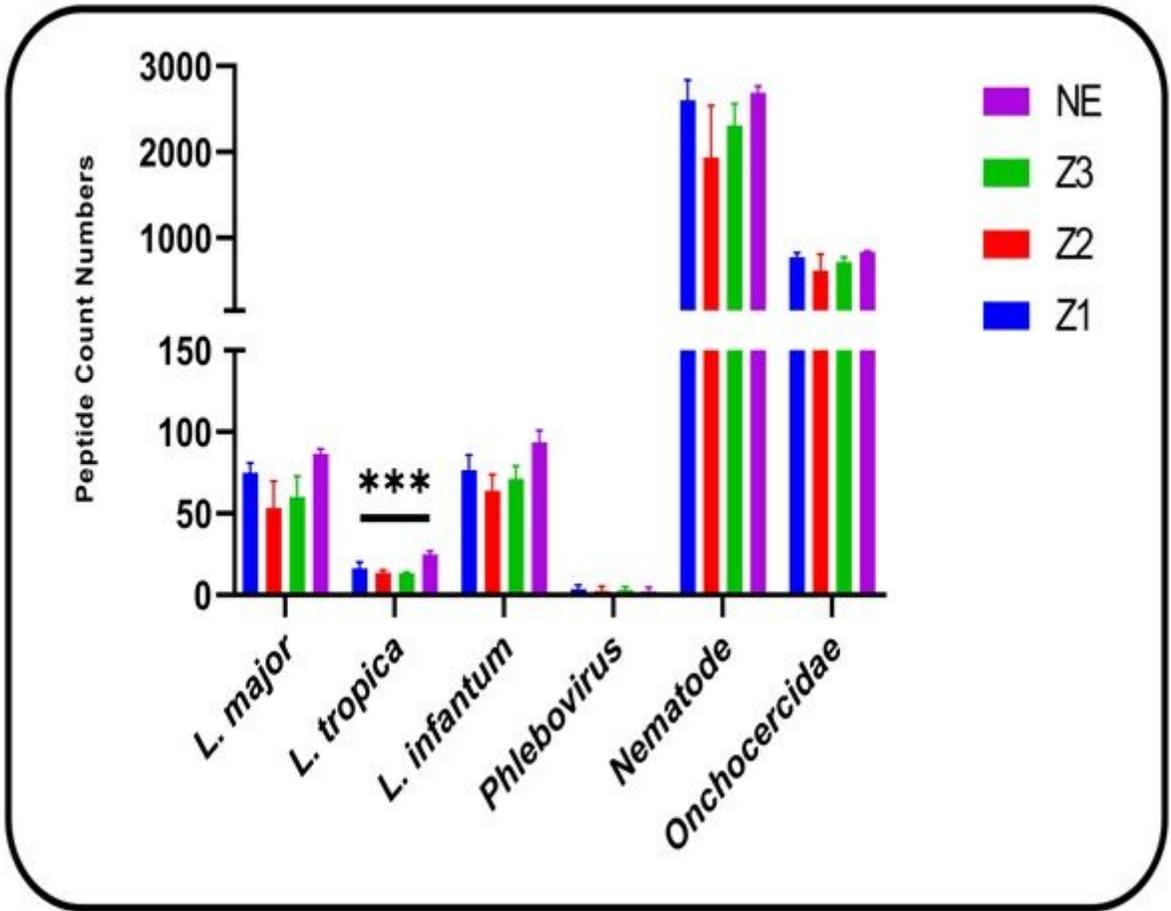


Figure 1

Number of peptide count of medically important pathogens (Leishmania and phlebovirus) and entomopathogenic parasite detected from *P. papatasi* in Morocco.

*Leishmania major*

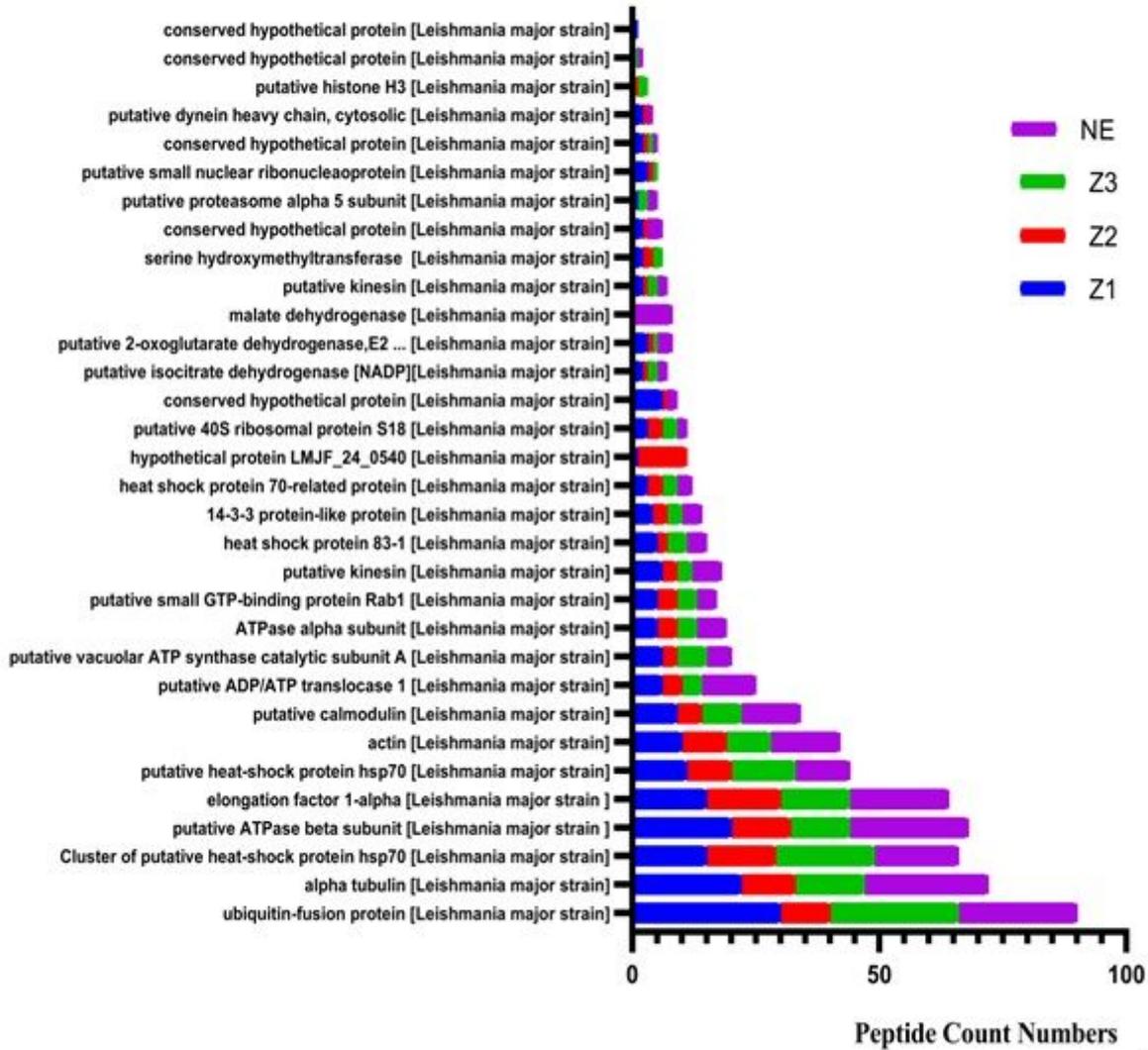
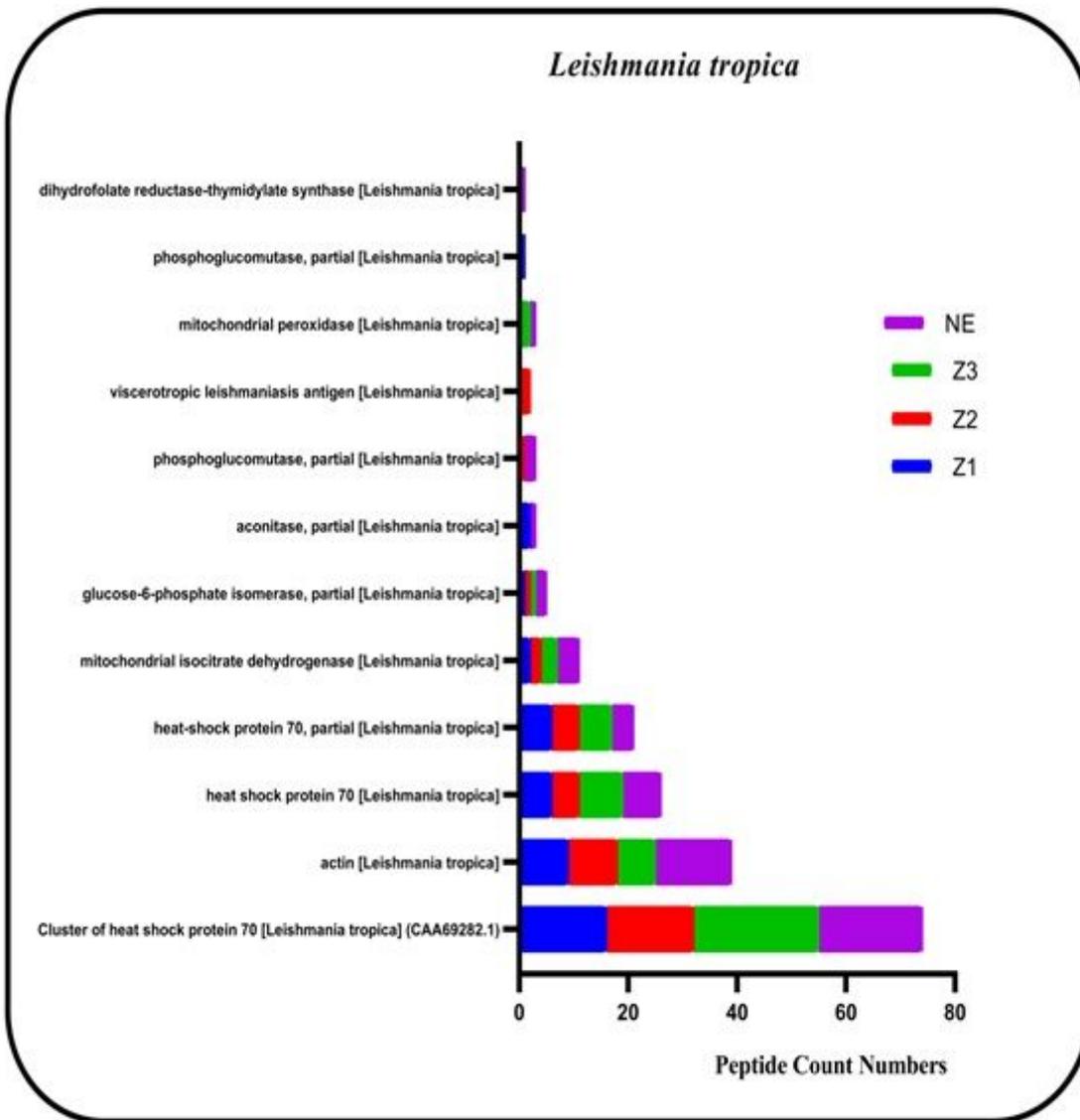


Figure 2

L. major proteins with high spectrum count numbers for P. papatasi.



**Figure 3**

*L. tropica* proteins with high spectrum count numbers for *P. papatasi*. Locality abbreviations: Z1, Errachidia; Z2, Imintanout; Z3, Zagora; NE, Marrakech

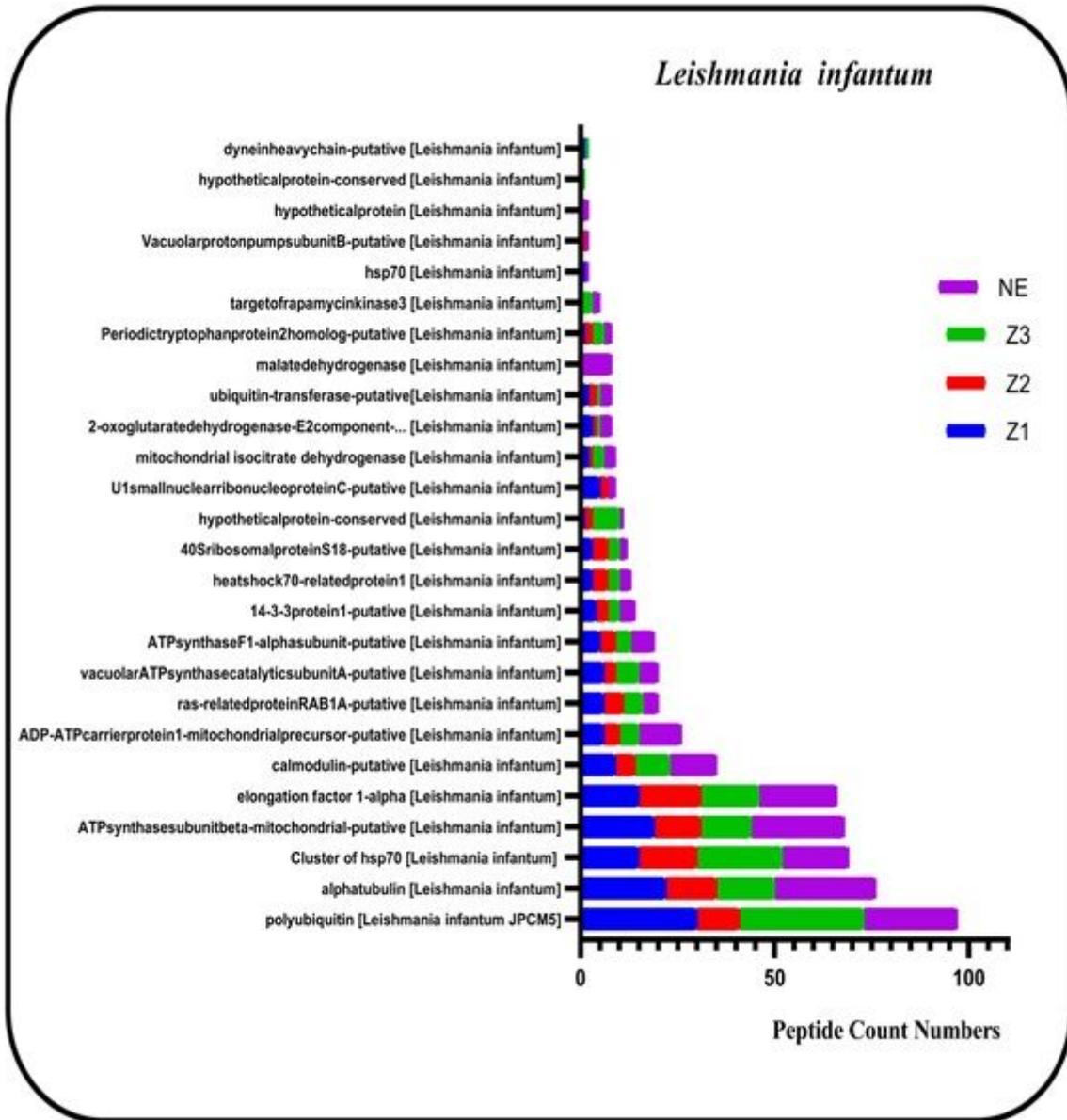
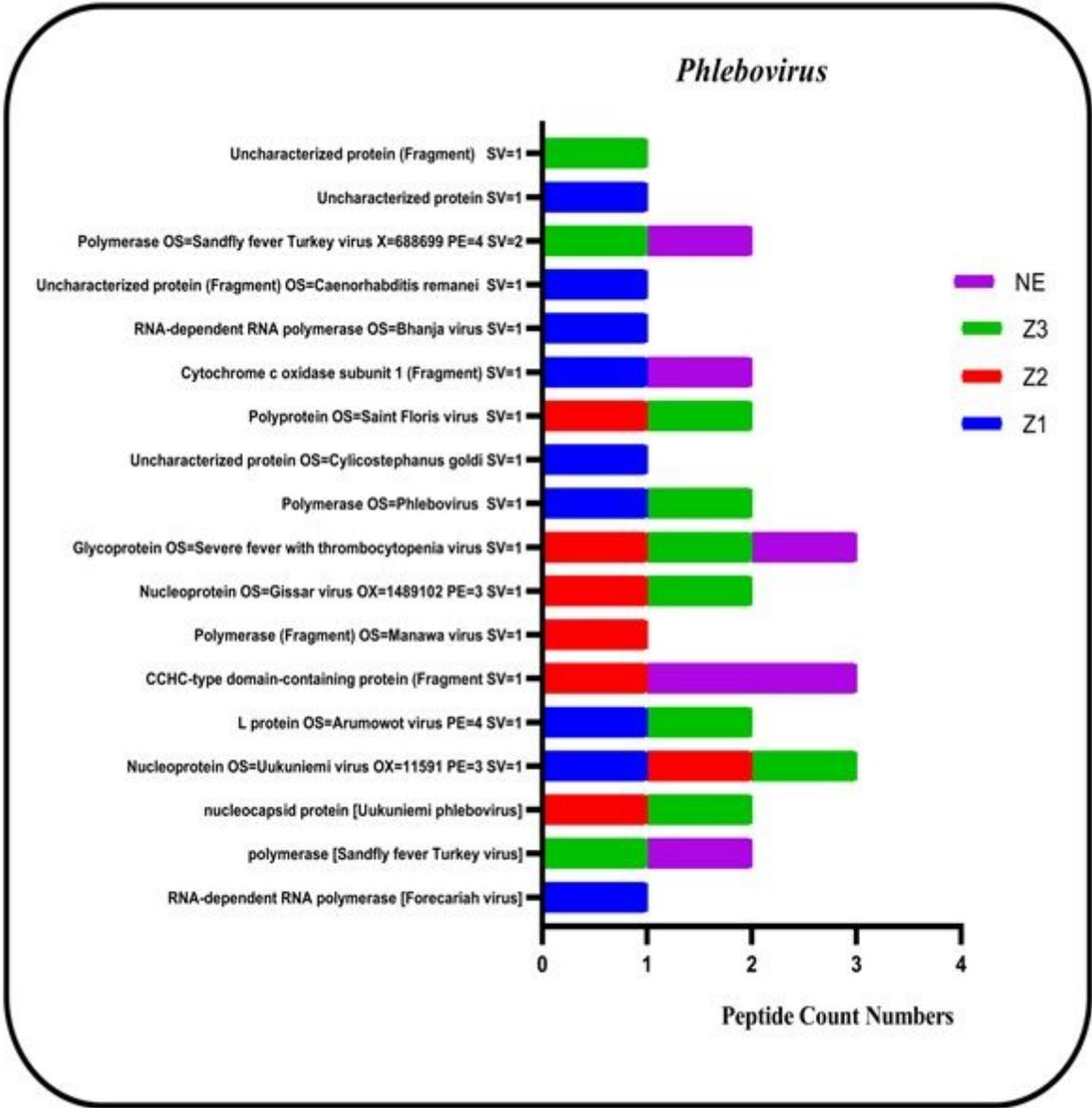


Figure 4

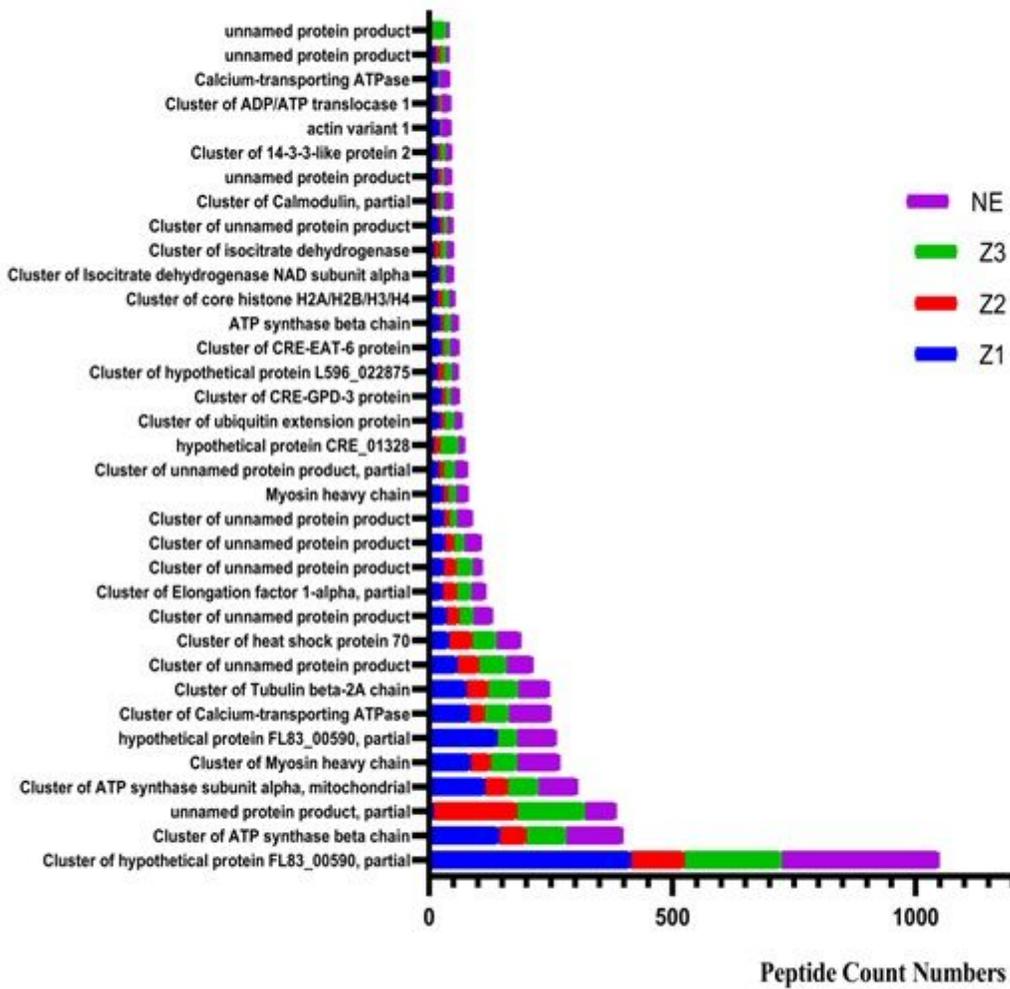
*L. infantum* proteins with high spectrum count numbers for *P. papatasi*. Locality abbreviations: Z1, Errachidia; Z2, Imintanout; Z3, Zagora; NE, Marrakech



**Figure 5**

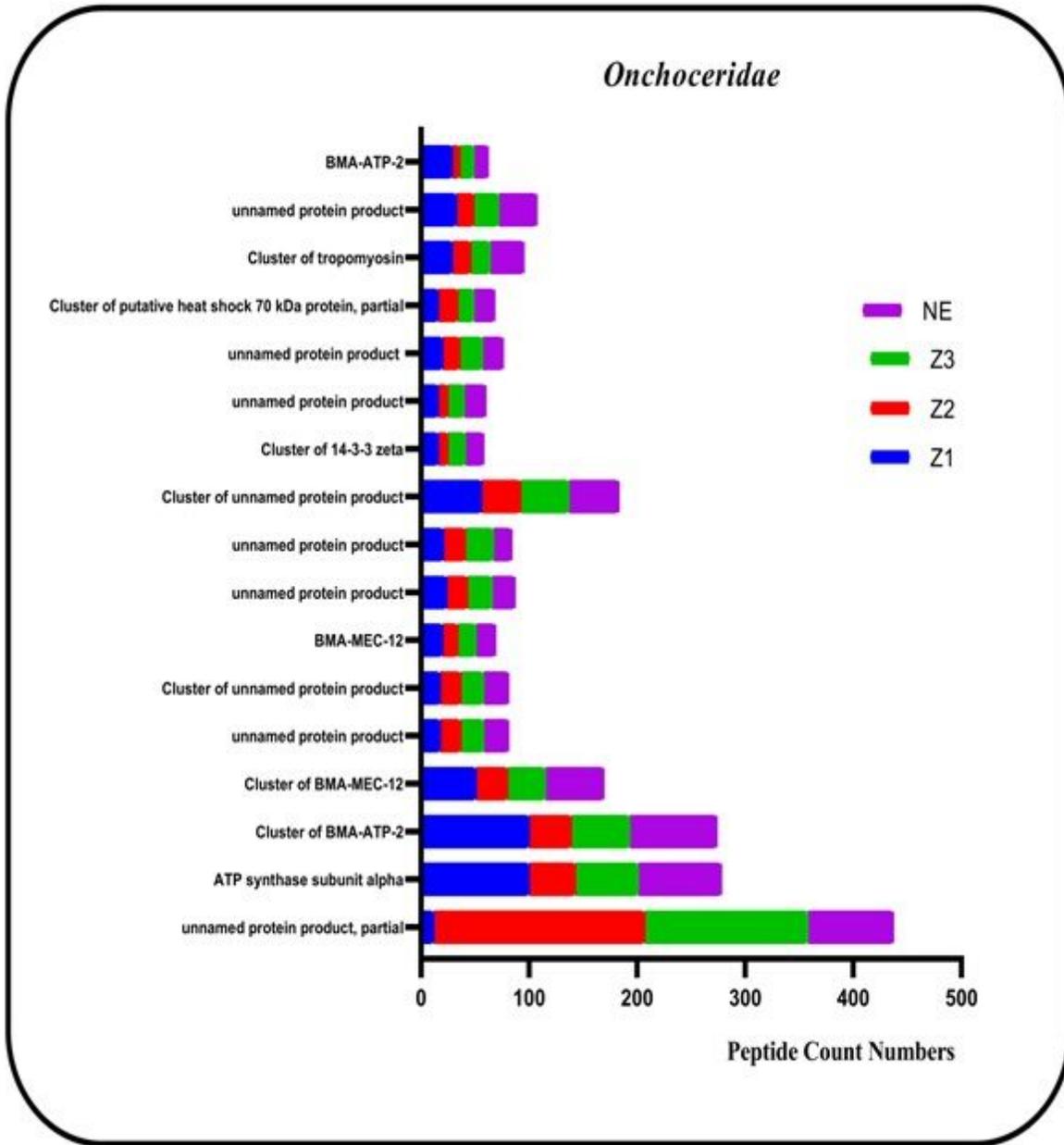
Phlebovirus proteins with high spectrum count numbers for *P. papatasi*. Locality abbreviations: Z1, Errachidia; Z2, Imintanout; Z3, Zagora; NE, Marrakech

## *Nematodes*



**Figure 6**

Nematodes proteins with high spectrum count numbers for *P. papatasi*. Locality abbreviations: Z1, Errachidia; Z2, Imintanout; Z3, Zagora; NE, Marrakech



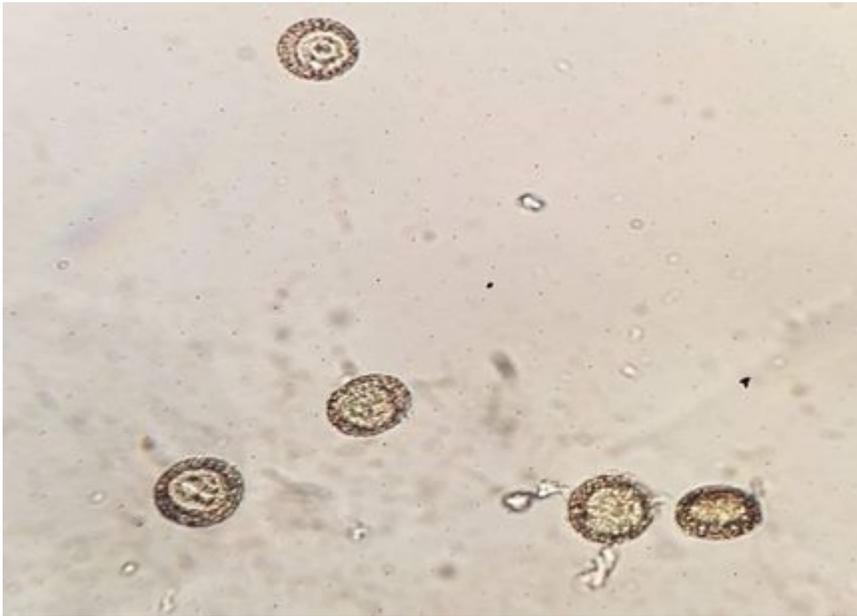
**Figure 7**

Onchoceridae proteins with higher number of spectrum count for *P. papatasi*. Abbreviations: Z1, Errachidia; Z2, Imintanout; Z3, Zagora; NE, Marrakech



**Figure 8**

Female of *P. sergenti* from Imintanout locality infected with a single female *Tetrakonematidea* (a) and *Microfilarea* (b)



**Figure 9**

Eggs of a *Tetrakonematidae* isolated from *P. sergenti* Morocco

## Supplementary Files

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