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Background  
The burden of parasitic diseases appears to be increasing throughout the world. This is due to climatic changes, human activities, population movement and globalization but also to the development of drug resistance by parasites and insecticide resistance by important parasite vectors such as mosquitoes and sandflies.  
During the last years the Parasitology lab’s research interests are focused on the Protozoan parasites *Leishmania* and *Toxoplasma* and the epidemiology of a number of important zoonoses using GIS technology, in Greece and Cyprus.

A. *Leishmania*. Is a protozoan parasite causing leishmaniases, which are vector-borne diseases. They have been endemic in southern Europe for centuries using phlebotomine sandflies (genus *Phlebotomus*) as vectors. In Greece two species of Leishmania are found: *L. infantum*, responsible for zoonotic visceral leishmaniasis in humans (VL) and canine leishmaniasis in dogs (which play the role of reservoir host for *L. infantum*) (CanL) and the anthropoontic *L. tropica* causing cutaneous leishmaniasis (CL) in humans. The most virulent and most drug resistant species, the anthropoontic *L. donovani*, was recently introduced in Cyprus (and Europe)(Antoniou et al., 2008), causing both VL and CL. It is widely accepted that the leishmaniases are dynamic diseases. As the conditions of transmission change (environmental, demographic, human behavior and health condition),
epidemiological studies and control measures to safeguard public health are vital.

A1. Epidemiology of Leishmaniasis: It was necessary to carry out a study to realize the spread of endemic \textit{L. infantum} and the risk of introduction of non endemic \textit{Leishmania} species to Greece and Cyprus. The work was done in collaboration with medical doctors and veterinarians from the two countries and the use of available data from KEELPNO regarding human cases. Serological results of dogs and human cases were mapped using GIS technology to assess the geographical distribution of the disease and its vectors. Isolation and typing of the parasite from humans and dogs revealed the \textit{Leishmania} species and zymodemes present in the two countries. Typing of the isolates was done using the newly developed K26-PCR assay (which is capable of discriminating species/subspecies of the \textit{L donovani} complex, based on the amplicon size (Haralambous et al., 2007) and multilocus enzyme electrophoresis (Rioux et al., 1990).

![Figure 1, 2, 3. Geographical distribution of human leishmaniasis cases in Greece and Cyprus in relation to percent dog seropositivity, \textit{Leishmania} spp., and zymodemes as well as vector species distribution. (Ntais et al., 2013, 2014, Mazeris et al., 2010).](image)

An important aspect of the epidemiology of vector born diseases is the vector biology and its geographical distribution. In the case of the leishmaniases, sandflies play the role of vectors. Nine proven, or potential, vector species (\textit{Phlebotomus ariasi}, \textit{P. perniciosus}, \textit{P. perfiliewi}, \textit{P. neglectus}, \textit{P. tobbi}, \textit{P. kandelaki}, \textit{P. balcanicus}, \textit{P. papatasi} and \textit{P. sergenti}) are indigenous in Europe six of which are found in Greece and/or Cyprus. The seasonal dynamics of important \textit{Phlebotomus spp} have being studied by our laboratory in Crete and Cyprus (Alt\textit{en} et al., 2015, 2016) to reveal the climatological conditions that favour vector activity and so the risk of infection. The taxonomy of \textit{Phlebotomus spp} using morphological and
molecular tools has/is also being studied. A new method is under development, with the collaboration of the Department of Parasitology, Faculty of Science, Charles University in Prague, for the identification of sandfly species by MALDI-TOF MS. This method was used for the first time on sandflies and proved successful and promising (Dvorak et al., 2014).

Figure 4. MALDI-TOF MS protein profiles of five different species of the genus *Phlebotomus* showing species-unique peaks for conclusive species identification. The peaks in the mass spectra represent peptides or small proteins obtained from the sand fly bodies using acidic extraction (Dvorak et al., 2014).

A. 2. *Leishmania* biology. Macrophages engulf and destroy invading pathogens in order to protect the host. However, a number of microbes has succeeded not only to avoid death within the macrophage but to develop different strategies in order to use the macrophage as a host cell for survival and protection against the host immune system. Often, however, a simultaneous infection by more than one microbe may occur in a single cell. *Leishmania* and *Toxoplasma*, both obligate intracellular protozoan parasites of man and animals, use macrophages as host cells. A first study the coinfection of human and mouse macrophages with these parasites, *in vitro* and *in vivo*, was carried out in order to understand the biology of these parasites and the pathology they cause in multiple infections which may help in the development of prophylaxis and treatment (Christodoulou et al., 2011).
**Figure 5.** Double infection of macrophages (THP-1 cell series) with *Leishmania infantum* and *Toxoplasma gondii*. The parasites were stained with double or triple immunofluorescence staining and examined by confocal laser fluorescence microscopy. *L. infantum*: green sign *T. gondii*: red sign cellular membranes: blue sign (Christodoulou et al., 2011).

*Leishmania* uses Phlebotomine sandflies as vectors where the promastigote stage develops, reproduces and becomes infective. Therefore the reproductive power of the promastigotes determines the inoculum size of the isolate. Ten *Leishmania* strains from Cyprus: two *L. donovani* and eight *L. infantum* were used to study the proliferation capacity of the promastigotes *in vitro*. Population increase during a 6-day culture period was assessed quantitatively and qualitatively by following the division history of each population during the same period by CFSE staining and flow cytometry. The strains exhibited different proliferation rates which may represent their fitness capabilities and their ability to synchronize the multiplication activity of individual members in the population for the production of a sizeable inoculum in time for the vector’s blood meal (Messaritakis et al., 2010).

**Fig. 6.** Single parameter overlaid histograms of CFSE fluorescence intensity of promastigotes as they migrated from channels 1–5 during the 6-day culture period, indicating the on-going cell divisions (Messaritakis et al., 2010).
*Leishmania* lives within the lysosome of the phagocytic immune cells inactivating the enzymes contained. The ability of an isolate to survive within the macrophage and its rate of multiplication in this environment is an important factor determining the infectivity potential of the isolate and the manifestation of the disease. This capacity of the parasite is measured as the percentage of infected cells and the mean value of parasites per cell. The infectivity potential, of clinical isolates of *L. infantum* infecting THP-1 cells *in vitro*, was studied by flow cytometry and light microscopy. The percentages of cells in a sample containing a specific number of parasites, as recorded by light microscopy, were used in flow cytometry to manually gate the mean fluorescence intensity which corresponded to the percentage of cells with that number of parasites. The gating obtained, was then used as a “standard reference curve” to evaluate results by flow cytometry compared to those obtained by light microscopy. The results showed that flow cytometry can be used as a rapid, cost effective, easy and reproducible method to study the infectivity potential of isolates, either in biological, epidemiological, or clinical tests, particularly for the assessment of drug efficiency trials (Kanellopoulos et al., 2014).

![Flow cytometry results for isolate GD1. All x axes indicate the mean fluorescence intensity (MFI) of the cells and the y axes the number of cells. THP-1 cells, not infected with Leishmania amastigotes (control), are given in histogram A1. The overall infectivity results obtained by FC are shown in A2, where the percentage of non-infected cells is 80.6% and the percentage of cells containing 1-2, 3-4, 5-6, 7+ parasites per cell is estimated according to the MFI of the cells. Histograms in Box B, of A2 and A3, show the percentages of infected THP-1 macrophages with: 1-2, 3-4, 5-6, 7+ parasites per cell; in A2 as the overall percentage of the infected cells in the population (indicated by different colors) and in A3 as gates of the percentages of infected cells with the different numbers of amastigotes per cell (taking into](image)
A. 3. An important goal of the parasitology laboratory is the study of *Leishmania*'s resistance to drugs. Resistance of pathogens to drugs is a growing concern regarding many diseases. Parasites like Leishmania, Plasmodium and Entamoeba histolytica; and neoplastic cells, present the multidrug-resistant phenotype rendering chemotherapy ineffective. The acquired resistance of *Leishmania* to antimony has generated intense research on the mechanisms involved but the question has not yet been resolved. To test the hypothesis that drug efflux in *Leishmania*, as measured by flow cytometry using the fluorescent dye Rhodamine-123, is largely dependent on the number of efflux pumps an isolate can express, the amount of Pgp 170 molecules was assessed in ten field isolates (5 “resistant” and 5 “susceptible”) using: Western Blotting, Confocal and Transmission Electron Microscopy, and proteomics. Their survival after exposure to three antileishmanial drugs, in vitro, was also evaluated and clinical data were compared to the in vitro results. The MDR gene, expressing the transmembrane efflux pump Pgp 170, appears to play a key role in the phenomenon of drug resistance. When “susceptible” versus “resistant” parasites were compared, it was shown that the higher the number of Pgp 170 molecules the higher the Rhodamine-123 efflux from the parasite body and, when exposed to the drug, the number of efflux pumps increased. However, the rate of this increase was not linear and it is possible that there is a maximum number of Pgp 170 molecules an isolate can express. Nevertheless, the phenomenon is a complex one and other factors and proteins are involved like the HSP-70 group proteins, detected in the “resistant” isolates, which may play a significant role (Messaritakis et al., 2013).

![Figure 8. Rhod-123 efflux in *Leishmania* promastigotes observed by Flow Cytometry. High Rhod-123 efflux in the “resistant” dog isolate D5 (a). Low...](image-url)
Rhod-123 efflux in the “susceptible” dog isolate D1 (b). Measurements were taken every 30 minutes, for two hours (Messaritakis et al., 2013).

Figure 9. Detection of Pgp 170 molecules, by Transmission Electron Microscopy, after immunogold labeling. *Leishmania* amastigote (inside a THP-1 infected cell): cytoplasm of the THP-1 cell (a); *Leishmania infantum* body (b). Black spots (as indicated by white arrow) show Pgp 170 molecules (gold granules after immunogold labeling using the C219 monoclonal antibody) (Messaritakis et al., 2013).

Figure 10. Number of Pgp molecules in a “resistant” and a “susceptible” isolate before/after exposure to Glucantime. The number of Pgp 170 molecules was found in higher numbers in the “resistant” compared to the
“susceptible” isolate. This number increased, in both isolates, after exposure to Glucantime (Messaritakis et al., 2013).

Although resistance to drugs is a complex phenomenon, the rate of efflux of the fluorescent dye Rhodamine-123 from the parasite body, using flow cytometry, is an indication of the isolate’s ability to efflux the drug thus avoiding death. After confirming this statement with the previous work, the rate of efflux of 275 Leishmania strains, isolated from patients and dogs from Greece and Cyprus, was measured and mapped to study the geographical distribution of the MDR 1 expression as an indication of the drug resistance of the parasite. The map showed that out of the seven prefectures, where dogs presented high efflux rates, five also had patients with high efflux rates. In one prefecture the highest number of isolates with efflux slope “α” >1, in both human and dog isolates, was found; a fact which may suggest that spread of drug resistance is taking place. The virulence of the Leishmania strains, assessed after infecting THP-1 human macrophages, fluctuated from 1% to 59.3% with only 2.5% of the isolates showing infectivity > 50%. The most virulent strains were isolated from Attica and Crete (Tsirigotakis et al., 2016).

Figure 11. The geographical distribution of MDR 1 gene expression in 275 Leishmania strains, isolated from patients and dogs from Greece and
Cyprus, is shown. It was measured by the Rhodamine-123 efflux potential of the isolates, using flow cytometry. The isolates were characterized as of high or low efflux potential (slope “α” >1, or low slope “α” < 1, respectively) and mapped using the geographical information system software (GIS, Redlands, CA; ArcGIS 10) (Tsirigotakis et al., 2016).

A. 4. Leishmania, vaccine trials on dogs
A study, conducted in collaboration with the Universities of Warwick and Cambridge, UK, aimed to conduct safety and immunogenicity trials of a DNA/Modified vaccinia virus Ankara vaccine expressing recombinant Leishmania DNA encoding TRYP, in the natural reservoir host of Leishmania infantum, the dog. A cohort of 22 uninfected outbred dogs were vaccinated and followed-up for 4 months post-vaccination. The vaccine was shown to be safe and showed no clinical side effects. TRYP vaccinated dogs demonstrated significantly higher TRYP specific total IgG and IgG2 subtype titres than in controls, and positive in vivo intradermal reactions at day 156 in the absence of natural infection (Carson et al., 2009a, b).

B. 1. Toxoplasma epidemiology and risk in pregnancy.
The objective of this work was to study the incidence of toxoplasmosis in pregnant women in Crete and Cyprus.
In Crete: 5532 pregnant women were screened serologically over a period of 5 years and followed until delivery according to the designed protocol. Cases with suspected acute toxoplasmosis, at risk of delivering congenitally infected infants, were followed in collaboration with the gynecologists and after delivery the absence of congenital infection in infants was confirmed by serology and clinical evaluation. The developed protocol allowed differentiation between acute and latent toxoplasmosis, safe management of the cases at risk and assisted in avoidance of unwarranted pregnancy terminations (Antoniou et al., 2004, Galanakis et al., 2007).
Fig. 12. Protocol for serologic monitoring of pregnant women and handling those at risk of delivering congenitally infected infants (Antoniou et al., 2004). At the same time, the prevalent genotypes of Toxoplasma gondii parasites isolated from pregnant women and patients from Crete and Cyprus were typed (Messaritakis et al., 2008).

In Cyprus: The prevalence of toxoplasmosis in Cyprus was studied for the first time: in young women (16-18 years of age), in pregnant women and in sheep and goats. The aim was to assess the prevalence of the disease and the associated risk factors in order to propose measures to avoid infection especially for the young generation (Liassides et al., submitted for publication).

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