Geographical Distribution of MDR1 Expression in *Leishmania* Isolates, from Greece and Cyprus, Measured by the Rhodamine-123 Efflux Potential of the Isolates, Using Flow Cytometry

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Abstract. Leishmaniasis, a neglected vector-borne disease caused by the protozoan parasite *Leishmania*, is encountered in 98 countries causing serious concerns to public health. The most alarming is the development of parasite drug resistance, a phenomenon increasingly encountered in the field rendering chemotherapy ineffective. 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. The rate of efflux measured 275 *Leishmania* strains, isolated from patients and dogs from Greece and Cyprus, was measured and mapped to study the geographical distribution of the multidrug resistance (MDR) gene 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, out of the 59 prefectures studied, the highest number of isolates with efflux slope $\alpha > 1$, 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 human macrophages of the THP-1 cell line, 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.

INTRODUCTION

In the last 35 years leishmaniasis in Greece is spreading, with human and dog cases encountered in 41 and 43 out of the 54 prefectures, respectively.^{1–3} Two species of *Leishmania* (Kinetoplastida: Trypanosomatidae) are the causative agents of the disease, *Leishmania infantum* (zymodemes MON-1 and MON-98) responsible for visceral (VL) and canine leishmaniasis (CanL) and *Leishmania tropica* (MON-300, MON-58, and MON-57) responsible for cutaneous leishmaniasis (CL).^{1,4} The Republic of Cyprus, on the other hand, presents an unusual situation in which two distinct leishmaniasis transmission cycles run in parallel: in dogs with *L. infantum* (MON-1 and MON-98) and in humans with the newly introduced *Leishmania donovani* (MON-37).^{5–7} Natural transmission of the parasite may be zoonotic (*L. infantum*) using the dog as reservoir host or anthroponotic (*L. tropica* and *L. donovani*), and it takes place by the bite of insect vectors and blood-sucking phlebotomine sandflies. Although leishmaniasis is spreading geographically^{8–11} favored by the changing conditions, constituting a serious public health threat, the development of parasite resistance to drugs presents an alarming situation.

Leishmania, *Plasmodium*, *Entamoeba histolytica*, and neoplastic cells present the multidrug-resistant phenotype.^{12–15} The multidrug resistance (MDR) gene expresses a transmembrane efflux pump (the P-glycoprotein molecule, Pgp 170^{16–18}), which appears to play a key role in the phenomenon of drug resistance.^{15,18} Control of the disease relies principally on chemotherapy, both in patients and dogs,¹⁹ and antimony has been the main drug to combat the disease in the

last 65 years. Antimony-resistant *Leishmania* strains have been reported from many endemic areas, worldwide, reaching epidemic proportions in the state of Bihar, India^{20,21} and the phenomenon appears to be intensifying and geographically spreading. To safeguard public health, there is an urgent need not only to identify what *Leishmania* species and vectors are present in an area but also to follow their drug resistance to assess the risk of leishmaniasis and to administer the right drugs and drug regimens to avoid the development of resistant parasites.

It is possible to evaluate the drug resistance of an isolate using flow cytometry (FCM), since the rate of efflux of the fluorescent dye Rhodamine-123 (Rhod-123), an established substrate for Pgp 170, is largely dependent on the number of efflux pumps an isolate can express.^{15,18} This pump is effective for a diverse group of lipophilic compounds^{15–17} and the higher the number of the Pgp 170 molecules on the cell membrane, the greater the ability of the isolate to expel the compound from its body.^{14,18,22–24}

Our aim was to evaluate the MDR1 expression of *Leishmania* in Greece and Cyprus and present it geographically to assess the distribution of drug resistance and give the clinicians the knowledge required for effective treatment, which will safeguard against the expansion of this phenomenon. For this purpose, the rate of efflux of Rhod-123 of 211 *Leishmania* strains isolated from patients and dogs in Greece and of 64 strains isolated from patients and dogs in Cyprus were evaluated using FCM. The isolates were placed into two groups, depending on their rate of efflux: 1) < 1 , 2) > 1 and mapped, according to their characterization, to evaluate *Leishmania* drug resistance in each prefecture. Various factors were examined, statistically, to identify their association to the drug efflux rate of the isolates studied.

MATERIALS AND METHODS

Parasites. Two hundred and seventy-five *Leishmania* strains, isolated from patients (45 *L. infantum* and two *L. tropica*

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from Greece; five *L. donovani* from Cyprus) and from dogs (163 *L. infantum* and one *L. tropica* from Greece; 59 *L. infantum* from Cyprus) were used. Freshly thawed parasites (exponential phase promastigotes), which had undergone two passages before been frozen at -80°C , were used in all experiments. They were maintained in supplemented RPMI 1640 culture medium containing 25 mM HEPES buffer, supplemented with 2 mM glutamine (GIBCO Invitrogen, USA), 10% heat-inactivated fetal bovine serum (GIBCO Invitrogen, Grand Island, NY), 100 IU/mL penicillin, 100 g/mL streptomycin (Roche Diagnostics, Indianapolis, IN), and 5% filtered human urine²⁵ at $26 \pm 1^{\circ}\text{C}$.²⁶

Serological testing of patients and dogs by immunofluorescence. Patient and dog sera, from which the parasites were isolated were tested serologically using anti-human or anti-dog anti-immunoglobulin G antibodies, respectively, by an indirect immunofluorescent antibody test (*Leishmania* SPOT IF, bioMérieux, Marcy-l'Étoile, France). A series of 2-fold serum dilutions, starting from 1/100 for patient and 1/40 for dog sera were performed.^{1,27}

Study of the rate of efflux of Rhod-123, using FCM. The rate of efflux of the fluorescent probe Rhod-123 (Sigma-Aldrich Inc., St. Louis, MO) was tested in all 275 isolates with and without verapamil hydrochloride (blocker of MDR to check the specificity of the pumps) using FCM (Beckman Coulter Epics Elite Flow Cytometer, equipped with argon-ion laser tuned to 630 nm), as described in Messaritakis and others.¹⁸ The mean fluorescence intensity (MFI) of 10,000 events was measured every 30 minutes, for 2 hours, in triplicates, to follow the rate of Rhod-123 efflux for each isolate. Measurements were considered only if the percentage of dead parasites in a run was $< 5\%$. Data analysis was performed using the Kaluza software (2000–2015 Beckman Coulter, Inc., Nyon, Switzerland). Each isolate was characterized by measuring MFI changes in time (the rate of efflux; slope α), in the promastigote stage and placed into two groups, accordingly: slope $\alpha < 1$; slope $\alpha > 1$.

Study of the virulence of the strains using THP-1 human macrophages. Freshly thawed cell cultures of the human monocytic cell line, THP-1 (Sigma-Aldrich, Inc.) were maintained in supplemented RPMI 1640 culture medium at 37°C , 5% CO_2 , and 80% humidity.^{28,29} They were infected with exponential phase *L. infantum* or *L. donovani* or *L. tropica* promastigotes of each of the 275 isolates at a ratio of five parasites to one host cell, in triplicates. They were then incubated overnight at 37°C , 5% CO_2 , and 80% humidity. The cultures were used for infection if dead cells were less than 5%, using the trypan blue exclusion assay.³⁰

Following overnight incubation, cytospin preparations were made using 100 μL from each infected cell culture, in triplicates. The slides were left to dry at room temperature before staining with Diff-Quick (Polysciences Europe GmbH, Eppelheim, Germany). The percentage of infected macrophages was determined microscopically at $\times 1,000$ magnification, performing three counts of 100 cells for each cytospin preparation to obtain the average. The infected THP-1 cells were assessed for overall percentage of infected cells and the percentage of cells infected with 0-1-2-3-4-5-6-7 + amastigotes, so that the mean number of parasites per cell for each sample could be estimated.³¹

Statistical analysis and mapping of the results. The rate of efflux of each isolate (slope α) was statistically related to the

origin of the isolate (dog/human), geographical origin, clinical symptoms, human/dog seropositivity, the original amount of the dye influxed (MFI on baseline), and isolate's virulence using IBM SPSS v22 software, Armonk, NY.^{32–34} No statistical analysis was performed for slope α on different *Leishmania* species due to the small number of isolates other than *L. infantum*.

Virulence of the isolates was categorized into two groups ($< 25\%$ and $> 25\%$), and the Mann–Whitney *U* test was used to test statistically significant differences between slope α in the two groups both in humans and dogs, the origin of the isolates (dog/human), and the presence of clinical symptoms. Human and dog seropositivity were categorized into three groups (A: 0–1/200, B: 1/400–1/1,600, C: $> 1/1,600$ and A: 0–1/160, B: 1/320–1/2,560, C: $> 1/2,560$, respectively) to test for statistically important differences between slope α and host seropositivity. A 5% significance level was chosen throughout the study.

To evaluate drug resistance of *Leishmania* isolates in each prefecture, the isolates were mapped using the geographical information system software (ArcGIS 10; GIS, Redlands, CA).

Ethical considerations. Collection and isolation of the parasites from Greece and Cyprus has been described previously.^{1,5} In all experimental trials, the samples used were anonymized. The experiments and procedures described have been approved by the Institutional Animal Care and Use Committee of the University of Crete Medical School and conform with the European Union Directive 2010/63/EU regarding use of animals and biological specimens in research, as well as the relevant Hellenic legislation (Presidential Decree 160/91, under the Code Numbers 31 EE 05, 31 EHP 04, and 31EPI 020). Written informed consent was obtained from the patients involved and the dog owners, according to the aforementioned national legislations.

RESULTS

Rate of efflux (slope α) of the isolates. Efflux appeared to be blocked by verapamil hydrochloride in the 275 isolates ($P < 0.05$), and influx (MFI on baseline) was found to be correlated to the rate of efflux, both in the presence and absence of verapamil hydrochloride ($P < 0.01$ in both cases).

FCM assays showed the rate of Rhod-123 efflux from the parasite body (slope α) to vary from -0.01 to -6.0 between the 275 isolates. Please note that, from now on, slope α values will be presented without the negative sign, for convenience. The highest rate was presented by two isolates from Cyprus: an *L. donovani* strain (slope $\alpha = 6.0$), isolated from a 9-month-old girl from Paphos with VL symptoms³⁵ and an *L. infantum* strain, isolated from a dog (slope $\alpha = 4.26$) with severe CanL symptoms from Paphos, whereas the lowest was found in a human isolate from Attica (slope $\alpha = 0.01$) and in a dog isolate from Central Greece (slope $\alpha = 0.02$). The majority of the isolates (80%) presented an efflux rate between 0.01 and 1.14. Overall, *L. donovani* isolates presented a greater slope α than *L. infantum* and *L. tropica* (Table 1), but this cannot be statistically supported due to the small number of *L. donovani* and *L. tropica* isolates available. No statistical difference in slope α was found between parasites isolated from patients or dogs, the presence of clinical symptoms in the hosts, or their serological profile.

TABLE 1

The MDR1 gene expression in 275 *Leishmania* strains, isolated from patients and dogs from Greece and Cyprus, measured by the Rhodamine-123 efflux potential of the isolates using flow cytometry

	Species	Slope $\alpha < 1$	Slope $\alpha > 1$	Total
No. of isolates from dog origin	<i>Leishmania infantum</i>	Greece: 154	Greece: 9 (5.5%)	163
		Cyprus: 43	Cyprus: 16 (27.1%)	59
	<i>Leishmania tropica</i>	Greece: 1		1
Total dog		198 (88.8%)	25 (11.2%)	223
No. of isolates from patients	<i>L. infantum</i>	Greece: 44	Greece: 1	45
	<i>L. tropica</i>	Greece: 1	Greece: 1	2
	<i>Leishmania donovani</i>	Cyprus: 1	Cyprus: 4	5
Total human		46 (88.5%)	6 (11.5%)	52
Grand total 275		244 (88.7%)	31 (11.3%)	

MDR = multidrug resistance. The isolates were placed into two groups, depending on their rate of efflux: slope $\alpha < 1$ or > 1 .

The higher the amount of Rhod-123 influxed by the parasite (MFI on baseline), the higher was the rate of efflux, in both dog ($r = 0.838$, $P < 0.05$) and human ($r = 0.719$, $P < 0.05$) isolates. High efflux rates (> 1) were observed in Attica and Crete in Greece and in Paphos, Limassol and Nicosia in Cyprus regarding patients and in Attica, Crete, Peloponnese and Central Macedonia in Greece and in Limassol, Paphos and Nicosia in Cyprus regarding dogs (Figure 1).

Virulence of the isolates. Virulence of *Leishmania* strains tested after infecting human macrophages (of the THP-1 cell line), fluctuated from 1% to 59.3%. Infectivity of 78.4% of the strains was $< 25\%$ and only 2.5% showed infectivity $> 50\%$: *L. infantum* from 1% to 59.3%; *L. donovani* from 5.8% to 17.3%; and *L. tropica* from 14.3% to 17.0%.

Binary logistic regression (univariate and multivariable logistic regression models) and χ^2 test showed that increased infectivity was presented by isolates from Attica ($P = 0.002$) and by isolates from patients in relation to dogs ($P = 0.023$). The most virulent strains were isolated from a patient and a dog in Attica and from three dogs in Crete, with infectivity rates $> 50\%$. In 94% of the isolates from Cyprus, infectivity rates were lower than 25% and none of the isolates presented virulence $> 50\%$.

The human isolate with the highest Rhod-123 efflux rate (*L. donovani* from Cyprus; slope $\alpha = 6$) presented a relatively low virulence (5.67%), as did the two dog isolates (*L. infantum*), which presented the highest Rhod-123 efflux (slope α : 4.26 and 3.53), both isolated in Cyprus and both presented virulence of 3.67%. The mean number of amastigotes per infected cell ranged from 1 to > 10 amastigotes. The highest and the lowest number of amastigotes per cell were found in two dog isolates from Crete with severe CanL symptoms.

DISCUSSION

Resistance of pathogens to drugs is an escalating phenomenon in many diseases rendering chemotherapy ineffective. We know that resistance of *Leishmania* to the antileishmanial drug glucantime, after 65 years of use, has become a serious problem in combating the disease worldwide and there are areas where it can no longer be used for effective treatment. Yet, the mode of action of antimony is still poorly understood.¹⁹

Multidrug resistance commonly takes place via the overproduction of ATP-binding cassette (ABC) transporter proteins, which act as broad-specific drug efflux pumps. Two ABC transporters are implicated in *Leishmania* drug resistance. The PgpA is involved in arsenic and antimony compounds and in the MDR.¹⁶ It has been shown that *Leishmania*, under arsenite and methotrexate selection, overamplifies and expresses

genes (e.g., ItpgpA) that are part of the gene family encoding the Pgp.^{18,36–38} A member of this gene family (*ldmdr1*) can induce a drug-resistant phenotype in *Leishmania*³⁹ and has been molecularly characterized in *L. donovani*.^{40,41}

The efflux rate of Rhod-123 from the parasite body, measured by FCM, indicates the ability of the isolate to actively and selectively expel the drug, thus escaping death.¹⁶ It is expected that the higher the pump activity, the lesser MFI inside the parasite body, which can be measured by the Rhod-123 accumulation assay as the loss of MFI from the body of the isolate in time (slope α).¹⁸ Verapamil hydrochloride, blocker of MDR,⁴² confirmed the specificity of the pumps since efflux appeared to be blocked in its presence and influx was correlated to the rate of efflux, both in its presence and absence.

Messaritakis and others¹⁸ related the efflux rate of *Leishmania* isolates to the number of Pgp molecules, using the C219 monoclonal antibody, and found them, consistently, to be higher in the isolates with a higher rate of efflux, as shown by FCM. This was also true for the isolates if they were exposed to Glucantime as a result of which, the number of pumps as well as the efflux rate of the isolates, increased.¹⁸ The efflux rate, therefore, may depend on the number and the effectiveness of the Pgp 170 efflux pumps each isolate expresses,⁴³ but also on other proteins.¹⁸ Other pumps are known to be expressed in parallel, like the influx pump aquaglyceroporin 1 (AQP1),⁴³ which may explain the paradox of the greater MFI on baseline in resistant isolates.

The Pgp 170 activity, expressed as slope α in the 275 isolates studied, varied from 0.01 to 6. Overall, 11.3% of the isolates presented slope $\alpha > 1$ (Table 1) with *L. donovani* isolates proportionally presenting a greater slope α than *L. infantum* and *L. tropica*, although the number of isolates of the three *Leishmania* species could not be compared statistically due to the uneven number of available isolates. Since 80% of the *L. donovani* isolates tested were found to overexpress the MDR phenotype, this pathogen should be treated alertly. Most of the patients in Cyprus, whether they had developed VL or CL had relapses after treatment (personal communication, Maria Koliou, Cyprus Ministry of Health). A greater percentage of *L. infantum* canine isolates from Cyprus was found to have slope $\alpha > 1$ compared with those from Greece (27.1% and 5.5%, respectively) (Table 1). Of the 31 isolates with slope $\alpha > 1$, 20 originated from Cyprus and 15 were from Paphos. This is an indication that, in Cyprus, the use of antileishmanial drugs may be more frequent than in Greece. This could be explained by the fact that, since there were no human leishmaniasis

FIGURE 1. The geographical distribution of multidrug resistance (MDR1) 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 $\alpha > 1$, or low slope $\alpha < 1$, respectively) and mapped using the geographical information system software (ArcGIS 10; GIS, Redlands, CA).

cases in the island, no strict rules were observed regarding dog treatment of the disease.

The map of Greece and Cyprus (Figure 1) illustrates that patients with slope $\alpha > 1$ are found in prefectures with dogs having slope $\alpha > 1$. In Cyprus, however, the human patients are infected by *L. donovani* and the dog cases by *L. infantum*. Nevertheless, the finding of both parasite species in one out of the 20 dogs examined⁶ suggests that the epidemiological cycle of the two *Leishmania* spp. may be more complex than originally considered. If sandfly vectors, biting both humans and dogs, are present in the island, this will allow the two parasites to meet in the dog and sandfly giving them the opportunity to generate hybrids. The fact that Paphos had the highest number of isolates with slope $\alpha > 1$ of all 59 prefectures studied may suggest that this is already taking place, affecting the spread of drug resistance. Indeed, the highest efflux rate

observed in this study was presented by two isolates from Paphos prefecture: an *L. donovani* strain isolated from a VL patient³⁵ and an *L. infantum* strain isolated from a dog (slope α 6 and 4.26, respectively).

No molecular markers of resistance are yet available to distinguish "resistant" from "susceptible" isolates, information that would help clinicians decide on treatment to avoid relapses and resistance development. Rhod-123 is increasingly used as a tracer dye for MDR studies, and the optimal parameters for its active and passive uptake into cells have been characterized.⁴²

The possible relationship of infectivity and slope α in *Leishmania* is further evaluated; they are two different biological concepts, which may be affected by different factors. The majority of the strains (78.4%) presented $< 25\%$ infectivity and only 2.5% strains presented $> 50\%$ infectivity, whereas in 94% of the isolates from Cyprus infectivity rates

were lower than 25% and none of the isolates presented virulence > 50%. Isolates from Attica and Crete showed the highest infectivity, whereas *L. infantum* appeared more virulent compared with *L. donovani* and *L. tropica* (1–59.3%, 5.7–17.3%, and 14.3–17.0%, respectively) but this may be due to the small number of *L. donovani* and *L. tropica* strains available for experimentation. Interestingly, higher infectivity was presented by isolates from patients in relation to that from dogs, which may be associated with host suitability and adaptation, but in vivo experiments are needed to confirm this.

Each isolate's population members are not clones and may behave differently,⁴⁴ but the isolate's overall behavior will determine the outcome of chemotherapy. In addition, a mixed infection may be present in a patient with two or more species/subspecies of the parasite, showing a different degree of drug resistance each, further complicating chemotherapy.⁴⁵

Resistance, to be confronted, must be clearly understood. As the disease is spreading geographically, it appears that resistant *Leishmania* strains can also spread via the vectors.⁴⁶ Interestingly, 65% of the Mediterranean *Leishmania* strains have been shown to have amplified *MDR1* genes.⁴⁷ Continuous surveillance must, therefore, be maintained to monitor the risk from the ongoing emergence and spread of drug resistance, which poses a threat to public health. The use of drugs for the treatment of the disease in humans and reservoir hosts must be such that selection of resistant strains is avoided.

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