Comparative study on a multiparametric serological approach to feline *Leishmania* infection

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Background

Leishmania infantum is a sandfly-transmitted zoonotic protozoan parasite responsible for canine (CanL) and human leishmaniasis in the Mediterranean basin. Although dogs have been pointed as primary reservoirs of *L.infantum*, there is a growing number of epidemiological studies reporing infection among domestic cats in this region [1–3].

In fact, feline leishmaniasis (FeL), albeit less prevalente when compared to CanL, should be considered a relevant vector-borne disease in cats. This could be partially explained by a more effective immune cellular response against *Leishmania* infection compared to dogs, as reported for other vector-borne pathogens in cats [4]. In the absence of pathognomonic clinical signs, FeL poses a diagnostic challenge. Molecular tests have demonstrated to have limitations in the context of CanL diagnosis, namely high costs and low sensitivity when performed on peripheral blood samples, and the same scenario is expected for FeL. Concerning serological approaches, only Indirect fluorescence test (IFAT) has been validated for diagnosis [5]. Other serologic methods commonly used in CanL diagnosis, such as the direct agglutination test (DAT) and Enzyme-Linked Immunosorbent Assays (ELISA), are yet to be validated for FeL diagnosis [6–7].

Herein, we are, for the first time, evaluating in controlled settings, the potential of different serological approaches to identify anti-*Leishmania* antibodies in cats. The evaluated tests include: IFAT (cut-off 80); DAT (cut-off 100); ELISA based on distinct *L. infantum* specific antigens, including recombinant antigens and *L. infantum* cytosolic and excreted proteins. A *Leishmania* non-related and ubiquitous antigen was introduced to address *non-Leishmania* specific responses. Parasite identification by PCR was performed in a sub-group of samples of interest.

Materials

- 224 feline samples from *L. infantum* endemic regions of Portugal;
- 41 feline samples from *L. infantum* non-endemic region of Azores (AZO) and Madeira (MAD);
- 5 FeL samples from cats with clinical signs of FeL and laboratory confirmation by IFAT, DAT and/or PCR.
- 8 *Leishmania*-specific ELISA antigens:
  - –*Leishmania* promastigote soluble antigens (SPLA)
  - –Recombinant *L. infantum* cytosolic peroxiredoxins (CPX)
  - –Recombinant K39 protein (rK39)
  - –Total *Leishmania* excreted proteins (EXO)
  - –Recombinant K28 protein (rK28)
  - –*Leishmania* secreted extra-cellular vesicles (EVS)
  - –Recombinant KDDR protein (rKDDR)
  - –Vesicle-depleted *Leishmania* excreted proteins (VDE)
- Direct Agglutination Test (DAT)
- Indirect Immunofluorescence Test (IFAT; homemade slides from *L. infantum*)
- Rt-PCR from whole-blood DNA

Methods

The study was conducted in four steps:

1. Address *Leishmania* –specific seroreactivity
   1.1) Characterize positive controls: Characterize the seroreactivity patterns against *Leishmania*-specific ELISA antigens and positivity titters for IFAT and DAT in the FeL positive control samples. Determine a seropositive profiles.
   1.2) Characterize negative controls: Characterize the sera seroreactivity of cats natural from non-endemic regions when exposed to *Leishmania*-specific ELISA antigens, IFAT and DAT. Determine a seronegative profile.
2. Explore possible seropositivity cut-offs
   2.1) populational approach: average + 3*Stdev of the OD values obtained against each ELISA antigen in the negative control group (n=40) of cats from AZO and MAD cats
   2.2) ROC-curve: 5 FeL controls vs. 40 feline samples from AZO and MAD
3. Screening for *Leishmania* spp. exposure using ELISA antigens, DAT, IFAT and PCR in a population of cats (n=224) living in *L. infantum* endemic regions of Portugal.
4. Address the agreement between of results obtained by the studied serological methods and PCR. Statistical analysis performed by GraphPad Prism version 8 (GraphPad Software, San Diego, California, USA), Microsoft Excell and idostatistics.com

1. Addressing *Leishmania*-specific seroreactivity

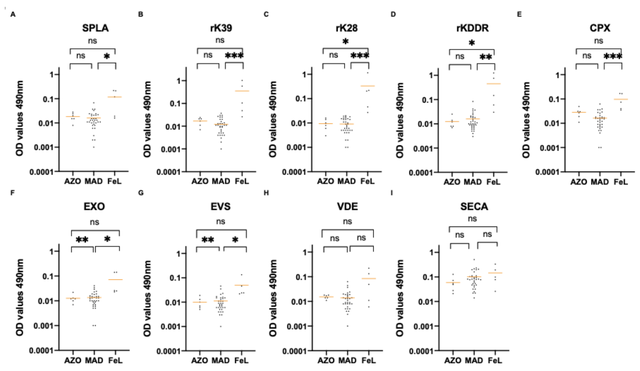

Fig. 01. Analysis of *Leishmania* specific seroreactivity among two groups of cats natural from FeL non-endemic regions of Azores (n=6) and Madeira (n=34), and a group of FeL cases (n=5). Each plotted dot represents the average results of triplicate measures obtained in two comparable and independent assays. Optical densities (OD, measured at 490 nm) against 8 *Leishmania* specific antigens are represented: A. SPLA; B. rK39; C. rK28; D. rKDDR; E. CPX; F. EXO; G. EVS; H. VDE) and a non-related antigen (I. SECA). The horizontal yellow line represents the average seroreactivity for each antigen. Kruskal-Wallis test with Dunn's multiple comparisons was used to compare differences between average results. ns, non-significant; * p < 0.0332; ** p< 0.0021; *** p <0.0002; **** p< 0.0001.

2. Cut-offs for ELISA *Leishmania*-specific antigens

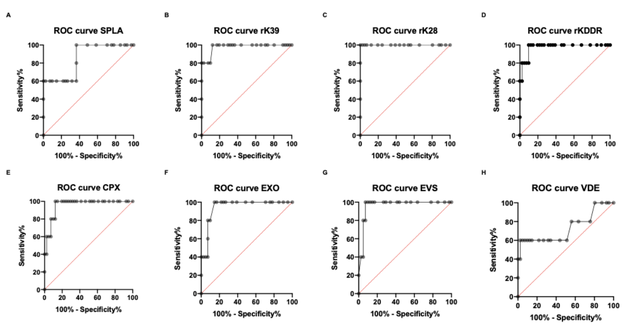

Fig. 02. . Receiver operation characteristics curve (ROC curve) of the sensitivity and specificity of possible cut-off values for optical densities (OD) measured against each *Leishmania*-specific ELISA antigen. Negative control group included sera from cats natural from FeL non-endemic regions of Azores (n=6) and Madeira (n=34). Positive control group included sera from FeL cases (n=5). A. ROC curve for SPLA; B. ROC curve for rK39; C. ROC curve for rK28; D. ROC curve for rKDDR; E. ROC curve for CPX; F. ROC curve for EXO; G. ROC curve for EVS; H. ROC curve for VDE.

AZO+MAD (n=40)	SPLA	rK39	rK28	rKDDR	CPX	EXO	EVS	VDE	SECA
Median	0.013	0.011	0.008	0.01	0.0155	0.012	0.0085	0.013	0.078
Mean	0.016	0.013	0.009	0.015	0.018	0.013	0.011	0.014	0.103
Stdev	0.012	0.009	0.006	0.014	0.013	0.008	0.009	0.012	0.092
Cut-off 2*STdev	0.041	0.031	0.020	0.044	0.045	0.030	0.028	0.038	0.288
Cut-off 3*Stdev	0.053	0.040	0.026	0.058	0.058	0.038	0.037	0.050	0.380
ROC curve cut-off (40 neg_5 pos)	0.016	0.026	0.024	0.029	0.033	0.023	0.022	0.047	

Table. 01 – Cut-off values for each ELISA *Leishmania*-specific antigen (SPLA, rK39, rK29, rKDDR, CPX, EXO, EVS, VDE) and SECA, according to the method applied to determine the cut-offs: population approach based on average OD+3*STDev (green) vs. ROC curve (yellow).

3. Screening exposure to using 8 ELISA *Leishmania*-specific antigens *Leishmania*

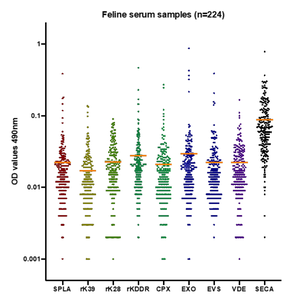

Fig.03. Seroreacctivity measured in optical densities (ODs) read at 490 nm for each ELISA *Leishmania*-specific antigen (SPLA, rK39, rK28, rKDDR CPX, EXO, EVS, VDE) and SECA in a group of 224 cats from *L. infantum* endemic regions of Portugal. Each dot represents the average result of two independent assays performed with technical triplicates for each sample by antigen. Horizontal orange lines represent the median OD value for each antigen. The Y axis represents OD values in log10 units.

Molecular detection and seropositivity to *Leishmania* by ELISA, IFAT and DAT

Table. 02. *Leishmania*-specific positivity in the studied group (n=224). Each column represents seropositivity to the different individual tests: ELISA with *Leishmania*-specific antigens (SPLA, rK39, rK29, rKDDR, CPX, EXO, EVS, VDE); DAT (titter >= 100); IFAT (titter >= 80) and qPCR. **Pannel A.** ELISA Seropositivity considering a population cut-off based on average OD+3*STDev for each antigen. **Pannel B.** ELISA seropositivity considering cut-offs based on ROC curve analysis, for each antigen.

A. Population based cut-off

	SPLA	rK39	rK28	rKDDR	CPX	EXO	EVS	VDE	DAT	IFAT	PCR
Tested samples	229	229	211	228	229	229	229	212	208	208	170
Total positive	12	20	65	19	13	31	24	24	18	63	6
% positivity	**5.24**	**8.73**	**30.81**	**8.33**	**5.68**	**13.54**	**10.48**	**11.32**	**8.65**	**30.29**	**3.53**

B. ROC Curve based cut-off

	SPLA	rK39	rK28	rKDDR	CPX	EXO	EVS	VDE	DAT	IFAT	PCR
Tested samples	229	229	211	228	229	229	229	212	208	208	170
Total positive	112	38	70	19	33	72	66	25	18	63	6
% positivity	**48.91**	**16.59**	**33.18**	**8.33**	**14.41**	**31.44**	**28.82**	**11.79**	**8.65**	**30.29**	**3.53**

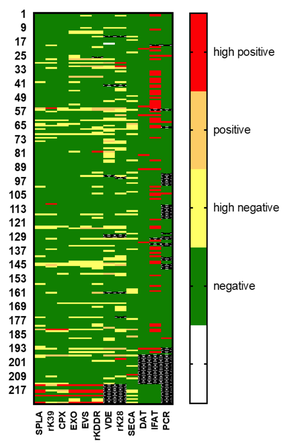

Fig.04. Colour-coded positivity analysis for the serological and molecular tests applied to the study group (n=224). Each column represents the evaluated individual tests, including the 8 *Leishmania* specific antigens assayed by ELISA (SPLA, rK39, rK28, rKDDR, CPX, EXO, EVS and VDE), DAT, IFAT and PCR. Each line represents an individual sample. Seropositivity to ELISA was determined by the population approach. Four categories of seroreactivity were determined, including: cats with high positive OD values (ODs > 1.5x cut-off, depicted in red); positive OD values (ODs between 1 and 1.5x cut-off, depicted in orange), high negative (ODs between 0.5 and 1 x cut-off, depicted in yellow) and negative (ODs < 0.5 x cut-off, depicted in green). Positivity to DAT was considered for titters equal to or above 100 and depicted in red. Positivity to IFAT was considered for titters equal to or above 80 and depicted in red. PCR positive samples are depicted in red. Black crosses represent samples not yet tested.

4. Agreement between the diferent serological methods and PCR for *Leishmania* detection

Cohen´s *k* agreement coeficient between IFAT, DAT, PCR and *Leishmania* -specific (SPLA, rK39, rK28, rKDDR, CPX, EXO, EVS, VDE) and non-specific ELISA antigens (SECA)

A. Population cut-offs for ELISA antigens. IFAT cut-off 80. DAT cut-off 100.

	SPLA	rK39	rK28	rKDDR	CPX	EXO	EVS	VDE	SECA
DAT	0.543	0.426	0.025	0.496	0.409	0.246	0.262	0.099	0.052
IFAT	0.137	0.185	0.398	0.090	0.061	0.045	0.238	0.338	0.015
PCR	0.309	0.183	0.174	0.114	0.172	0.113	0.155	0.152	0.155

B. ROC curve cut-offs for ELISA antigens. IFAT cut-off 80. DAT cut-off 100.

	SPLA	rK39	rK28	rKDDR	CPX	EXO	EVS	VDE	SECA
DAT	0.373	0.001	0.199	0.404	0.255	0	0.122	0.095	0.052
IFAT	0.259	0	0.377	0.092	0.057	0.031	0.318	0.379	0.015
PCR	0.309	0.183	0.174	0.114	0.172	0.113	0.155	0.153	0.155

no agreement
fair agreement 0.01 - 0.20
slight agreement 0.21 - 0.40
moderate agreement 0.41 - 0.60
substantial agreement 0.61 - 0.80
almost perfect agreement 0.81 - 1.00

Fig.05 . Cohen's *kappa* coefficient values for DAT, IFAT, PCR, *Leishmania*-specific ELISA antigens (SPLA, rK39, rK28, rKDDR, CPX, EXO, EVS, VDE) the *Leishmania* non-specific antigen SECA . *K* values below 0: no agreement; *k* values between 0 and 0.20: slight agreement; *k* values between 0.21–0.40: fair agreement; *k* values 0.41–0.60: moderate agreement; *k* values between 0.61– 0.80: substantial agreement; *k* values between 0.81 to 1.00: almost perfect agreement.

Cohen´s *k* agreement coeficient between IFAT, DAT, PCR and *Leishmania* -specific antigens

	SPLA	rK39	rK28	rKDDR	CPX	SECA	EXO	EVS	VDE
SPLA		0.580	0.425	0.619	0.769	0.267	0.725	0.089	0.067
rK39			0.426	0.451	0.167	0.245	0.044	0.306	0.420
rK28				0.250	0.162	0	0.042	0.220	0.371
rKDDR					0.499	0.240	0.620	0.704	0.350
CPX						0.289	0.107	0	0.128
SECA							0.184	0.191	0.128

no agreement
fair agreement 0.01 - 0.20
slight agreement 0.21 - 0.40
moderate agreement 0.41 - 0.60
substantial agreement 0.61 - 0.80
almost perfect agreement 0.81 - 1.00

Fig.06. Cohen's *kappa* coefficient values for the combination of *Leishmania*-specific antigens (SPLA, rK39, rK28, rKDDR, CPX, EXO, EVS, VDE) and the *Leishmania* non-specific antigen SECA, considering the population cut-offs to determine seropositivity. For *k* values below 0: no agreement; *k* values between 0 and 0.20: slight agreement; *k* values between 0.21–0.40: fair agreement; *k* values 0.41–0.60: moderate agreement; *k* values between 0.61– 0.80: substantial agreement; *k* values between 0.81 to 1.00: almost perfect agreement.

Cohen's *k* agreement coefficient between IFAT, DAT and PCR

	PCR	DAT	IFAT
PCR		0.122	0.087
DAT			0.101

no agreement
fair agreement 0.01 - 0.20
slight agreement 0.21 - 0.40
moderate agreement 0.41 - 0.60
substantial agreement 0.61 - 0.80
almost perfect agreement 0.81 - 1.00

Fig.07. Cohen's *k* coefficient values for DAT, IFAT and PCR. Values below 0 are considered poor agreement; values between 0 and 0.20 — Slight Agreement; 0.21–0.40 — fair agreement; 0.41–0.60 — moderate agreement; 0.61– 0.80 — substantial agreement; 0.81 to 1.00 — almost perfect agreement.

Discussion and conclusions

- A wide range of *Leishmania*-specific seroreactivities were observed in FeL cats. Seropositivity to *Leishmania* specific antigens SPLA, rK39, CPX, EXO, EVS varied according to the cut-offs established by poupulation vs. ROC curve.
- Seropositivity cut-offs derived from a population approach allowed the identification of seropositive samples with increased agreement with DAT and IFAT.
- Poor to slight agreement was observed between IFAT, DAT and PCR. However, moderate to substancial agreement was registered between *Leishmania*-specific recombinante antigens and DAT.
- Variable degrees of agreement were recorded between the ELISA *Leishmania* specific antigens. Moderate agreement was observed between rK39, rK28 and SPLA; rK28, rKDDR, VDE and rK39; CPX and rKDDR. Substancial agreement was observed between SPLA and rKDDR, CPX and EXO; rKDDR, EXO and EVS.
- The preliminary data points out to poor agreement between the IFAT, DAT and PCR. However, DAT and ELISA antigens SPLA, rK39, rKDDR and CPX presente moderate agreement
- Combination of recombiannt *Leishmania*–specific antigens in ELISA improves the assay sensitivity and specificity in detecting anti-*Leishmania* antibodies in this species.
- A better understanding *L. infantum* infection progression in cats is needed for proper interpretation of the obtained serological data.

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