

The use of *Escherichia coli* total antigens as a complementary approach to address seropositivity to *Leishmania* antigens in canine leishmaniosis

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SUMMARY

Canine leishmaniosis (CanL) is a major veterinary concern and a public health issue. Serological data are essential for disease management. Several antigens used in serological assays have specificity related problems preventing relevant seropositivity values establishment. Herein we report significant seropositivity level disparity in a study cohort with 384 dogs from eight countries, for antigens traditionally used in CanL – soluble promastigote *Leishmania* antigens (SPLA) and K39 recombinant protein (rK39): 43·8 and 2·9% for SPLA and rK39, respectively. To better understand the reasons for this disparity, CanL-associated serological response was characterized using, for complement serological evaluation, a ubiquitous antigen – soluble *Escherichia coli* antigens (SECAs). Using cohorts of CanL dogs and dogs without clinical evidences of CanL from non-endemic regions of Portugal, the serological response of CanL animals followed specific trend of seropositivity rK39 > SPLA > SECA absent in non-diseased animals. Using receiver operating characteristic curve analysis, these characteristic trends were converted in ratios, SPLA/SECA, rK39/SECA and rK39/SPLA, that presented high predictive for discriminating the CanL cohort that was potentiated when applied in a scoring system involving positivity to four out of five predictors (rK39, SPLA, SPLA/SECA, rK39/SECA and rK39/SPLA). In fact, this approach discriminated CanL with similar sensitivity/specificity as reference antigens, diminishing seropositivity in European cohort to 1·8%. Ultimately, non-related antigens like SECA and seropositivity ratios between antigens enable different perspectives into serological data focusing on the search of characteristic serological signatures and not simple absolute serology values contributing to comprehensive serological status characterization.

Key words: Canine leishmaniosis, rK39, serosurveillance, *Leishmania*, ELISA, seropositivity.

INTRODUCTION

Canine leishmaniosis (CanL), caused by *Leishmania infantum* (syn. *Leishmania chagasi*), is endemic in the Mediterranean basin, South America and Southern Asia, being a major veterinary concern (Saridomichelakis, 2009; Ready, 2010; Noli and Saridomichelakis, 2014; Wylie *et al.* 2014). These protozoa may also induce human visceral leishmaniasis, making CanL not just a veterinary problem but also a public health issue due to the zoonotic potential of the infection (Michalsky *et al.* 2007; Sousa

et al. 2011; Noli and Saridomichelakis, 2014). CanL control is based on vaccination, transmission prevention by the insect vectors and also diseased animals treatment (Palatnik-de-Sousa, 2012; Courtenay *et al.* 2014; Silva *et al.* 2014). The CanL diagnosis is based on clinical signs and demonstration of infection by direct visualization of parasites and/or genetic material in conjunction with quantitative serology (Michalsky *et al.* 2007; Paltrinieri *et al.* 2010; Solano-Gallego *et al.* 2011). Of paramount importance for disease management is the detection of dogs that have no leishmaniosis specific signs but are infected. It is well established that infected dogs without clinical signs are capable of perpetuating the zoonotic life cycle of the parasite (Molina *et al.* 1994; Moreno and Alvar, 2002). Therefore,

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low-accuracy serological assays can have significant impact in the global strategy to combat disease progression originating seropositivity levels that are not translatable into meaningful epidemiological data influencing policies associated with specific control measures. In fact, cumulative evidence point to a low specificity (Sp) of several serological assays that hamper the detection of *Leishmania* infection in dogs (Gomes *et al.* 2008; Morales-Yuste *et al.* 2012; Arruda *et al.* 2016; Elshafie *et al.* 2016; Fraga *et al.* 2016). Moreover, detection of *L. infantum* vectors across central European countries like Switzerland, Germany and Hungary (Naucke *et al.* 2008; Ready, 2010), in conjunction with global warming, might open new areas to endemicity, through the adaptation of the vectors to new latitudes (Geisweid *et al.* 2013; Millan *et al.* 2014). In fact, disease is advancing northwards, with reports of infected animals in traditionally non-endemic areas (Saari *et al.* 2000; Dujardin *et al.* 2008; Ready, 2010; Miro *et al.* 2012; Carvalho *et al.* 2015; Maia and Cardoso, 2015). Therefore, more than ever, it is essential to have adequate serosurveillance systems and tools to monitor and control infection progression into new regions.

Herein we evaluate serological response of an uncharacterized cohort of animals from distinct geographical backgrounds to two known *Leishmania* antigens [soluble promastigote *Leishmania* antigens (SPLA) and K39 recombinant protein (rK39)] to determine the adequacy of these antigens for serological surveys in non-endemic regions. To exclude any inter-laboratory variability, we used CanL-positive and negative cohorts of dogs to determine the cut-offs that are able to distinguish diseased from non-diseased animals for both antigens. The generated cut-offs were applied to the test cohort and levels of seropositivity compared. The unrelated soluble *Escherichia coli* antigen (SECA) was also used to address the Sp of the serological response to the *Leishmania* antigens. Finally, the ratios between the cut-off normalized responses to the antigens were evaluated for their capacity to predict disease in the control cohorts and then used as part of a scoring system that addresses seropositivity with clinical value.

MATERIALS AND METHODS

Canine sera

Group CanL+ ($n = 29$): sera from dogs living in geographical regions of Portugal where CanL is endemic, which were brought to a veterinary hospital, clinic or anti-rabies campaign and that presented at least two clinical signs compatible with the disease (*viz.* lymphadenomegaly, alopecia, dermatitis, skin ulceration, keratoconjunctivitis, onychogryphosis, lameness, epistaxis, anorexia and weight loss). These animals were also seropositive

for anti-*Leishmania* antibodies by the direct agglutination test (DAT) (cut-off titre = 400) and/or positive for the presence of amastigotes in bone marrow or lymph node aspirates.

Group CanL- ($n = 121$): sera from dogs that visited a veterinary clinic in a Portuguese region considered to be non-endemic for CanL, Serra da Estrela mountain in central Portugal. All were seronegative by DAT (titre < 100).

Group Europe ($n = 384$): sera from dogs without clinical information, with unknown serological status, that visited veterinary clinics in several countries of Europe, namely Denmark ($n = 50$), France ($n = 50$), Germany ($n = 47$), Hungary ($n = 47$), the Netherlands ($n = 42$), Poland ($n = 50$), Portugal ($n = 50$) and the UK ($n = 48$).

Antigens

Three different antigens were used for enzyme-linked immunosorbent assay (ELISA): SPLA, SECA and *Leishmania* rK39. For SPLA, *L. infantum* promastigotes were obtained as previously described (Santarem *et al.* 2010). Parasites were washed three times with phosphate-buffered saline (PBS), pH 7.4 and centrifuged at 3500 *g* for 10 min at 4 °C. The pellet was resuspended in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor and submitted to 10 freeze–thaw cycles for rupture of the parasites. This suspension was centrifuged at 13 000 *g* for 30 min at 4 °C and the supernatant was recovered, quantified by DC (detergent compatible)TM Protein Assay (BioRad, Munich, Germany), and stored at –80 °C in single aliquots.

The rK39 antigen obtained from Dr Steven Reed, from Infectious Disease Research Institute (Seattle, Washington, USA) was suspended in mili-Q water, quantified and stored at –80 °C in single-use aliquots.

For SECA, *E. coli* DH5- α strain was plated in an agar plate and incubated at 37 °C overnight (O/N). Then, a colony was transferred to 10 mL of Luria Bertani broth (Sigma-Aldrich, St. Louis, USA) and incubated at 37 °C O/N. The next day, the optical density (OD) of the culture was measured at 600 nm (initial OD should be nearly 0.1) and left to grow for 3 h. The culture was then centrifuged at 4000 *g* for 5 min. The pellet was resuspended in 1 mL of PBS containing 1 mM of PMSF and submitted to 10 freeze–thaw cycles for rupture of the bacteria. The suspension was then centrifuged at 13 000 *g* for 30 min at 4 °C and the supernatant was recovered, quantified by DC (detergent compatible)TM Protein Assay (BioRad, Munich, Germany) and stored at –80 °C in single-use aliquots.

ELISA

Ninety-six-well flat-bottom microtitre plates (Greiner Bio-One, Frickenhausen, Germany) were

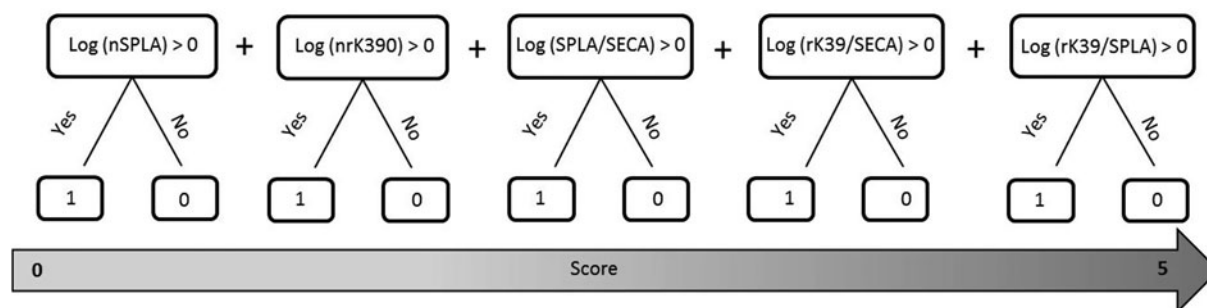


Fig. 1. Evaluation of seropositivity of each individual serum evaluated as a cumulative score. Each individual serum was 'scored' as $nSPLA + nrK39 + nSECA + SPLA/SECA + rK39/SECA + rK39/SPLA$ using the following premise: each serology value above or equal to the cut-off values of $nSPLA$, $nrK39$, $nSECA$, $SPLA/SECA$, $rK39/SECA$ and $rK39/SPLA$ was transformed into a score of '1' originating a cumulative score ranging from 0 to 5 to each individual sample.

coated with $50 \mu\text{L}$ of 0.1 M carbonate buffer, $\text{pH} = 9.6$, with $10 \mu\text{g mL}^{-1}$ of either $SPLA$ or $SECA$, or $1 \mu\text{g mL}^{-1}$ of $rK39$. Plates were incubated O/N at 4°C and blocked with $200 \mu\text{L}$ of PBS low-fat milk (3%) at 37°C for 1 h. Next, plates were washed with PBS-Tween 0.05% (PBS-T), and the sera, positive and negative controls, diluted at 1 : 1500 in PBS-T low-fat milk (1%), were dispensed in triplicate ($100 \mu\text{L}$ per well) and incubated at 37°C for 30 min. After a washing step, $100 \mu\text{L}$ per well of conjugate secondary anti-dog IgG antibody conjugated with horseradish peroxidase (Sigma, St. Louis, USA) – diluted at 1 : 1176.5, was added and the plates were incubated at 37°C for 30 min. Plates were washed and incubated with 0.5 mg mL^{-1} of *o*-phenylenediamine dihydrochloride (Sigma, St. Louis, USA) for 10 min in dark. Reaction was stopped with $50 \mu\text{L}$ per well of HCl (3 M). Absorbance was read at 492 nm in an automatic reader (Synergy 2, BioTek Instruments, Winooski, USA).

All samples and antigens were assayed in at least two independent assays.

Statistical analysis

Receiver operating characteristic (ROC) curves were generated using sera from groups CanL+ and CanL–, using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. A 95% confidence interval for the area under the ROC curve was considered.

Cut-off values were inferred through these curves for each antigen [by choosing the best compromise between sensitivity (Se) and Sp associated with the ROC curve], and values of Se, Sp, false negatives (FN), false positives (FP), positive predictive value and negative predictive value were calculated for the samples for each group (Parikh *et al.* 2008). Optical densities of each sample were normalized by division with the corresponding cut-off value ($nSPLA$, $nrK39$ and $nSECA$) and the logarithm of this ratio was applied for graphical representation [$\log(nSPLA)$, $\log(nrK39)$ and $\log(nSECA)$]. The

cut-off normalized values (n) were used to assess the ratio between the antigens: $SPLA/SECA$ (ratio between $nSPLA$ and $nSECA$), $rK39/SECA$ (ratio between $nrK39$ and $nSECA$) and $rK39/SPLA$ (ratio between $nrK39$ and $nSPLA$). A score evaluation method was established based on the cut-off values inferred from the ROC curves for the ratios and the single antigens. A binary score (1 or 0) was applied to represent seropositivity (arbitrary value of 1) or seronegativity (arbitrary value of 0) to the five serological parameters previously established and whose cut-off values was determined by ROC curve analysis: $rK39$, $SPLA$, $rK39/SECA$, $SPLA/SECA$ and $rK39/SPLA$. Total cumulative score for each individual sample ranged from '0' to '5' (Fig. 1).

RESULTS

ROC curve analysis was performed to define cut-off values for $rK39$ and $SPLA$ using sera from dogs of CanL+ and CanL– groups (Supplementary Fig. S1A, S1B and Table S1). Cut-off values for $rK39$ and $SPLA$ were 0.127 and 0.074, respectively. These cut-offs enabled a Se of 100% (29/29) for both antigens with Sp of 100% in both cases (0/121) (Supplementary Table S1). When the cut-offs were applied to the European cohort, they originated a global seropositivity of 43.8% (168/384) and 2.9% (11/384) for the individual antigens, $SPLA$ and $rK39$, respectively (Table 1, Fig. 2A–C). If seropositivity to both antigens was used as a requisite, then 2.3% (9/384) of the cohort was seropositive (Table 1).

Observing the generated seropositivity data disparity for antigens that excelled at discriminating CanL from non-CanL dogs, two possibilities were considered: either the $SPLA$ was detecting FP or $rK39$ was detecting FN. Considering that both antigens presented very high Sp and Se, the cut-off stringency was increased to decrease the number of FP (Supplementary Table S2 and Fig. 3A). For seropositivity associated with both antigens to have

Table 1. Seropositivity levels in the different study cohorts for SPLA and rK39 antigens

	SPLA (%)	rK39 (%)	SPLA \wedge rK39 ^a (%)	SPLA \vee rK39 ^b (%)
CanL+	100 _(29/29)	100 _(29/29)	100 _(29/29)	100 _(29/29)
CanL-	0 _(0/121)	0 _(0/121)	0 _(0/121)	0 _(0/121)
Europe	43.8 _(168/384)	2.9 _(11/384)	2.3 _(9/384)	44.3 _(170/384)

^a Positive for both antigens simultaneously.

^b Positive for at least one of the antigens.

epidemiological relevance, then the increase in cut-off stringency should enable a decrease of FP associated with SPLA leading seropositivity levels similar to rK39. In the European cohort, using two cut-offs (cut-off value $\times 2$) for rK39, the number of seropositive animals is reduced to just one, PT30 (Fig. 3B). For SPLA, 66/384 (17.2%) animals remained seropositive when two cut-offs were used (Supplementary Table S2). Comparing these SPLA-seropositive animals with the original 11 animals seropositive for rK39, only six animals were seropositive to rK39 and SPLA using two SPLA cut-offs (Fig. 3B). From 3 to 7 SPLA cut-offs, only three animals were seropositive for both antigens – when compared once again to the original rK39-positive animals (Fig. 3B). The sample with highest seropositivity to both antigens was PT30 (Fig. 3B).

The geographic distribution of seropositive animals was not random, ranged from 0 to 86%. Poland presented (43/50) seropositive animals, while France presented 0/50 animals to SPLA (Fig. 4 and Supplementary Table S2). Considering the individual countries, rK39-seropositive animals were mostly from Portugal: 66.3% (7/11) (Fig. 4A, Supplementary Fig. S2A). Still, three animals were from non-endemic countries [two from Denmark (DK44 and 50) and one from Poland – PL9] and a final one from France (FR2) (Fig. 3). For SPLA, although there was also an over-representation of dogs from Portugal (i.e. 12.5%; 21/168), this was not as evident as for rK39 (Supplementary Fig. S2). As the groups were proportional, the basal cohort serological response was compared to evaluate if existed a regional influence in the response to the antigens (Supplementary Tables S3 and S4). For rK39, the dogs from Germany had a basal median response significantly different from Hungary and Portugal, while the animals from Portugal were significantly different from all the other cohorts (Supplementary Fig. S2A and Table S3). For SPLA, more countries had significant differences, with the cohort from Poland being the most divergent (Supplementary Fig. S2 and Table S4). As SPLA is an undefined antigen mixture, these variations in seropositivity might correspond to an unspecific response.

To address serological response Sp, an undefined ubiquitous non-related antigen was used, *E. coli*

DH5- α , to evaluate if the serological response pattern was similar to SPLA suggesting a general unspecific response (Fig. 4A and Supplementary Fig. S2B). The SECA antigen had some predictive value to distinguish the control cohorts (Se of 81.5% and Sp of 71.9%) (Fig. 4B and Supplementary Table S1). Notwithstanding, the seropositivity profile against SECA was distinct from the SPLA (Fig. 4A, Supplementary Fig. S2B and Tables S4 and S5). To further address the relationship between these antigens, correlations in the control cohorts and also in each individual country were done (Supplementary Table S6). In the CanL+ cohort, the highest correlation was obtained for rK39 *vs* SPLA ($r = 0.499$, $P < 0.01$; $r^2 = 0.249$), whereas the lowest was for rK39 *vs* SECA ($r = -0.010$, $P = 0.952$, $r^2 = 0.0001$). In the CanL- cohort, the highest correlation value was for SPLA *vs* SECA ($r = 0.356$, $P < 0.001$, $r^2 = 0.127$). At the country level, the most common significant correlation was SPLA *vs* SECA (3/8), while rK39 correlated positively with SPLA in two countries and with SECA in one country (Supplementary Table S6). The cohort comparison at a country level (absolute values and correlation data) suggests distinct basal responses to the different antigens influencing the serology absolute values.

To bypass the regional effects on the seropositivity, the ratios between the antigens were analysed for their capacity to predict CanL in the control cohorts. For this, the median normalized single-antigen reactivity in the CanL+ control group was compared (Figs 2 and 4B). The n rK39 and n SPLA responses were higher than n SECA in CanL+ cohort. Moreover, this was characteristic of the individual sera from the CanL+ cohort: (27/29) for n SPLA $>$ n SECA and (26/29) n rK39 $>$ n SECA (Supplementary Table S7). Likewise, the response to rK39 was higher than to SPLA in (24/29) of the cohort (Supplementary Table S7). Therefore, these ratios, [$\log(n$ SPLA/ n SECA) $>$ 1; $\log(n$ rK39/ n SECA) $>$ 1; $\log(n$ rK39/ n SPLA) $>$ 1] were characteristic of CanL dogs (Supplementary Fig. S3). Consequently, ROC curves analysis was performed for each of the aforementioned individual ratios (Supplementary Fig. S1D–F). The predictive value of these ratios was considerable, although not superior overall to the reference antigens alone (Supplementary Table S1). The Sp of the ratios

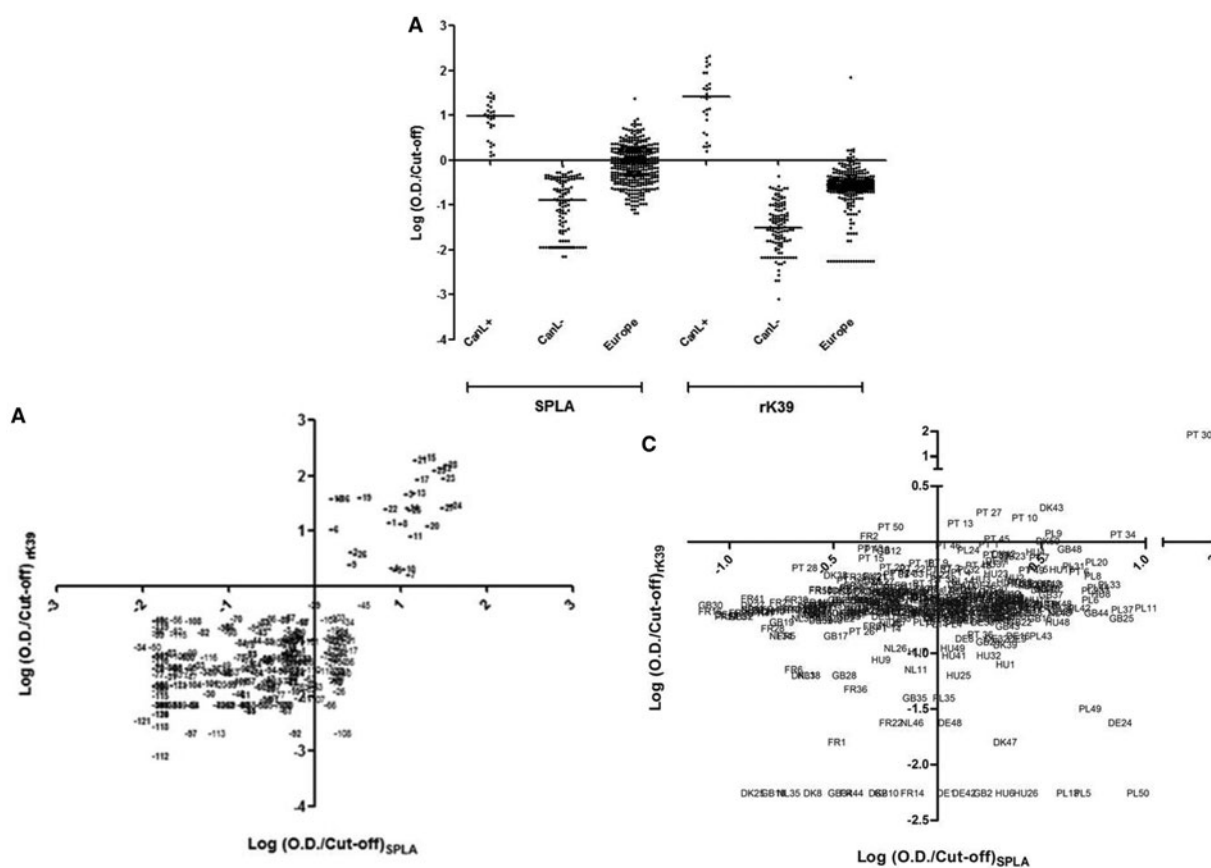


Fig. 2. Reactivity of CanL+, CanL– and European dog sera against SPLA and rK39 antigens (A). Correlation of the seroreactivity against SPLA and rK39 antigens in CanL+ and CanL– cohorts (B) and in European cohort (C). Results are expressed as the logarithm of the optical density (OD) at 492 nm normalized by the cut-off value for each antigen (*n*). Bars represent the median for the cohort and each single dot represents the dataset for an individual dog. The data depicted were generated from the average of at least two independent assays done in triplicate.

was 94.2, 92.6 and 72.7%, for *n*SPLA/*n*SECA, *nr*K39/*n*SECA and *nr*K39/*n*SPLA, respectively (Supplementary Tables S1 and S8). The lower Se of the assays can be attributed to artefactual responses due to low serological responders that create ratios that are above the cut-off level (see PL38, FR9 and DE47 in Supplementary Table S9). Therefore, the capacity of these ratios to predict disease requires the determination of positivity to reference antigens to exclude the aforementioned artefactual values. Taking this in consideration, a scoring system was created to address not only the positivity to the reference antigens but also to the characteristic ratios described above (Fig. 1). The Cohen’s κ coefficient was determined for the possible scores to define the best predictive value when compared to both reference antigens. The scores of 4 or 3 presented the best predictive value (Supplementary Table S10). We excluded the score 3 as it enabled positive results that were negative for both reference antigens (Fig. 1). This approach (using a score ≥ 4) resulted in an overall Se (96%) and Sp (100%) similar to the combined reference antigens (Table 2). When this score system was applied to our cohorts (Table 2), it reduced the number of seropositive animals in the

European cohort to seven (PT10, PT30, PL9, PL24, DK43, DK50 and FR2) with an overall seropositivity of 1.8% (7/384) (Table 2 and Supplementary Tables S9–12). This represents a diminution of 36% in seropositivity when compared with rK39 alone. We also report a diminution of 22% in seropositivity when compared with rK39 and SPLA combined (Table 1). Only one animal in Europe presented a perfect score of 5 (PT30), the most common score in the CanL+ cohort [82.8% (24/29)], with the remaining six animals presenting a score of 4. These seven seropositive animals from the European cohort obtained after application of the score methodology were all rK39-seropositive animals with the exception of one sample from Poland (PL24) (Supplementary Fig. S4 and Table S9).

DISCUSSION

In the present study, the used antigens, SPLA and rK39, are established antigens with reported high sensitivities and specificities and are the base of several reference tests. The cut-off values for two antigens, using a cohort of CanL+ dogs and a

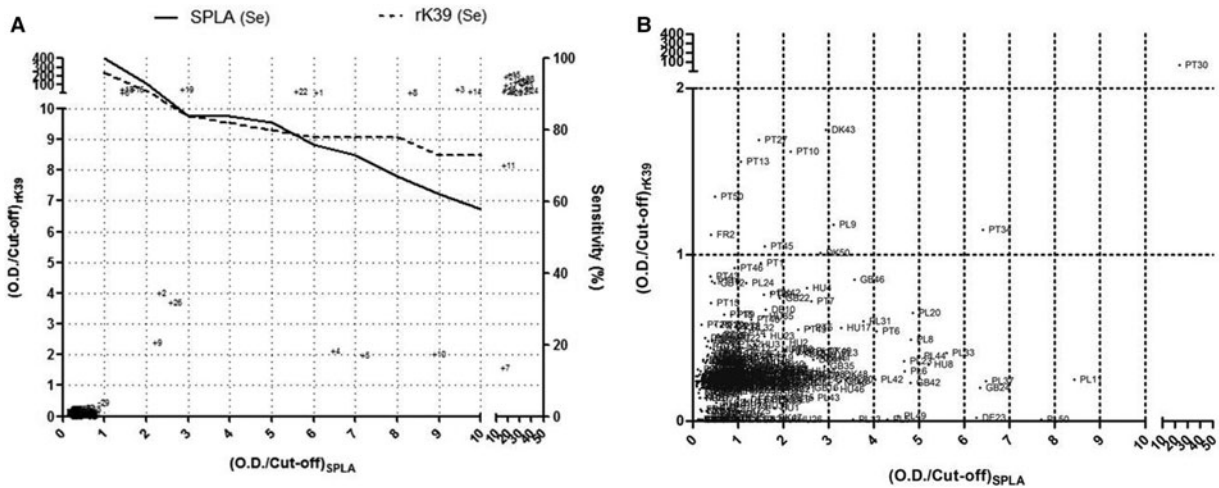


Fig. 3. Representation of seroreactivity against SPLA and rK39 antigens in CanL+ and CanL+ cohorts (A), and European cohort (B). Vertical and horizontal dotted lines represent the number of cut-offs ($n \times$ cut-off) for each antigen. The cut-off-dependent sensitivity is represented in (A) for SPLA and for rK39. Results are expressed as the logarithm of the optical density (OD) at 492 nm normalized by the cut-off value for each antigen (n). Each single dot represents the dataset for an individual dog. The data depicted were generated from the average of at least two independent assays done in triplicate.

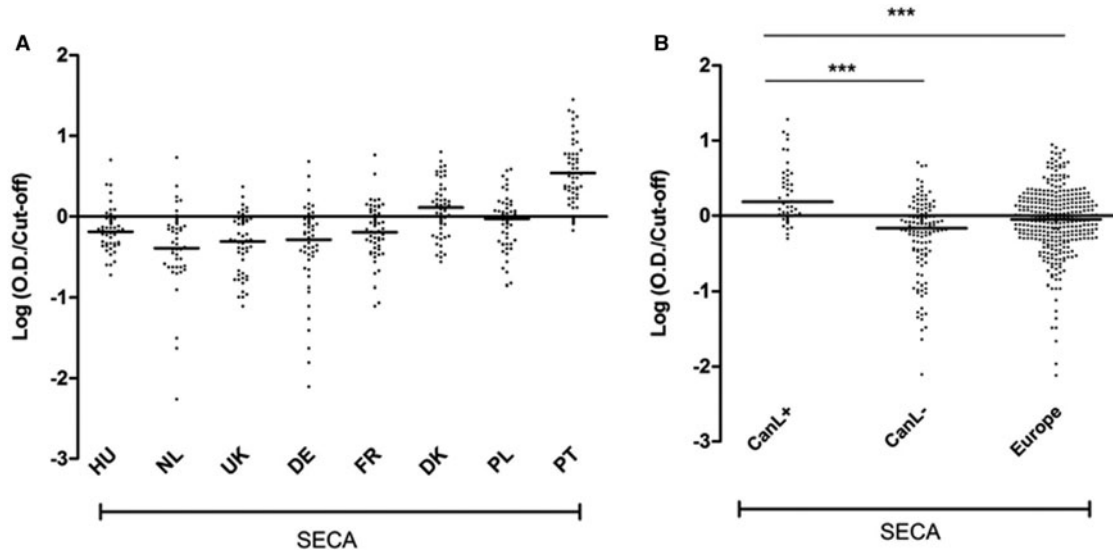


Fig. 4. Reactivity of dog sera against SECA antigen for: (A) individual countries Hungary (HU), The Netherlands (NL), UK, Germany (DE), France (FR), Denmark (DK) and Poland (PL). Results are expressed as the logarithm of the optical density (OD) at 492 nm normalized by the cut-off value for each antigen (n) and (B) CanL+, CanL- and European cohort. Bars represent the median for the cohort and each single dot represents the dataset for an individual dog. The data depicted were generated from the average of at least two independent assays done in triplicate. Statistical analysis was done by one-way ANOVA and Kruskal–Wallis tests. Statistical significance between groups is ‘extremely significant’ ($***0.0001 \leq P < 0.001$).

cohort of CanL- dogs, were determined. The obtained cut-offs were similar in absolute value to the ones obtained in previous work from our group (Santarem *et al.* 2010), in a clear indication that both antigens are capable of consistently discriminating CanL+ from CanL- dogs. When the newly determined cut-offs were applied to a cohort of animals from Europe without clinical characterization, a seropositivity that was distinct for both antigens (43.8% for SPLA and 2.9% for rK39) was

obtained. This large difference in seropositivity cannot be attributed to laboratory inter variability, as all assays were performed in the same laboratory, including the ROC curve analysis. The CanL seropositivity levels reported for endemic Mediterranean regions range between 5 and 30% (Moreno and Alvar, 2002). The overall seropositivity levels obtained for the non-endemic countries was not in accordance with epidemiological data that do not support significant seropositivity levels

Table 2. Seropositivity levels in the different study cohorts using the scoring system

	Score		
	'5' ^a (%)	'4' ^b (%)	Total ^c (%)
CanL+	82.8 _(24/29)	13.8 _(4/29)	96.5 _(28/29)
CanL-	0.0 _(0/121)	0.0 _(0/121)	0.0 _(0/121)
Europe	0.3 _(1/384)	1.6 _(6/384)	1.8 _(7/384)

^a 5: seropositivity to all serological parameters defined in Fig. 1.

^b 4: seropositivity to four out of five serological of the parameters defined in Fig. 1.

^c Total: seropositivity to at least four out of the parameters defined in Fig. 1.

in the non-endemic countries from the European cohort (Solano-Gallego *et al.* 2011). The rK39 seropositivity levels are more in the range of what is reported from the endemic regions, but still non-endemic seropositive samples were identified in Denmark and Poland. These positive animals might be associated with canine population mobility associated with travelling, an issue of growing concern for CanL (Menn *et al.* 2010; Bart *et al.* 2013). *Leishmania infantum* is endemic in southern European countries (Dujardin *et al.* 2008) that are a highly valued vacation destination generally during the peak of the transmission season. In these conditions, the risk for exposure to the parasite can be as high as 0.23% for dogs coming from northern Europe (Teske *et al.* 2002). In fact, the few reported CanL cases in northern Europe are restricted to dogs that have travelled to endemic areas of the Mediterranean basin during periods when phlebotomine sand fly exposure is high, mostly between March and November, or that have to be relocated from those areas (Saari *et al.* 2000).

SPLA is known to have cross-reactivity with other diseases like babesiosis, Chagas, ehrlichiosis, rickettsiosis and toxoplasmosis, probably due to some epitope conservation generating unspecificity (Gomes *et al.* 2008; Saridomichelakis, 2009; Silvestre *et al.* 2009; Zanette *et al.* 2014; Peixoto *et al.* 2015). This cross-reactivity was suggested upon correlation of the data from SPLA with SECA (Supplementary Table S6). In the cohorts from three countries, there was a significant correlation between SECA and SPLA, more than between SPLA and rK39. This suggests that, in the absence of a *Leishmania*-specific serological response, the SPLA might also be recognized by other non-related infectious diseases or immune disorders. This is also suggested by the correlation existing in the CanL- control group for these two antigens. Still, the distinct pattern of basal recognition between SPLA and SECA (Fig. 4A and

Supplementary Fig. S2B) is not suggestive of general unspecific recognition due to immunological disorders, like polyclonal activation. Intriguingly, it will also be important to address if the apparent lack of Sp of SPLA might also be true for other assays that use parasite total antigens, such as IFAT or DAT. The rK39 was less prone to cross-reactivity (Fig. 3B). In fact, the general basal serological response was more uniform. This is in agreement with the recognized value of rK39 as a good serological marker for leishmaniosis (Porrozzini *et al.* 2007). Still, regional differences in antigen performance have been already reported, the performance of rK39 (for the human visceral leishmaniasis) is known to be diminished in sub-Saharan Africa (Pattabhi *et al.* 2010; Bezuneh *et al.* 2014; Mukhtar *et al.* 2015). Subtle genetic differences in *Leishmania* strains are sufficient to influence the performance of the recombinant antigens (Abass *et al.* 2015).

The adjustment of the cut-off stringency is often used to help in the diagnosis of CanL (Paltrinieri *et al.* 2010). Therefore, we increased the cut-off stringency (at the cost of decreased Se) (Fig. 3A). Two cut-offs decreased the global seropositivity for SPLA and rK39 to 66/384 (17.2%) and 1/384 (0.26%), respectively. Still, a significant difference in readout between both antigens was apparent, as there was no correspondence between SPLA- and rK39-seropositive animals. In fact, the increase in SPLA cut-off stringency also led to a loss of rK39-positive animals (standard cut-off), instead of having an enrichment of double positive sera as would be expected for seropositivity related to CanL. Still, it must be referred that existence of dogs with different clinical stage of the disease might also contribute to distinct levels of seropositivity to rK39 and SPLA. It was shown for naturally infected dogs that rK39, although adequate for detecting symptomatic infections, presented lower capacity to evaluate sub-clinical infections (Santarem *et al.* 2010). This could justify the failure in enrichment for double positive animals with the cut-off increase. Still, as most countries in the study are non-endemic (exception of Portugal and France), natural exposure to the parasite and asymptomatic infections should not be a decisive factor for the lack of correspondence in seropositivity levels.

To address the possibility of unspecificity of SPLA in the European cohort, SECA was used as a measure of the humoral response that is not specifically directed to *Leishmania*. Although SECA has some predictive value (probably as a consequence of some level of conservation at the epitope level or due to, in severe cases of the disease, polyclonal activation and effects of co-infections), it presented low Sp (71.9%). Upon cut-off normalization, it was also evident the clear relationships between the antigens. The median normalized response of the animals of

the CanL+ control group was highest for rK39 and lowest for SECA (Figs 2A and 4B). This characteristic seropositivity pattern $rK39 > SPLA > SECA$ was also evident for the individual animals (Supplementary Table S8). This was suggestive that the relation between the antigens might have predictive value for the detection of CanL. In fact, the relationship between the three antigens ($nSPLA/nSECA$, $nrK39/nSECA$, $nrK39/nSPLA$) presented significant predictive power, as determined by ROC curve analysis (Supplementary Table S1). Still, their direct application in the European cohort did not result in a diminution of seropositivity (Supplementary Table S8). This was not unexpected because the relation is independent on absolute values. Therefore, very low serological results might be seropositive using this approach. Therefore, the unrestricted use of antigen seropositivity ratios present a significant risk of irrelevant artefactual values. Still, these data clearly established that the ratios between the antigens followed a pattern that is in conjunction with positivity to one or both reference antigens was found to be predictive of CanL+ (Fig. 1).

When applied to the European cohort, the score approach originated a global seropositivity of 1.8% (7/384). These seven animals were indistinguishable from the CanL+ cohort in at least four evaluated characteristics (one presented a perfect score of 5). This was the animal sorted upon the increase of rK39 threshold (PT30). Only one animal from Poland (PL24), which was classified with a score of 4, was not positive for rK39 alone. This sample was positive for SPLA and for the three ratios evaluated in the score system ($nSPLA/nSECA$, $nrK39/nSECA$ and $nrK39/nSPLA$). Although the absolute value of the OD to rK39 was below the cut-off (0.127) for this sample, this difference was only 0.021 (see PL24 in Supplementary Table S9). This is a clear example of problems associated with using absolute values. Subtle differences can place a serum in opposite sides of the threshold. For example, PT45 was seropositive for rK39, having 0.134 of average OD. Still, in our score system, it was negative with only a score of 3 due to a disproportionately high response to SECA and SPLA (Supplementary Table S9). The high reactivity to SECA prevented positive ratios and consequently preventing the higher score. This very high relative reactivity to SECA was not characteristic of our CanL+ cohort. PT34 is another interesting sample with overall high responses to all tested antigens with average ODs of 0.659 for SECA, 0.447 SPLA and 0.147 for rK39. Once again, this profile is not characteristic of the infected cohort and might reflect auto-immunity or other complications or a different stage of disease.

In the CanL+ group also, some samples are mentionworthy. The CanL+ 4 is the sample that

prevents a 100% Se for the score approach. This is the sample with the highest SECA average OD (2.455). This might be due to an already advanced stage of the disease resulting in the loss of the characteristic serological pattern of active disease due to other co-infections or even polyclonal activation.

The presented data demonstrate that the evaluation of seropositivity is a complex issue that is often oversimplified. We report that complex mixtures of antigens like SPLA are prone to cross-reactivity making them more unreliable for serosurveillance studies than rK39. Recombinant antigens like rK39 were less afflicted by regional-specific serological background and still might be more sensitive to subtle strain-specific changes in the native antigen. The use of ratios associated with serological responses to specific antigens can be used to create more accurate serological profiles that might contribute for identification of infected animals. Disease-specific serological footprints might be exploited to better understand the immune responses of clinically and sub-clinically infected and/or merely exposed animals, enabling early warning system for disease development and more accurate serosurveillance studies. Further studies with different recombinant antigens and experimentally infected animals and cohorts of animals with different clinical status will validate these observations and help to overcome the reported failure of complex antigens mixtures (SPLA) and recombinant proteins (rK39) in establishing meaningful seropositivity levels.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182017000713>.

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ETHICAL AND REGULATORY GUIDELINES

This study observed Portuguese legislation for the protection of animals (Law no. 92/1995, from 12 September). According to the European Directive of 24 November 1986, article 2 d, non-experimental, agricultural or clinical veterinary were excluded. The Animal Ethics Committee of the Associate Laboratory IBMC-INEB approved the animal protocol used. Serum samples were collected during vaccination campaigns, and informed consent was obtained from all dog owners before sample collection.

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