Metadata of the article that will be visualized in OnlineFirst

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Article Sub-Title		
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Journal Name	Veterinary Research Communications	
Corresponding Author	FamilyName	**Zatelli**
	Particle	
	Given Name	**Andrea**
	Suffix	
	Division	Department of Veterinary Medicine
	Organization	University of Bari
	Address	Valenzano, 70010, Bari, Italy
	Phone	
	Fax	
	Email	andrea.zatelli@uniba.it
	URL	
	ORCID	
Author	FamilyName	**Iatta**
	Particle	
	Given Name	**Roberta**
	Suffix	
	Division	Interdisciplinary Department of Medicine
	Organization	University of Bari
	Address	70124, Bari, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	
Author	FamilyName	**Paltrinieri**
	Particle	
	Given Name	**Saverio**
	Suffix	
	Division	Department of Veterinary Medicine and Animal Sciences
	Organization	University of Milan
	Address	26900, Lodi, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	
Author	FamilyName	**Cavalera**
	Particle	
	Given Name	**Maria Alfonsa**
	Suffix	
	Division	Department of Veterinary Medicine
	Organization	University of Bari

	Address	Valenzano, 70010, Bari, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	
Author	FamilyName	**Scavone**
	Particle	
	Given Name	**Donatella**
	Suffix	
	Division	Department of Veterinary Medicine and Animal Sciences
	Organization	University of Milan
	Address	26900, Lodi, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	
Author	FamilyName	**Otranto**
	Particle	
	Given Name	**Domenico**
	Suffix	
	Division	Department of Veterinary Medicine
	Organization	University of Bari
	Address	Valenzano, 70010, Bari, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	

Schedule	Received	5 Aug 2022
	Revised	
	Accepted	23 Oct 2022

Abstract

Canine leishmaniosis (CanL) by *Leishmania infantum* (*L.i.*) and heartworm disease by *Dirofilaria immitis* (*D.i.*) are common zoonotic vector-borne diseases (VBDs) characterized by a variety of pathological and clinical signs. The immunopathology in both VBDs is extremely complex, and their clinical manifestations are strongly dependent on the type of immune response elicited by the parasites. In particular, the formation of circulating immune complexes (CICs) plays an important role in the pathogenesis of these VBDs. Based on the international guidelines, dogs with high anti-*L. infantum* antibody titres and one or more clinical and/or laboratory signs related to CanL require anti-*Leishmania* treatment. Consequently, the CICs measurement could be used for improving the clinical staging process of CanL. The aim of the study was to assess the CICs level by a competitive inhibition enzyme immunoassay, in healthy or sick dogs seropositive to *L.i.* and in healthy dogs positive to *D.i.*. Out of 51 enrolled dogs, 11 were included in Group A (seronegative to *L.i.*, *D.i.* negative and healthy), 15 in Group B (exposed to *L.i.*, *D.i.* negative and healthy), 12 in Group C (seropositive to *L.i.*, *D.i.* negative and sick) and 13 in Group D (seronegative to *L.i, D.i.* positive and healthy). The comparison of CIC level in canine sera revealed a significant difference among groups ($P < 0.001$), with the highest concentration (i.e., median = 104.6 µg/mL) in dogs with CanL. The findings of the study highlight the CICs measurement as a useful tool in the clinical staging of CanL for avoiding misclassification of dogs as leishmaniotic, thus not requiring anti-*Leishmania* therapy, as well as the possibility of results misuse in geographical areas where both leishmaniosis and heart-worm disease are endemic.

Keywords (separated by '-') *Leishmania infantum* - Natural infection - CIC concentration - Dogs - *Dirofilaria immitis*

Footnote Information

Veterinary Research Communications
https://doi.org/10.1007/s11259-022-10031-8

RESEARCH

Assessment of circulating immune complexes in canine leishmaniosis and dirofilariosis

Roberta Iatta[1] · Saverio Paltrinieri[2] · Maria Alfonsa Cavalera[3] · Donatella Scavone[2] · Domenico Otranto[3] · Andrea Zatelli[3]

Received: 5 August 2022 / Accepted: 23 October 2022

Abstract

Canine leishmaniosis (CanL) by *Leishmania infantum* (*L.i.*) and heartworm disease by *Dirofilaria immitis* (*D.i.*) are common zoonotic vector-borne diseases (VBDs) characterized by a variety of pathological and clinical signs. The immunopathology in both VBDs is extremely complex, and their clinical manifestations are strongly dependent on the type of immune response elicited by the parasites. In particular, the formation of circulating immune complexes (CICs) plays an important role in the pathogenesis of these VBDs. Based on the international guidelines, dogs with high anti-*L. infantum* antibody titres and one or more clinical and/or laboratory signs related to CanL require anti-*Leishmania* treatment. Consequently, the CICs measurement could be used for improving the clinical staging process of CanL. The aim of the study was to assess the CICs level by a competitive inhibition enzyme immunoassay, in healthy or sick dogs seropositive to *L.i.* and in healthy dogs positive to *D.i.*. Out of 51 enrolled dogs, 11 were included in Group A (seronegative to *L.i.*, *D.i.* negative and healthy), 15 in Group B (exposed to *L.i.*, *D.i.* negative and healthy), 12 in Group C (seropositive to *L.i.*, *D.i.* negative and sick) and 13 in Group D (seronegative to *L.i, D.i.* positive and healthy). The comparison of CIC level in canine sera revealed a significant difference among groups ($P < 0.001$), with the highest concentration (i.e., median = 104.6 µg/mL) in dogs with CanL. The findings of the study highlight the CICs measurement as a useful tool in the clinical staging of CanL for avoiding misclassification of dogs as leishmaniotic, thus not requiring anti-*Leishmania* therapy, as well as the possibility of results misuse in geographical areas where both leishmaniosis and heart-worm disease are endemic.

Keywords *Leishmania infantum* · Natural infection · CIC concentration · Dogs · *Dirofilaria immitis*

Introduction

Visceral leishmaniosis caused by *Leishmania infantum* and heartworm disease (HWD) by *Dirofilaria immitis* are common zoonotic vector-borne diseases (VBDs) with a broad geographical distribution in temperate and tropical regions (Otranto et al. 2009).

Dogs are the main hosts and reservoirs of both parasites which are transmitted in the Mediterranean basin mainly by phlebotomine sand flies of the genus *Phlebotomus* and mosquitoes of the genus *Aedes*, *Anopheles* and *Culex*, respectively (Latrofa et al. 2018; Panarese et al. 2020).

For both VBDs the immunopathology is extremely complex, and their clinical manifestations are strongly dependent on the type of immune response elicited by the parasites. In this scenario, the formation of circulating immune complexes (CICs), as a consequence of prolonged presence of pathogen antigens and the production of high antibody levels, plays an important role in the pathogenesis of these VBDs and others such as ehrlichiosis and anaplasmosis (Harrus et al. 2001; Ravnik et al. 2014).

In canine leishmaniosis, *L. infantum* causes from chronic and subclinical conditions to overt clinical disease according to the host immune response (Paltrinieri et al. 2010). Indeed, resistant dogs develop an effective cell-mediated immune response (Th1), with the production of proinflammatory cytokines (i.e., IFN-γ and TNF-alfa), limiting the infection

✉ Andrea Zatelli
andrea.zatelli@uniba.it

[1] Interdisciplinary Department of Medicine, University of Bari, 70124 Bari, Italy

[2] Department of Veterinary Medicine and Animal Sciences, University of Milan, 26900 Lodi, Italy

[3] Department of Veterinary Medicine, University of Bari, Valenzano, 70010 Bari, Italy

and associated inflammation by increasing the leishmanicidal activity of macrophages. Contrarily, a dominant humoral response in susceptible dogs induces the release of Th2 associated-cytokines (i.e., interleukins IL-4 and IL-13), and the activity of regulatory T and B cells via IL-10. Therefore, the continuous antigenic stimulation and the antibody production result in the formation of circulating immune complexes (CICs) depositing in the target organs causing glomerulonephritis, vasculitis, polyarthritis, and uveitis (Day 2016; Roura et al. 2021). Moreover, the severity of the clinical presentation has been assumed to be related to high antibody level (Proverbio et al. 2014) along with the high concentration of CICs (Parody et al. 2019; Gizzarelli et al. 2020; Cacheiro-Llaguno et al. 2021). Conversely, in dogs affected by HWD, *D. immitis* is present in the cardiovascular system and the shedding of antigens by microfilariae (mcf) and adult worms is responsible for a wide range of pathological changes observed in the pulmonary vascular bed and lung parenchyma (McCall et al. 2008). Furthermore, the mcf circulation induces a higher expression of IL-4 and IL-10 than in dogs with occult infection, suggesting that their presence is associated with immune tolerance towards infection. Albeit few reports describe the circulation of immune complexes in dogs with HWD, their clinical implications need further investigations (Matsumura et al. 1986). Interestingly, in a recent study on the assessment of seasonal variation of anti-*L. infantum* antibody titres in dogs from a hyperendemic area, increased antibody titres were detected in asymptomatic animals during the transmission season (Cavalera et al. 2021). At the same time dogs are either exposed to sand fly bites developing a strong anti-saliva antibody response, therefore the stimulation of both saliva and parasite antigens may up-regulate the individual immune response (Quinnell et al. 2018). In this scenario the presence of asymptomatic dogs could be related to a low antigen-antibody ratio with a limited production of soluble CICs, and subsequent reduced inflammatory response, without the appearance of organ damage and clinical disease (Day 1999). According to international guidelines, dogs with high antibody titres and one or more clinical and/or laboratory signs related to CanL require anti-*Leishmania* treatment (Oliva et al. 2010; Paltrinieri et al. 2010). Therefore, the CICs measurement could be used for improving the clinical staging process, monitoring the disease progression and the response to treatment, and avoiding misclassification of dogs as leishmaniotic. Considering the almost overlapping endemicity of *L. infantum* and *D. immitis* infections in canine population from some regions of the Mediterranean basin (Mendoza-Roldan et al. 2020; Panarese et al. 2022) and the paucity of reports regarding the implications of CICs in the pathogenesis of canine HWD (Matsumura et al. 1986), the determination of CICs must be considered. Thus, the aim of this study was to determine the CICs level in dogs clinically healthy or sick and seropositive to *L. infantum*, and in dogs clinically healthy, seronegative to *L. infantum* and positive to *D. immitis*.

Materials and methods

Study design and availability of data

From February 2020 to February 2022, medical records and sera of dogs of any sex, age, and breed which were evaluated in concluded (Cavalera et al. 2021) or still ongoing (data unpublished) clinical and parasitological trials conducted in Apulia region (southern Italy), were retrospectively collected based on established criteria. Dogs were included if they scored *L. infantum* seronegative (Ab titre $\leq$ 1:80) or seropositive (1:80 < Ab titres < 1:2560) by indirect immunofluorescent antibody test (IFAT), and *D. immitis* positive by modified Knott's test or negative by both modified Knott's test and SNAP 4Dx Plus test (IDEXX Laboratories, Inc., Westbrook, ME, USA). The modified Knott's test and the IFAT for anti-*Leishmania* antibodies detection were performed as described in Panarese et al. 2020 and Otranto et al. 2009. For all included animals, data on clinical examination and laboratory test results (i.e., complete blood count [CBC], serum biochemical profile including C-reactive protein, and serum protein electrophoresis [SPEP]) were collected for classifying leishmaniotic dogs as sick or clinically healthy. Dogs were excluded if: (a) vaccinated for leishmaniosis; (b) seropositive to other vector-borne pathogens (VBPs), including *Ehrlichia canis* and *Anaplasma phagocytophilum* tested by indirect immunofluorescent antibody test (IFAT); (c) based on physical examination and laboratory results, suspected or known to be affected by diseases influencing the immune response and/or the inflammatory markers (e.g., neoplastic, auto-immune and heart diseases, diabetes mellitus and insipidus, hypo-, and hyperadrenocorticism or hyper- and hypothyroidism, chronic dermatopathies). All animals that had been administered drugs that can modify the immune and inflammatory response (e.g., glucocorticoids) in the previous three months were also excluded.

Based on the previous criteria, the animals were subdivided into 4 groups according to *L. infantum* IFAT results and clinical-pathological evaluation as reported in Paltrinieri et al. 2010, along with the results for *D. immitis* detection: group A including dogs *L. infantum* seronegative, with normal physical examination and without any clinical-pathological abnormalities and *D. immitis* negative; group B, dogs exposed to *L. infantum* with normal physical examination, without any clinical-pathological abnormalities and *D. immitis* negative; group C, seropositive to *L. infantum* with antibodies titre higher than 1:640 and sick dogs with one or more clinical signs related to

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leishmaniosis and/or clinical-pathological abnormalities, and *D. immitis* negative; group D, seronegative to *L. infantum* with normal physical examination, without any clinical-pathological abnormalities and *D. immitis* positive.

CICs level assessment

The quantitative measurement of CICs (µg/ml) was assessed in canine serum samples collected as follows: a 5 mL blood was sampled from the cephalic vein and placed in clot activator tube, then transported to the laboratory within 4 h, serum was obtained by centrifugation (1500 g for 15 min) and an aliquot (i.e., 100 µl) was promptly stored at -20 °C until CICs level assessment.

A competitive inhibition enzyme immunoassay technique method (Dog CIC ELISA - Cusabio Technology LLC, USA) was performed according to the manufacturer's instructions. Briefly, standards and samples diluted at 1:400 were pipetted into the wells, pre-coated with dog CICs, with a horseradish peroxidase (HRP) conjugated antibody specific for dog CICs. Following a wash to remove any unbound reagent, a substrate solution was added to the wells and the color intensity developed which is inversely proportional to the canine CICs concentration in samples is spectrophotometrically measured.

In order to check the performance of the kit, intra-assay imprecision was assessed by testing and reading in quadruplicate two pools of 3 samples each that in a preliminary test had high and low CICs concentration respectively. The inter-assay imprecision was assessed by reading 9 samples with variable amount of CICs in two separate sessions of tests. The accuracy of CICs measurement was indirectly evaluated by a linearity under dilution (LUD) test prepared by diluting the pool with high concentration of CICs that was diluted with distilled water (i.e., 80%, 60%, 40%, 20%).

Statistical analysis

Statistical analyses were performed using the Analyse-it software (Analyse-it Ltd. Leeds, UK). In both intra- and inter-assay tests, imprecision was determined by measuring the coefficient of variation (CV) using the formula: CV = SD/mean x 100. The correlation between expected and obtained results of the LUD tests was assessed using a linear regression test, followed by a test of lack of fit. The results of CICs level recorded in different groups of dogs, as described above, were compared to each other using a non-parametric ANOVA test for independent samples (Kruskal Wallis), followed by a Wilcoxon signed rank test as a post-hoc test. A P value less than 0.05 was considered statistically significant.

Results

Out of 313 dogs enrolled in previous or ongoing studies, records of 51 mixed breed dogs (i.e., 18 females and 33 males) aging from 3 to 15 years (median 7.8 years), met all the study inclusion criteria. Out of 51 enrolled animals, 11 were included in Group A, 15 in Group B, 12 in Group C, and 13 in Group D with a parasitic load higher than 3000mcf/100µl.

Using the pools of sera with low and high concentrations of CICs (11.5 and 71.6 µg/mL, respectively) the median CVs regarding intra-assay imprecision were 8.4% and 18.2% respectively, while regarding inter-assay imprecision, the median CV recorded in the 9 samples read in duplicate accounted for 17.7%, with values ranging from 0.3 to 42.8%. The linearity under the dilution test provided excellent results ($r^2 = 0.942$; $P = 0.004$).

A significant difference in the concentration of CICs among groups has been observed ($P < 0.001$) (Fig. 1). In particular, dogs in group C (i.e., sick) had the highest concentration of CICs (119.2 ± 43.0 µg/mL; median: 104.6 µg/mL; interquartile range: 78.0-159.0 µg/mL) compared with dogs of group B (47.6 ± 12.4; 47.6; 40.2–55.4; $P < 0.001$), group D (52.1 ± 27.3; 46.8; 33.6–73.7, $P < 0.001$), and group A (65.2 ± 21.2; 57.3; 55.4–71.7, $P < 0.001$). In the latter group, two CICs values were outliers (i.e., 98.97 and 106.19 µg/mL). The other groups were not significantly different from each other, except for a significant difference ($P = 0.024$) between Group B (exposed dogs) and group A (dogs negative for both the parasites).

Discussion

This study describes the variation of CICs level in a canine population from a southern region of Italy as characterized by a significant increase in leishmaniotic dogs (group C) compared with those clinically healthy but seropositive to *L. infantum* and dogs seronegative to *L. infantum* and positive to *D. immitis*. The diagnosis of CanL may be challenging and a multiple approach (i.e., clinical examination, assessment of clinicopathological findings, direct and indirect detection of the parasite) is required for determining the clinical stage of patients and enabling a suitable treatment. Therefore, the quantification of CICs may help in improving the clinical staging of CanL in particular in dogs with high anti-*L. infantum* antibody titres but without any clinical signs related to leishmaniosis. To date, the assessment of CICs level has been used for revealing the pathologic stage in naturally infected dogs (Parody et al. 2019), and for evaluating their prognostic value in

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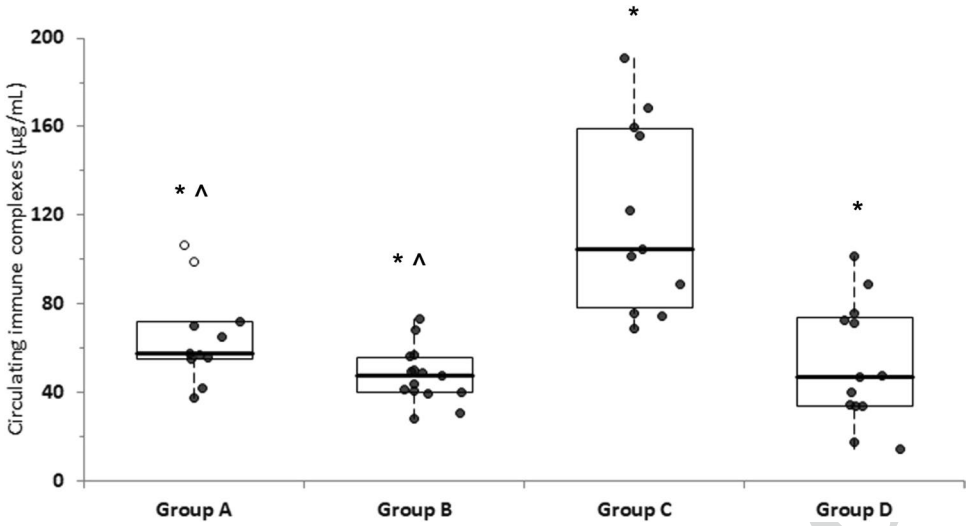

AQ4 **Fig. 1** Distribution of results recorded in the four groups of dogs. Group A: clinically healthy dogs seronegative for *L. infantum* and microfilaria (mcf) negative; Group B: clinically healthy dogs seropositive for *L. infantum* and mcf negative; Group C: dogs with clinical signs consistent with leishmaniosis, seropositive for *L. infantum* and mcf negative; Group D: clinically healthy dogs seronegative for *L. infantum* but *D. immitis* mcf positive. The boxes indicate the first to third interquartile range (IQR), the horizontal lines indicate the median value, and whiskers extend to further observation within the first quartile minus 1.5 x IQR or to further observation within the third quartile plus 1.5 x IQR. Black dots indicate values that were not classified as outliers; white dots indicate the near outliers (values exceeding the third quartile ± (1.5 x IQR). Statistical differences in the concentration of CICs among groups are indicated with symbols (*$P < 0.001$; ^$P = 0.024$)

naturally and experimentally infected dogs (Gizzarelli et al. 2020). Moreover, the effect of vaccination with LetiFend® in reducing the CICs level as a mechanism to control CanL has been also described (Cacheiro-Llaguno et al. 2020).

In this study, the concentration of CICs was measured using an ELISA method that, based on the data regarding precision and accuracy generated in the current study, can be considered sufficiently accurate and precise to be used in studies based on group comparison, as herein described, or in clinical practice: in particular, both intra- and inter-assay imprecision, despite higher than those reported by the producer of the kit (< 8% and < 10%, respectively), are in accordance with those considered as acceptable for most of the tests routinely employed in clinical pathology (Ricós et al. 1999). Using this analytical method, significant differences among dogs in group C and the other groups were recorded, and the magnitude of changes between this and the other groups is higher than the intrinsic variability of the method, suggesting that the differences between groups really depend on a different concentration of CICs rather than on the imprecision of the method. In turn, the detection of this difference among groups indicates that the deposition of CICs in target organs may be among the causes of tissue damage leading to vasculitis, uveitis and renal failure. Indeed, dogs in group C showed higher CICs concentration compared with the exposed ones. Therefore, dogs seropositive to *L. infantum* with clinical-pathological signs associated with other diseases and living in endemic regions for leishmaniosis, could be wrongly considered sick thus receiving unnecessary anti-*Leishmania* therapy. Indeed, asymptomatic *L. infantum*-seropositive dogs living in a hyperendemic area for CanL can present an increase of antibody titres during the sand fly season (Cavalera et al. 2021) due to uninfected and *L. infantum*-infected sand fly bites leading to an up-regulation of the humoral immune response. As a consequence, the limited availability of *L. infantum* antigens and the low level of antibodies may reduce the formation of soluble CICs leading to a reduced inflammatory response and absence of clinical signs (Day 1999; Roura et al. 2021).

The absence of differences in CICs level between dogs seronegative to *L. infantum* and positive to *D. immitis*, those negative to both parasites and exposed dogs supports the hypothesis that in dogs with HWD the formation of immune complex occurs in situ as part of the pathogenesis of glomerulonephritis despite being a less common clinical manifestation than cardiopulmonary and hepatic lesions (Abramowsky et al. 1981; Grauer et al. 1987; Paes-De-Almeida et al. 2003).

The significant difference in CICs level between exposed dogs and those negative to both parasites even with similar concentrations (47.6 vs. 57.3 µg/mL), but significantly lower than leishmaniotic dogs, may be due to CICs concentrations of the outliers recorded in two dogs with other conditions not revealed during the clinical examination such as

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autoimmune diseases (Weissmann 2009; Baba et al. 2019), allergic diseases (Park and Nahm 1998) or cancer (Beneduce et al. 2008; Madki et al. 2021).

This study highlights the importance of CICs measurement as a useful tool in the clinical staging process of CanL, particularly in dogs with high antibodies titres and clinical-pathological signs associated with other diseases, that may be misclassified as leishmaniotic and do not require anti-*Leishmania* therapy. Furthermore, the findings of low level of CICs in clinically healthy dogs seronegative to *L. infantum* but positive to *D. immitis* avoid the possibility of results misuse in geographical areas where both leishmaniosis and HWD are endemic such as the regions of the Mediterranean basin.

Finally, potential limitations of the study should be considered including the long storage period of the serum samples that may have affected the CIC level determination although the results herein obtained denoted their stability at -20 °C. Furthermore, the assessment of CIC level in dogs coinfected by *L. infantum* and *D. immitis* herein missed for avoiding results overlapping and the sera collection over a long timeframe including transmission and non-transmission seasons thus different exposures to vector bites of dogs that could have resulted in changes in their immune response. Further studies will be carried out by enrolling dogs coinfected by both parasites and dogs during different vector seasons.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Roberta Iatta, Domenico Otranto, Saverio Paltrinieri, Andrea Zatelli. The first draft of the manuscript was written by Roberta Iatta, Andrea Zatelli. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval This study was approved by the Ethics Committee of the Department of Veterinary Medicine, University of Bari, Italy (approval number 12/20).

Competitive interests The authors have no relevant financial or non-financial interests to disclose.

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