Leishmaniasis: The prevalence in the Netherlands and the relation between height of DAT titre and different staging systems.

Research Project Veterinary Medicine Utrecht University

A.E.H. Albers, BSc
5605342
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Supervisor:
Dr. C.J. Piek
Department of Clinical Sciences of Companion Animals
Faculty of Veterinary Medicine, Utrecht University

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Aim 1: Abstract

Objective: To determine the (sero)prevalence of Canine Leishmania, Ehrlichia and Dirofilaria *immitis*, non-endemic vector borne diseases, in dogs imported from endemic areas to the Netherlands.
Study Design: This is a prospective study.
Animal: Dogs.
Methods: Seven dogs imported from Mediterranean endemic areas by several animal welfare organisations were clinically examined within six weeks after import. Blood samples were taken to determine the prevalence of Canine Leishmaniasis, Ehrlichiosis and Dirofilariasis. DAT titres for CanL antibodies and Knott's tests were performed, blood samples for Ehrlichiosis were stored for tests in the future.
Results: Of the seven dogs, only one dog had a positive DAT titre (1:5120). The prevalence at this point of the study was determined and set on 14,3%. Also, one of the seven dogs was tested positive on heartworm antigen, giving a prevalence of 14,3% as well.
Conclusion: The prevalence of both Canine Leishmania and Dirofilaria *immitis* in the Netherlands in this point of the study was set on 14,3%.
Key words: Dog, Canine, Leishmaniasis, Leishmania *infantum*, seroprevalence, DAT antibody titre, Dirofilaria *immitis*, Ehrlichiosis, vector borne disease.
Abbreviations: Canine Leishmaniasis (CanL); Leishmania *infantum* (*L. infantum)*; immunofluorescence antibodies test (IFAT); direct agglutination test (DAT); enzyme linked immunosorbent assay (ELISA); Utrecht University Clinic of Companion Animals (UUCCA); Stray Animal Foundation Platform (SAFP); capillary refill time (CRT); haematocrit (Ht); content of hemoglobin in reticulocytes (CHr); ethylenediaminetetraacetic acid (EDTA); University Veterinary Diagnostic Laboratory (UVDL).

Aim 2: Abstract

Objective: To determine the relation between the level of the DAT antibody titre and several different staging systems used to classify a patient based on severity of clinical signs and haematological and biochemical results of Canine Leishmaniasis.
Study Design: This is a retrospective study.
Animal: Dogs.
Methods: Positive and negative titres (n = 109) as well as height of the DAT titre (n = 39) were compared to clinical signs and haematological and biochemical parameters. All dogs were born or had travelled to endemic areas and had clinical signs suggesting infection with CanL and were clinically examined and blood samples were taken. Also, all of these dogs had a DAT titre performed. Retrospectively, all dogs were assigned to fitting stages in five different clinical staging systems, according to severity of clinical signs, and haematological and biochemical alterations.
Results: For the first part of the aim, most common alterations in dogs with a negative DAT titre (n = 26) were decreased endurance (61,5%), lymphadenopathy (57,5%) and anaemia (69,2%). In dogs with a positive titre (n = 83), most common clinical signs were lymphadenopathy (66,3%), decreased endurance (61,4%), alopecia (43,4%) and hypergammaglobulinemia (73,5%). For the second part of the aim, of the 39 dogs 15 dogs had a positive DAT titre (>1:40). Most common clinical signs and haematological and biochemical alterations found in dogs with a positive DAT titre were lymphadenopathy (66,3%), hypergammaglobulinemia (73,5%) and anaemia (67,5%). This study shows that in all used clinical staging systems no correlations were found between the height of the DAT titre and clinical signs, except for the system of CLWG (1). Besides, height of the DAT titre was not related to any of the haematological or biochemical parameters used in this study.
Conclusion: The DAT titre is not significantly related to clinical signs, haematological and biochemical parameters used in this study. However, a positive correlation was found between DAT titre and severity of clinical signs according to the staging system of CLWG.
Key words: Dog, Canine, Leishmaniasis, Leishmania infantum, DAT antibody titre, clinical staging system.
Abbreviations: Canine Leishmaniasis (CanL); Leishmania *infantum* (*L. infantum)*; immunofluorescence antibodies test (IFAT); direct agglutination test (DAT); enzyme linked immunosorbent assay (ELISA); polymerase chain reaction (PCR); University Veterinary Diagnostic Laboratory (UVDL); Utrecht University Clinic of Companion Animals (UUCCA); capillary refill time (CRT); haematocrit (Ht); urea-protein-creatinine ratio (UPC ratio); Canine Leishmaniosis Working Group (CLWG); ethylenediaminetetraacetic acid (EDTA).

Introduction

Leishmaniasis, Ehrlichiosis and Dirofilariasis are all vector-borne diseases endemic in mostly Southern European countries. Despite the fact that over the years the number of imported dogs and dogs traveling from those endemic countries to non-endemic countries increases, little is known about the prevalence of these diseases in the Netherlands (2)(3)(4). Radstake *et al*. (2017) reported that 12608 dogs were imported to the Netherlands in 2016, meaning that there is a lot of demand these dogs (4). The main focus of this study was Canine Leishmaniasis (CanL).

CanL, caused by *Leishmania infantum* (*L. infantum*) is a zoonotic vector-borne disease. It was first described in dogs in 1908 and is endemic in more than 70 countries, including regions of southern Europe, Africa, Asia and South and Central America (5). CanL is transmitted by the sand fly of the genus *Phlebotomus* in its flagellated infective promastigote form to a mammal. In the mammal, the intracellular amastigote form develops and replicates (6). Infection in cats, wild canids and horses has been reported in these endemic areas as well. However, dogs are considered the main reservoir of *L. infantum* (7). Due to socioeconomic and climate factor changes, the distribution of *L. infantum* has spread more northward in Europe (6). Several reports of CanL in non-endemic countries such as Germany, the United States, Denmark and Austria have been published. Despite those reports, the prevalence in all these countries is unknown, but it is important to veterinarians and potential dog owners, since infected dogs may contribute to the maintenance of the parasite within the canine population and the fact that it is spreading to the North of Europe, where it could occur in a new non-endemic area (7)(8).

CanL is a systemic disease that may cause damage to any organ, tissue or body fluid (6). Clinical presentations in infected dogs can manifest as subclinical infection, self-limiting illness or life-threatening disease (7). Most dogs develop the cutaneous signs, causing skin lesions and alopecia generalized or localized over the face, ears and limbs (7). Other clinical signs of CanL are non-specific, such as generalized lymphadenopathy, loss of body weight, change in appetite and decreased endurance (6). These clinical signs can develop as early as after 2 months after infection, but within 7 years (9). The clinical spectrum is broad and the clinicopathological alterations based on blood analysis, biochemical profile and urinalysis are multiple and non-specific (6). The variety of clinical signs is due to the complexity of the disease, affecting different organs, and the diversity of immune responses of the individual hosts (5). The role of the protective immunity and disease susceptibility is highly important. The protective immunity would consist of CD4 T-cell mediated by γ-interferon, interleukine-2 and tumor necrosis factor α-release. These factors induce activation of macrophage anti-*Leishmania*. The macrophages produce nitric oxide which mediates intracellular killing of *Leishmania* amastigotes by apoptotic cell death (7)(10). The susceptibility is associated with the production of a marked humoral non-protective immune response and a reduced or depressed cell mediated immunity with a mixed Th1 and Th2 cytokines response (7).

Since not all infected dogs show clinical signs, diagnosis of the disease is important, yet complex, due to the various clinical presentations and individual immune responses. The most commonly used techniques for the detection of CanL are quantitative serological techniques such as an immunofluorescence antibody test (IFAT), an enzyme-linked immunosorbent assay (ELISA), or a direct agglutination test (DAT) for the detection of specific serum antibodies (11). High levels of antibodies confirm infection with CanL in dogs with clinical signs and/or clinicopathological alterations compatible with CanL (12). However, a low level of antibodies does not exclude CanL infection (7). Further work-up is necessary by other methods such as cytology, histopathology or polymerase chain reaction (PCR) (13). According to the LeishVet guidelines for practical management of CanL, diagnostic approach for sick or healthy dogs living in non-endemic areas that have travelled to endemic areas should include quantitative serology three months after the beginning of exposure (6).

Seroconversion is important when testing dogs for infection with CanL. Some dogs can remain seronegative long after being infected with CanL (14). Due to the long incubation period, sick dogs are more likely to be tested seropositive (15). Seroconversion could be used to investigate the best moment to test dogs traveling from endemic areas to non-endemic areas.

Several clinical classification systems of Leishmaniasis in dogs have been described and are used for monitoring the course of the disease, treatment and prognosis. Patients are staged in a certain moment in time, which can change as the disease progresses or improves (16). The classical clinical staging system by Mancianti *et al.* (1988) is based only on the physical exam, consisting of asymptomatic, oligosymptomatic and polysymptomatic, but this system does not consider clinicopathological abnormalities and disregards subclinical infected dogs (16)(17). Meléndez-Lazo *et al.* (2018) compared five of these clinical staging systems, including Mancianti's system. They stated that the criteria for these systems should be simple and clinical, with the use of uncomplicated diagnostic methods. Besides that, they stated that parameters reflecting renal damage are important as well, because they have an impact on the prognosis of dogs with CanL (16).

Torres *et al.* (2011) stated that the antibody titre remains high for years, long after clinical signs abate (18). Although different studies show a correlation between the number of antibodies and clinical signs, not all authors agree on the usefulness of antibody titres as a mean of assessing the activity of the Leishmania infection at the time of diagnosis, during treatment, or flare-ups of the disease (19)(20). For example, Solano-Gallego *et al.* (2016) show a significant correlation between reduction of antibodies and clinical improvement using an in-house diagnostic ELISA (11) and Rodríguez *et al.* (2006) found that there was an association between slow, progressive reduction in ELISA antibody titre and a good clinical response to therapy (21). In contrast to these authors, Mateo *et al.* (2009) did not find a significant correlation between serological titres by IFAT results and clinical signs (22). A recent pilot (unpublished data) suggested that some of blood samples may have a DAT titre above 1:5120. The DAT can be executed in higher dilutions in positive samples (1:5120). To establish the maximum reaction titre, samples can be executed in serially dilution ranging from 1:40, up to 1:81920. These higher titres could be related to clinical, haematological and biochemical alterations.

The first aim of this study concerns the fact that despite the consequences of CanL on the quality of life of dogs, little is known about the prevalence in the Netherlands. Therefore, the aim was to investigate the prevalence of CanL, Ehrlichiosis and Dirofilariasis, non-endemic vector borne diseases, in dogs imported from endemic areas to the Netherlands. Since this was a prospective cohort study to which several students will contribute over time, seroprevalences of these diseases will be determined in the future, when concluding the study.

The second aim was to determine the relation between the level of the DAT antibody titre and several different staging systems used to classify a patient, based on severity of clinical signs and haematological and biochemical results of CanL.

Aim 1

Materials and Methods

The first aim of this study was to determine the prevalence of Canine Leishmaniasis, Ehrlichiosis and Dirofilariasis, non-endemic vector borne diseases, in dogs imported from endemic areas to the Netherlands.

Material

This was a prospective cohort study to which several students will contribute over time. The cohort was selected from dogs imported from Mediterranean endemic areas by several animal welfare organisations. These dogs were examined within six weeks after import and will be examined again in nine months to one year for clinical or laboratory evidence of these diseases.

Animals and inclusion and exclusion criteria

The inclusion criteria were:

A) Dogs must be at least six to seven months of age;
B) Dogs must be born in an endemic area;
C) Dogs must not yet have resided in the Netherlands for longer than six weeks;
D) The owner of the dog must agree to return to the Utrecht University Clinic of Companion Animals (UUCCA) after nine months for a second examination for determination of the seroprevalence.

The exclusion criteria were:

A) Dogs under the age of six months;
B) Dogs over the age of six months, who have resided in the Netherlands for longer than six weeks;
C) Dogs born in other area's then which are known endemic;
D) Dogs who only visited the UUCCA once, which makes it impossible to determine seroprevalence.

Ethical approval

The protocol has been discussed with the Utrecht Animal Welfare Body. Since the examinations and testing are part of regular veterinary care, the dogs are considered to be patients and not regarded as laboratory animals. Informed consent was collected from all owners (attachment 1).

Methods

The Stray Animal Foundation Platform (SAFP) collaborated to this study in selecting a list of 66 animal welfare organisations that import dogs from endemic areas to the Netherlands. These organisations were all contacted by e-mail and telephone in December 2018 to February 2019 and were asked to collaborate in this study. The organisations provided dogs that met the inclusion criteria and their new potential owners to collaborate on this study. Potential owners were informed by a letter and an informative brochure (attachment 2 and 3).

Physical exam and blood examination

Dogs will be examined twice; the first time was in the first six weeks of residence in the Netherlands. The second time will be nine months to a year later (by other students). The examinations consisted of a complete signalment, history, physical exam, dermatologic examination and laboratory testing. The exams were performed by last year veterinary students under supervision of a veterinary specialist in internal medicine. When indicated as such by abnormalities in the history or the physical exam, a

complete cardiac, ophthalmologic or orthopaedic examination was performed by a veterinary specialist.
The following data was sampled:

- Signalment: sex, breed, age, weight, chip number;
- Results of anamnesis: environmental history, time resided in the Netherlands, results of earlier laboratory tests, clinical signs or abnormalities (according to the owner);
- Results of physical examination: respiratory rate and character; heart/pulse rate; rectal temperature; coat and skin; colour, capillary refill time (CRT) and aspect of mucosal membranes; and palpation of lymph nodes;
- Results of dermatological examination: morphology, configuration, skin colour, smell, turgor, elasticity, sensibility, coat and nails;
- Laboratory results: DAT antibody titre, ehrlichia titre, dirofilarial antigen, Knott's test, haematocrit (Ht), reticulocytes, reticulocytes absolute, content of hemoglobin in reticulocytes (CHr), leukocytes, lymphocytes, monocytes, blastocysts, metamyelocytes, band cells, neutrophils, eosinophils, basophils, normoblasts and thrombocytes.

Laboratory data collection

Blood samples of 10 mL were collected from the jugular vein. The amount was divided into three tubes of 1 mL containing ethylenediaminetetraacetic acid (EDTA) and three Z Serum Sep Clot Activator tubes for 2,5 mL. The ADVIA 120 and ADVIA 2120i haematology systems were used to analyse the blood samples. Both systems were run with veterinary software.

The antibody titre for Leishmania was measured by the DAT method according to the recommendations by El Harith *et al.* (1989) (23). Blood sample analysis and determination of the Leishmania DAT titre was performed by haematological veterinary analysts at the University Veterinary Diagnostic Laboratory (UVDL), as well as analysis for antigens of adult heartworms using the FASTest®HW Antigen kit.

A Knott's test was performed on all blood samples within 24 hours after collecting the sample, as well as haematological analysis and a DAT antibody titre (attachment 5). The Knott's test was performed by last year veterinary students. To perform the Knott's test 1 mL of the EDTA sample was mixed with 9 mL formaldehyde (2%). This was spun down in a centrifuge for five minutes (1250 routes per minute) at 21°C. Then the sample was reduced until 1 mL was left. A single drop was put on a microscope slide. The remainder of the sample was mixed with several drops of methylene blue and one drop of that mixture was put on another microscope slide. Both samples were put under a microscope (10x and 40x magnification) to determine if Dirofilaria *immitis* larvae were visible. All blood samples were stored to test for serology for ehrlichiosis in the future.

Statistical analysis

All statistical analyses were performed using commercial statistical software (R studio, Version 1.1.463 – © 2009-2018 RStudio, Inc.). From the number of imported dogs (n=11300, 2015) a sample size of 241 dogs was calculated to be sufficient to detect a prevalence of 20% with a 95% confidence interval of 15-25%, using an online available calculator (24).

Results

Descriptive analysis

Seven dogs met the inclusion criteria. Of these seven dogs, three were male (all neutered) and four were female (all spayed). The mean age was 2,9 years (range 0,7 to 4,9 years old). Breed distribution was one Spanish Bullmastiff and six crossbreeds. Three dogs were born in Spain, two dogs were born in Portugal and two dogs were born in Greece. Six dogs were tested for vector borne diseases earlier in the country of birth, using different methods (table 1). One dog was already tested positive for IgG against CanL and one dog was tested positive on Ehrlichiosis and Dirofilariosis.

Table 1 Results of the dogs tested in country of birth (n = 7). ND: not determined, NA: not available, *: no further information was available on which test was used.

Dog Nº	Sex	Disease	Test	Outcome
1	Female	ND	ND	ND
2	Female	Leishmaniosis	IgG*	**Positive**
3	Female	Leishmaniosis	NA	Negative
		Ehrlichiosis	NA	**Positive**
		Dirofilariosis	NA	**Positive**
4	Female	Leishmaniosis	ELISA (antigen)	Negative
		Ehrlichiosis	ELISA (antibodies)	Negative
		Dirofilariosis	ELISA (antibodies)	Negative
5	Male	Leishmaniosis	ELISA (antigen)	Negative
		Ehrlichiosis	ELISA (antibodies)	Negative
		Dirofilariosis	ELISA (antibodies)	Negative
6	Male	Leishmaniosis	IgG	Negative
7	Male	Leishmaniosis	NA	Negative
		Ehrlichiosis	NA	Negative

Results in anamnesis

From the questions asked in the anamnesis, the following results were found; two dogs had signs of polyuria and/or polydipsia. One dog had a slight decrease in endurance. One dog was tested positive for antibodies against Leishmania prior to importation to the Netherlands and received treatment in form of allopurinol and glucantime. The other three dogs did not have any signs according to the owner.

Results in physical examination

No mayor abnormalities were found during the physical examination in respiration rate, heart rate, temperature, coat, skin and mucosal membranes. Two dogs had enlarged left and right popliteus lymph nodes. One dog had a firmer left popliteus lymph node in comparison to the right one. Three dogs had palpable axillaries accessories lymph nodes on left and right side.

Results in dermatologic examination

The following results listed in table 2 were found for the individual dogs during dermatologic examination.

Table 2 Results of dermatologic examination in individual dogs (n = 7).

Dog *Nº*	Description
1	Little bold spot on left elbow, scar on nose
2	Hypotrichosis on lips, nose and eyes, crustae on left and right front leg, little bold spot on right hindleg and left heel with exfoliation, squamae on the back, erythema around eyes, on ears and on right front leg, callus on both front legs
3	Scar with exfoliation and hyperkeratosis on right ear, mild exfoliation on the back, nails in the front are a bit long
4	Crustae on left pinnae, erythema and hypotrichosis of the interdigital skin on the right front paw, lichenification of the left and right heel, mild exfoliation on the back
5	Exfoliation on both ears, erythema and hypotrichosis of the interdigital skin on the right front paw, erythema of the interdigital skin on the left front paw, little bold spot and hyperkeratosis on left heel, mild exfoliation on the back
6	Scar on nose, mild exfoliation and hyperkeratosis on left pinnae, erythema and discoloured hairs of the interdigital skin on both front paws and right hind paw, discoloured hairs of the interdigital skin on left hind paw, mild exfoliation on left heel and on the back
7	Discoloured hairs under all four paws

Results in blood

The following results listed in table 3 were found in the blood of the individual dogs. One dog had leukocytes and neutrophils above laboratory references, one dog had lymphocytes above laboratory references and one dog had eosinophils above laboratory references and a Ht below laboratory references.

Table 3 Results in haematological parameters determined at the UVDL (n = 7).

Dog *Nº*	Ht (L/L) (0,41-0,61)	Reticulocytes (<1,5%)	Reticulocytes (absolute) (x10ˆ9/L) (5,2-126,5)	CHr (fmol) (1,43-1,71)	Leukocytes (x10ˆ9/L) (4,5-14,6)	Lymphocytes (x10ˆ9/L) (0,8-4,7)	Monocytes (x10ˆ9/L) (0,0-0,9)	Blastocytes (x10ˆ9/L) (<0,0)
1	0,56	NA	NA	NA	11,1	5,4	0,5	0
0	0,41	1	60,2	1,53	14,8	2,5	0,7	0
3	0,47	0,9	60	1,62	9,9	2,0	0,3	0
4	0,52	0,1	9,8	1,62	5,3	1,8	0,3	0
5	0,52	0,2	18	1,64	10,3	2,5	0,5	0
6	0,44	0,3	19,8	1,62	9,6	2,6	0,5	0
7	0,38	0,8	48,7	1,51	11,4	2,6	0,3	0

Dog *Nº*	Metamyelo-cytes (x10ˆ9/L) (<0,0)	Band cells (x10ˆ9/L) (0,0-0,3)	Neutrophils (x10ˆ9/L) (2,9-11,0)	Eosinophils (x10ˆ9/L) (0,0-1,6)	Basophils (x10ˆ9/L) (0,0-0,1)	Normoblasts (/100 leukocytes)	Thrombocytes (x10ˆ9/L) (144-603)
1	0	0	4,9	0,3	0	0	350
2	0	0	11,3	0,2	0,1	0	189
3	0	0	7	0,5	0	0	253
4	0	0	2,9	0,4	0	0	146
5	0	0	7,1	0,1	0	0	158
6	0	0	5,4	1,1	0	0	187
7	0	0	6,4	2,2	0	0	416

All dogs were tested at the UVDL for CanL antibodies, Dirofilaria *immitis* larvae and antigens of adult worms. In one dog, an ehrlichia titre was performed after examination, since this was indicated due to the clinical signs reported prior to the participation of this study. All results are listed in table 4.

Table 4 Results of the dogs tested at the UVDL (n = 7). NA: not available.

Dog *Nº*	Disease	Test	Outcome
1	Leishmaniosis	DAT titre	<1:40
	Dirofilariosis	Knott	Negative
		Antigen	NA
	Ehrlichiosis	IFAT titre	>1:80
2	Leishmaniosis	DAT titre	**1:5120**
	Dirofilariosis	Knott	Negative
		Antigen	NA
3	Leishmaniosis	DAT titre	<1:40
	Dirofilariosis	Knott	Negative
		Antigen	**Positive**
4	Leishmaniosis	DAT titre	<1:40
	Dirofilariosis	Knott	Negative
		Antigen	Negative
5	Leishmaniosis	DAT titre	<1:40
	Dirofilariosis	Knott	Negative
		Antigen	Negative
6	Leishmaniosis	DAT titre	<1:40
	Dirofilariosis	Knott	Negative
		Antigen	Negative
7	Leishmaniosis	DAT titre	<1:40
	Dirofilariosis	Knott	Negative
		Antigen	Negative

One of the seven dogs had a positive DAT titre (1:5120). From this cohort (n=7) a prevalence for CanL was calculated of 14,3% (1/7). Considering Dirofilariasis, only one dog had antibodies against Dirofilaria *immitis* adults. The prevalence for Dirofilariasis was calculated of 14,3% as well. The prevalence of Ehrlichiosis will be calculated in the future, since all serology samples are stored.

Discussion

The aim of this study was to determine the prevalence of Canine Leishmaniasis, Ehrlichiosis and Dirofilariosis, non-endemic vector borne diseases, in dogs imported from endemic areas to the Netherlands. At this point of the study, a prevalence of 14,3% was found for CanL as well as for Dirofilariosis.

Important to keep in mind, is that most of these dogs were selected by animal welfare organisations. All of these organisations have different methods for adopting dogs from the endemic areas. Most of them have a selection procedure for the dogs, which includes testing for several diseases in the country of birth before being placed in a shelter for adoption. Due to this, a selective bias occurred in the cohort.

No major abnormalities where found during physical examination, aside from some dogs with enlarged lymph nodes, which are common abnormalities for dogs with CanL infection (6). According to the results from the dermatologic exam, only one dog had several fitting skin lesions for a CanL infection (6). This dog had already tested positive before visiting the UUCCA and had received treatment.

The blood results showed that one dog had leukocytes and neutrophils above laboratory references, one dog had lymphocytes above laboratory references and one dog had eosinophils above laboratory references and a Ht below laboratory references. The dog with slightly higher leukocytes and neutrophils had a positive CanL titre and had already received treatment. Possible alterations found in haematological parameters in dogs with CanL could be leukocytosis and neutrophilia (25). These findings are common in dogs infected with CanL, and therefore could be fitting for this dog.

In this study, the prevalence of CanL, at the period of time the DAT titre was performed, was determined and set at 14,3%. Little is known about the (sero)prevalence of CanL in other non-endemic countries, which makes it hard to compare the result in this study with results found in other studies. Schäfer *et al.* (2019) did a retrospectively study on vector-borne diseases in dogs imported from Mediterranean regions and Southeastern Europe. Most dogs presented at their clinic had clinical signs. Even though their inclusion criteria were slightly different than the criteria used in this study, they found 6 out of 50 dogs that resides in Germany for 0 to 2 months, tested positive for L. *infantum* (26). This is results in an occurrence of 12%. However, the cohort in this study was too small (n = 7) to be representative for the population imported dogs from Southern European countries (n = 12608, in 2016 (4)) to the Netherlands. Besides, the fact that only one dog had a positive DAT titre does not mean that the other six dogs could not have been infected with CanL as well, due to the high prevalence of subclinical infections (6).

The (sero)prevalence of Dirofilaria *immitis* is also unknown. In Southern European countries where the disease is endemic, prevalences are found in high percentages, depending on the region of the country with the highest prevalence found in Italy (50-80%)(27). Schäfer *et al.* (2019) found 1 out of 50 dogs that resides in Germany for 0 to 2 months tested positive for Dirofilaria spp, a percentage of 2% (26), which is lower than the 14,3% found in this study.

Since this was a prospective cohort study to which several students will contribute over time, a more specific number of these (sero)prevalences will be determined in the future, when concluding the study.

Conclusion

The prevalence of both Canine Leishmaniasis and Dirofilariasis in the Netherlands in this point of the study was set on 14,3%. Since this was a prospective cohort study to which several students will contribute over time, a more specific number of these (sero)prevalences will be determined in the future, when concluding that study.

Aim 2

Materials and Methods

The second aim of this study was to determine the relation between the level of the DAT antibody titre and several different staging systems used to classify a patient based on severity of clinical signs and blood results of Canine Leishmaniasis.

To answer this question, two sub questions were made:

1. Is there a difference between clinical, haematological and biochemical manifestations found in dogs with positive (>1:40) and negative (<1:40) titre?
2. Is the height of the DAT antibody titre related to clinical, haematological and biochemical manifestations according to clinical staging systems?

Material

Animals and inclusion and exclusion criteria

The following inclusion criteria were used:

A) Dogs must be born in or have travelled to endemic areas;
B) Presence of clinical signs that warrant a differential diagnosis of CanL;
C) Dogs must have a leishmania DAT titre performed at the UVDL;
D) For the purpose of this study, a diagnosis of Leishmaniasis was defined as clinical signs of Leishmaniasis, in combination with either a positive titre or the confirmation of amastigotes in tissue samples;
E) Dogs must have a complete medical file, including history and the results of physical examination at the time point that the DAT is performed. The medical record must be detailed enough that in retrospect, the diagnosis of Leishmaniasis can be confirmed;
F) Therefore, information on the following clinical signs and biomarkers must be present:
    a. Birth in, or history of traveling to Southern European countries;
    b. State of awareness;
    c. Changes in appetite;
    d. Results of physical examination;
    e. Description of appearance of skin lesions, such as morphology, configuration and location;
    f. Condition of nails;
    g. Signs of kidney involvement/insufficiency;
    h. Signs of arthritis/joint stiffness.
G) Results of laboratory tests were collected from the same blood samples which were used to determine the antibody titre, within a range of 7 days before or after the height of the DAT titre was determined.

Exclusion criteria used for this aim were:

A) Dogs who did not have a history of travelling to endemic area's;
B) Dogs who did not have a medical file with results of physical examination and/or blood results at the time point that the DAT is performed;
C) Dogs who received treatment for CanL prior to their visit at the UUCCA[1].

Medical record review

Data extracted from the medical records of each dog at their first visit at the UUCCA included:

- Signalment: sex, breed, age when the dog was diagnosed;
- Results of anamnesis: clinical signs according to the owner, environmental history;

[1] These dogs were only excluded in the part of the study were height of DAT titre was compared to clinical, haematological and biochemical alterations, since this could have an influence on the results. All dogs who received treatment remained included in the part of the study were positive and negative titres were compared.

- Results of physical examination: respiratory rate and character; heart/pulse rate; rectal temperature; coat and skin; colour, CRT and aspect of mucosal membranes and palpation of lymph nodes;
- DAT antibody titre;
- Laboratory results: Ht, leukocytes, neutrophils, thrombocytes, protein spectre (alpha 1, alpha 2, beta 1, beta 2 and gamma globulin), albumin spectre, creatinine (in blood), urea-protein-creatinine ratio (UPC ratio) and urinary sediment.

Methods

All dogs were presented at the UUCCA, except for one dog, who was presented at a local veterinary practice, in the period between 2008 and 2018. All dogs had a leishmania titre performed at the UVDL between 2008 and 2018 using a direct agglutination test on a collected blood sample. Clinicopathological data from the dogs presented at the UUCCA was extracted from medical records in the veterinary software program Vetware (Vetware, v. 1.6.135-rc01, Canada). Clinicopathological data from the dog presented at a local veterinary practice was extracted from medical records, provided by the practice its selves, using informed consent. All dogs were retrospectively classified using five different staging systems i.e. the categories published by Mancianti *et al.* (1988)(17), Ciaramella *et al.* (1997)(28), Amusategui *et al.* (2003)(29), the Leishvet group (7) and the Canine Leishmaniosis Working Group (CLWG)(30).

To answer the first subquestion, dogs with positive and negative titres were compared to clinical, haematological and biochemical alterations.
To answer the second subquestion, only dogs with titres 1:40 – 1:2560 and titres 1:10240 are used, since not all dogs with titre 1:5120 are further serially diluted. Also, dogs who received treatment for CanL were excluded, due to possible changes in titre height and (patho)clinical presentation.

Clinical data collection

Clinical data was collected from medical records recorded in Vetware. Physical exams and anamnesis of the dogs were performed and recorded by multiple veterinary medicine students, as well as veterinarians at the UUCCA from 2008-2018. For classifying the dogs in the different staging systems, extra emphasis was added to the most common clinical signs of leishmania, including the different degrees of lymphadenopathy, weight loss, mucous membranes pallor, polyuria and polydipsia, vomiting, diarrhoea, cutaneous alterations, onychopathy, (kerato)conjunctivitis, lameness, fever, epistaxis, and immunocomplex lesions (such as vasculitis, arthritis, uveitis, glomerulonephritis). In some cases, a complete dermatologic, ophthalmologic or orthopaedic examination was performed by a veterinary specialist.

Laboratory data collection

All laboratory tests were performed by the UVDL. In short, blood samples of 5 mL were collected from the jugular vein into Z Serum Sep Clot Activator tubes and tubes containing EDTA. The ADVIA 120 and ADVIA 2120 haematology systems were used to analyse the blood samples. The urine analysis sediment and UPC ratio were determined on Olympus AU 680 (Beckman Coulter, Woerden, The Netherlands). The protein spectre was determined by protein electrophoresis (hydrogel beta-1 beta2, Hydriasis, Sebia, Surrey, The United Kingdom). All systems were run with veterinary software. The antibody titre for Leishmania was measured by DAT according to the recommendations by El Harith et al. (1989) (23). To establish the maximum reaction titre, three positive sera were serially diluted and tested, starting at a dilution of 1:40, up to 1:81920.

Assignment according to classification systems

Once all data was collected, all dogs were retrospectively placed into the most appropriate stage of the different staging systems following the criteria established by the different authors. Meléndez-

Lazo *et al.* (2018) reviewed and compared six clinical staging systems. Five of these are also used in this study (Mancianti *et al.* 1988, Ciaramella *et al.* 1997, Amusategui *et al.* 2003, Solano-Gallego *et al.* 2009, Paltrinieri *et al*. 2010) (16). Foglia Manzillo *et al.* (2013) was excluded from this study since the inclusion criteria for the different stages were not as clearly defined as the others (31). In all systems 'unclassified dogs' were defined as 'dogs who do not meet the criteria for classification in any possible way in the proposed staging method'. All staging systems are listed in table 5.

***Table 5* Criteria used for clinical classification of dogs with CanL as proposed by different authors**
Source: *(16)*

Study	Clinical classification based on clinical signs	Further diagnostic testing
Mancianti et al. (1988)	1. Asymptomatic dogs: absence of clinical signs 2. Oligosymptomatic dogs: lymphadenopathy, small weight loss and/or dull fur 3. Symptomatic dogs: all or some of the characteristic signs of the disease	None
Ciaramella et al. (1997)	1. Mild signs: mild lymphadenomegaly and/or weight loss 2. Clear signs: systemic lymphadenomegaly, splenomegaly, skin disorders and weight loss 3. Severe signs: all the above signs, plus chronic cutaneous changes and/or ocular lesions and/or severe weight loss	1. And/or Anemia 2. Anemia 3. Anemia and/or kidney involvement (azotemia)
Amusategui et al. (2003)	1. Initial stage: asymptomatic or with mild, non-specific clinical signs 2. Established disease: typical clinical signs of canine leishmaniosis 3. Advanced stage: severe organic complications (renal, hepatic, cardiac, etc.)	1. Slight dysproteinemia or a non-altered serum protein electrophoretogram, antibody titer > 1/100 ≤ 1/800 2. Dysproteinemia, antibody titer ≥ 1/400 3. Serious biochemical and hematological alterations; variable dysproteinemia and variable antibody titers.
Solano-Gallego et al. (2009) – **LeishVet**	I. Mild disease: mild clinical signs such as localized lymphadenomegaly and papular dermatitis II. Moderate disease: apart from signs listed in stage I may present: skin disorders, anorexia, weight loss, fever, and epistaxis III. Severe disease: apart of the signs listed in stages I and II, may present signs originating from immune-complex lesions IV. Very severe disease: dogs with clinical signs listed in stage III. Pulmonary thromboembolism	I. Usually no clinicopathological abnormalities II. Low to high positive antibody levels. Clinicopathological abnormalities such as mild non-regenerative anemia, hyperglobulinemia, hypoalbuminemia, serum hyperviscosity syndrome. a. Normal renal profile: creatinine < 1.4 mg/dL; non-proteinuric: UPC < 0.5 b. Creatinine < 1.4 mg/dL; UPC = 0.5–1 III. Severe disease: clinicopathological abnormalities listed in stage II. CKD IRIS (IRIS, 2015) stage I with UPC > 1 or stage II (creatinine 1.4-2 mg/dL) IV. Very severe disease: Medium to high positive antibody levels. Clinicopathological abnormalities listed in stage II, CKD IRIS stage III (creatinine 2–5 mg/dL). Nephrotic syndrome: marked proteinuria UPC > 5 and end-stage renal disease
Paltrinieri et al. (2010) – **CLWG**	A. Exposed B. Infected: dogs are clinically normal or have signs associated with other diseases C. Sick (clinically evident disease): One or more clinical signs common to leishmaniosis are present. Dogs without clinical signs but with laboratory alterations D. Severely sick: dogs with severe clinical illness. Concurrent problems that require immunosuppressive treatment; severe concomitant conditions; and clinical unresponsiveness to repeated courses of anti-*Leishmania* drugs E. a) Sick-unresponsive b) Sick-early relapse	A. Negative cytologic, histologic, parasitological, and molecular findings and low titer antibodies against *Leishmania spp.* B. Dogs in which parasites have been detected through direct diagnostic methods and with low-titer antibodies against *Leishmania spp.* C. Dogs with positive cytologic results regardless of serologic results and dogs with high antibody titers against *Leishmania* spp. Hematologic, biochemical, and urinary alterations common to leishmaniasis D. Evidence of proteinuric nephropathy or chronic renal failure
Foglia Manzillo et al. (2013)	1. Subpatent infections: absence of clinical signs attributable to CanL 2. Asymptomatic active infection: absence of clinical signs attributable to CanL 3. Symptomatic active infection: presence of clinical signs attributable to CanL	1. Subpatent infections: detection of parasite DNA in BM samples; IFAT titers < 1:160; negative lymph node culture; absence of clinicopathological signs attributable to CanL 2. Asymptomatic active infection: detection of parasite DNA in BM samples; IFAT titers ≥1:160; positive lymph node culture; absence of clinicopathological signs attributable to CanL 3. Symptomatic active infection: detection of parasite DNA in BM samples; IFAT titers > 1:160; positive lymph node culture; presence of clinicopathological signs attributable to CanL

Statistical analysis

All statistical analyses were performed using commercial statistical software (R studio, Version 1.1.463 – © 2009-2018 RStudio, Inc.). All continuous data was tested for normal distribution using the Shapiro-Wilk test. Spearman's coefficient of rank correlation (rho) was used to evaluate the degree of association between the DAT titre and the clinical stages from the different staging systems and the degree of association between the DAT titre and haematological and biochemical parameters. For all tests, significance was set as $P < 0,05$.

Results

Two hundred seventy dogs had a DAT titre performed from 2008 to 2019 at the UVDL. Of these dogs, fourteen dogs were excluded because they had an incomplete file, sixteen were excluded because the clinical signs were not suggestive of Leishmaniasis, 67 were excluded because they had no travel history to endemic areas and 64 were excluded because a different diagnosis was confirmed. There were 109 dogs that qualified for the inclusion in this study.

Determination of the difference between positive and negative DAT titre in clinical, haematological and biochemical manifestations

Descriptive analysis

Of the 109 dogs, 60 were males (18 intact, 42 neutered) and 49 females (4 intact, 45 spayed). The mean age was 5,1 years (range 6 months to 11,1 years). Breed distribution was 50,5% crossbreeds, 49,5% other breeds. Fifty-one dogs were born in Spain, fifteen in Greece, four in France, three in Italy, two in Turkey, two in Portugal, two in Mallorca, one in Cyprus and one in Ibiza. Forty-three dogs travelled to different countries including Spain, France, Italy and Portugal. Fifteen of these dogs were tested for co-infections and had a known co-infection with other vector borne diseases (such as ehrlichiosis, babesiosis or dirofilariasis), it is unknown if the other dogs were tested for these diseases. Twenty-six dogs already received treatment when the titre was performed, due to positive results in previously performed tests. Twelve of these dogs received treatment for more over a year. The DAT titre ranged from ≤ 1:40 to 1:20480.

Table 6 DAT titres (n = 109).

Height of DAT titre	Number of dogs
≤ 1:40	26
1:80	3
1:160	1
1:320	2
1:1280	3
1:2560	9
1:5120	62
1:10240	1
1:20480	2

To determine if there is a difference between clinical, haematological and biochemical manifestations found in dogs with negative (<1:40) and positive (>1:40) titres, all 109 dogs are compared. Twenty-six of these dogs had a negative titre, 83 dogs had a positive titre.

Most common clinical signs in dogs with a negative DAT titre were decreased endurance (61,5%), lymphadenopathy (57,5%) and fever (50,0%). Less commonly clinical signs were weight loss and dry exfoliative dermatitis (both 23,1%). In dogs with a positive titre, most common clinical signs were lymphadenopathy (66,3%), decreased endurance (61,4%) and alopecia (43,4%). Less commonly found clinical signs were ulcerations on the skin (18,1%) and epistaxis (6,0%). Cutaneous manifestations included dry exfoliative dermatitis, alopecia, erythema, crustae, squamae, ulcerative lesions and hyperkeratosis. One dogs with a negative titre suffered from a (cardio)vascular disorder, in form of a vasculitis. Also, two dogs with a positive titre suffered from (cardio)vascular disorders, one suffered from ventricular extra systoles and the other from sinus tachycardia. A neurological disorder was found in just one case, this dog suffered from some kind of epileptic attack and unconsciousness. All clinical signs are listed in table 7.

Table 7 Frequency of clinical signs of dogs with negative DAT titres (<1:40) and positive DAT titres (>1:40) (n = 109)

Clinical signs	Number of dogs with <1:40 (n = 26)	%	Number of dogs with >1:40 (n = 83)	%
Lymphadenopathy	15	57,7%	55	66,3%
Decreased endurance	16	61,5%	51	61,4%
Alopecia	8	30,7%	36	43,4%
Weight loss	6	23,1%	35	42,2%
Dry exfoliative dermatitis	6	23,1%	35	42,2%
Fever	13	50,0%	34	41,0%
Crustae	10	38,5%	32	38,6%
Change in appetite	6	23,1%	32	38,6%
Lameness	9	34,6%	28	33,7%
Diarrhoea	5	19,2%	24	28,9%
Vomiting	6	23,1%	24	28,9%
Polyuria / polydipsia	5	19,2%	19	22,9%
Erythema	5	19,2%	16	19,3%
Onychopathy	1	3,8%	15	18,1%
Ulcerative lesions on the skin	6	23,1%	15	18,1%
Mucous membranes pallor	5	19,2%	13	15,7%
Mucocutaneous / mucosal ulcerative or nodular lesions (oral, genital, nasal)	2	7,7%	10	12,0%
Splenomegaly	4	15,4%	9	10,8%
(Kerato)conjunctivitis	-	-	5	6,0%
Pustules	1	3,8%	5	6,0%
Epistaxis	2	7,7%	5	6,0%
Ulceration of footpads	1	3,8%	4	4,8%
Squamae	2	7,7%	4	4,8%
Hyperkeratosis	2	7,7%	4	4,8%
Atrophic masticatory myositis	1	3,8%	4	4,8%
Hyperpigmentation	2	7,7%	4	4,8%
Uveitis	3	11,5%	3	3,6%
Papular dermatitis	3	11,5%	3	3,6%
(Cardio)vascular disorder	1	3,8%	2	3,6%
Nodular dermatitis	1	3,8%	1	1,2 %
Blepharospasm	-	-	1	1,2%
Neurological disorder	-	-	1	1,2%

Haematological and biochemical alterations between positive and negative DAT titres are listed in table 8. Anaemia (69,2%) and leucocytosis (34,6%) are most commonly found in dogs with a DAT negative titre. Most dogs with a positive DAT titre had hypergammaglobulinemia (73,5%). Anaemia and hypoalbuminemia were present in respectively 67,5% and 42,2%. Thrombocytopenia was seen in only 6 dogs (7,2%) with a positive DAT titre.

Table 8 Haematological and biochemical signs of dogs with negative DAT titres (<1:40) and positive DAT titres (>1:40) (n = 109)

Haematological and biochemical signs	Number of dogs with titre <1:40 (n = 26)	%	Number of dogs with titre >1:40 (n = 83)	%
Hypergammaglobulinemia	6	23,1%	61	73,5%
Anaemia	18	69,2%	56	67,5%
Hypoalbuminemia	6	23,1%	35	42,2%
Proteinuria	6	23,1%	22	26,5%
Leucocytosis	9	34,6%	12	14,5%
Leukopenia	1	3,8%	7	8,4%
Thrombocytopenia	7	26,9%	6	7,2%

Determination of correlation between height of the DAT titre and clinical, haematological and biochemical manifestations

Descriptive analysis

Of the 109 dogs, 62 dogs with DAT titre 1:5120 were excluded from this part of the study, since none of these dogs had a DAT titre that was further serially diluted to a maximum titre reaction, given that all these titres were determined before the start of this research. Also, 8 dogs who received treatment for longer than two weeks, were excluded from this part of the study. Only dogs with DAT titres <1:40 – 1:2560 and titre 1:10240 were used, meaning that 39 dogs were included in this part of the study.

Of the 39 dogs, 18 were males (4 intact, 14 neutered) and 21 females (2 intact, 19 spayed). The mean age was 5,9 years (range 0,8 to 10,4 years). Breed distribution was 43,6% crossbreed and 56,4% other breeds. Thirteen dogs were born in Spain, four in Greece, two in France, two in Portugal, one in Italy, one in Cyprus and one in Ibiza. Twenty dogs travelled to different countries including France, Spain, Portugal and Germany. Five of these dogs were tested for co-infections and had a known co-infection with other vector borne diseases (such as ehrlichiosis, babesiosis or dirofilariosis). It is unknown if the other dogs were tested for these diseases. Titre distribution is shown in table 9.

Table 9 DAT titres (n = 39)

Height of DAT titre	Number of dogs
≤ 1:40	24
1:80	1
1:320	2
1:1280	3
1:2560	8
1:10240	1

In table 10, the clinical signs of these 39 dogs are shown. The most common clinical signs were decreased endurance (66,7%), lymphadenopathy (59,0%) and fever (41,0%). Less commonly found clinical signs were dry exfoliative dermatitis (23,1%), epistaxis (7,7%) and onychopathy (5,1%). Cutaneous manifestations included dry exfoliative dermatitis, alopecia, erythema, crustae, squamae, ulcerative lesions and hyperkeratosis.

Table 10 Frequency of clinical signs of dogs with DAT titres 1:40 – 1:10240 as highest serial dilution (n = 39).

Clinical signs	Number of dogs	%
Decreased endurance	26	66,7%
Lymphadenopathy	23	59,0%
Fever	16	41,0%
Lameness	11	28,2%
Vomiting	11	28,2%
Change in appetite	11	28,2%
Weight loss	11	28,2%
Crustae	10	25,6%
Alopecia	10	25,6%
Dry exfoliative dermatitis	9	23,1%
Diarrhoea	9	23,1%
Erythema	8	20,5%
Polyuria / polydipsia	7	17,9%
Ulcerative lesions on the skin	6	15,4%
Mucous membranes pallor	6	15,4%
Splenomegaly	6	15,4%
Uveitis	4	10,3%
Mucocutaneous / mucosal ulcerative or nodular lesions (oral, genital, nasal)	3	7,7%
Epistaxis	3	7,7%
Papular dermatitis	3	7,7%
Hyperkeratosis	2	5,1%

Onychopathy	2	5,1%
Vascular disorder	2	5,1%
Hyperpigmentation	2	5,1%
Pustules	2	5,1%
Squamae	1	2,6%
Nodular dermatitis	1	2,6%
Atrophic masticatory myositis	1	2,6%
(Kerato)conjunctivitis	1	2,6%

Haematological and biochemical alterations of the 39 dogs are listed in table 11. Most dogs had anaemia (61,7%). Hypergammaglobulinemia and leukocytosis were both present in 35,9%. Leukopenia was seen in only two dogs (5,1%).

Table 11 Haematological and biochemical signs of dogs with DAT titres 1:40 – 1:10240 as highest serial dilution (n = 39)

Haematological and biochemical signs	Number of dogs	%
Anaemia	25	64,1%
Hypergammaglobulinemia	14	35,9%
Leucocytosis	14	35,9%
Hypoalbuminemia	12	30,8%
Thrombocytopenia	12	30,8%
Proteinuria	10	25,6%
Leukopenia	2	5,1%

Urinary sediment was determined in 19 dogs and are listed in table 12. Of these 19 dogs, 9 dogs had an increased UPC ratio (> 1,00). Squamous epithelium, leukocytes and fatty casts were found most commonly in the urinary sediment (respectively 78,9%, 68,4% and 57,9%).

Table 12 Urinary sediment found in dogs with DAT titres 1:40 – 1:10240 as highest serial dilution (n = 19).

Sediment	Number of dogs	%	Increased UPC ratio
Squamous epithelium	15	78,9%	8
Leukocytes	13	68,4%	7
Fatty casts	11	57,9%	5
Erythrocytes	10	52,6%	5
Amorphous solid	7	36,8%	5
Granular casts	5	26,3%	3
Transitional epithelium	3	15,8%	2
Sperm	3	15,8%	2
Bilirubin	2	10,5%	1
Bacteria	2	10,5%	2
Triple phosphate crystals	1	5,3%	1

Table 13 shows the lowest value, highest value, mean, median, standard deviation (SD) and laboratory reference range of haematological and biochemical parameters. The mean of Ht and alpha 1 was lower than the laboratory references, the mean of beta 2, gamma globulin and the UPC ratio was higher than the laboratory references. All other parameters were within laboratory references. According to the Shapiro-Wilk test, only Ht, thrombocytes, alpha 2 and albumin (spectre) were normally distributed.

Table 13 Lowest value, highest value, mean and standard deviation of laboratory findings (n = 39).

Parameter (units)	Minimum value	Maximum value	Mean	Median	SD	Laboratory reference range
Ht (L/L)	0,1	0,55	0,37	0,39	±0,11	0,42 – 0,61
Leukocytes (x10^9/L)	1,00	46,7	13,01	12,10	±8,52	4,5 – 14,6
Neutrophils (x10^9/L)	0,60	38,3	9,57	8,60	±7,16	2,9 – 11,0
Thrombocytes (x10^9/L)	4	613	202,72	207,50	±146,68	144 - 603
Alpha 1 (g/L)	1	6	2,86	3,00	±1,13	5 – 10
Alpha 2 (g/L)	6	18	11,00	10,00	±2,94	4 – 13
Beta 1 (g/L)	2	7	3,59	3,00	±1,18	3 – 10
Beta 2 (g/L)	6	29	10,93	9,00	±4,54	4 – 10
Gamma globulin (g/L)	2	73	13,79	9,00	±14,80	3 – 9
Total Protein (serum) (g/L)	44	112	69,70	66,00	±17,10	55 – 72
Albumin (spectre) (g/L)	8	39	26,93	26,00	±9,33	26 – 37
Creatinine (blood) (µmol/L)	27	983	122,5	68,50	±188,20	50 – 129
UPC Ratio	0,06	12,6	1,87	1,11	±2,90	<1,00

Height of the titre compared to clinical signs

All 39 dogs were staged in the different categories of the five classification systems, which result is shown in table 14. In the Mancianti *et al.* system, most dogs were classified in the symptomatic stage (57,4%). In the systems of Ciaramella *et al* and Leishvet group, most dogs were staged 'unclassified' (respectively 35,9% and 30,8%). In the system of Amusategui *et al.* the initial stage was fitting for most dogs (61,5%) and in the system of CLWG most dogs were classified in stage A (33,3%). According to the Shapiro-Wilk test, none of the data was normally distributed.

Table 14 Distribution of the dogs in the five classification systems (n = 39)

System	Stage	Number of dogs	%
Mancianti et al.	Unclassified	6	14,9%
	Asymptomatic	5	12,8%
	Oligosymptomatic	6	14,9%
	Symptomatic	22	57,4%
Ciaramella et al.	Unclassified	14	35,9%
	Mild signs	8	20,5%
	Clear signs	9	23,1%
	Severe signs	8	20,5%
Amusategui et al.	Unclassified dogs	2	5,1%
	Initial stage	24	61,5%
	Established stage	10	25,6%
	Advanced stage	3	7,7%
Leishvet	Unclassified dogs	12	30,8%
	Stage I	7	17,9%
	Stage II	11	28,2%
	Stage III	7	17,9%
	Stage IV	2	5,1%
CLWG	Unclassified dogs	2	5,1%
	Stage A	13	33,3%
	Stage B	7	17,9%
	Stage C	12	30,8%
	Stage D	5	12,8%
	Stage E	0	0%

A positive significant correlation was found between the height of the DAT titre and the system of the CLWG. No significant relations between DAT titre and the other classification systems were found in any systems using Spearman's coefficient of rank correlation test (table 15).

Table 15 Rho and P-values of DAT titres and classification systems in dogs with DAT titres 1:40 – 1:10240 as highest serial dilution (n = 39).

Classification system	Spearman's correlation coefficient (r_s)	*P*-value
Mancianti et al.	0,292	0,0708
Ciaramella et al.	0,196	0,2313
Amusategui et al.	0,289	0,0747
LeishVet	0,123	0,4543
CLWG	0,348	**0,0302***

The boxplot (figure 1) shows the positive relation between the height of the DAT titre and the classification system of CLWG.

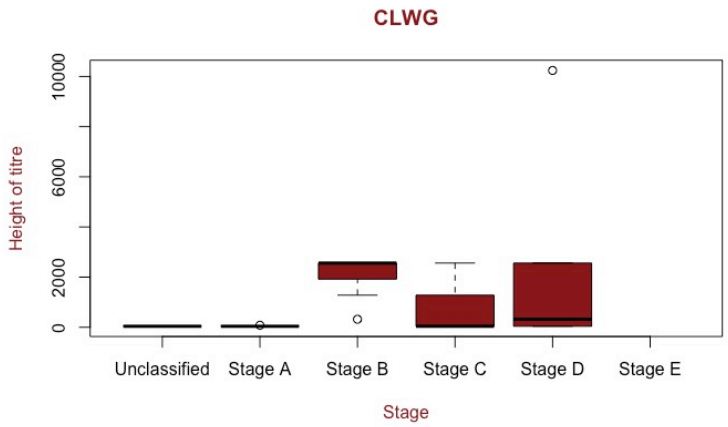


Figure 1 Boxplots of significant relation between height of DAT titres and classification systems of CLWG. The black line in the middle of the red boxes represent the mean of the parameter and dots represent the outliers.

Height of the titre compared to haematological and biochemical parameters

None of the data was normally distributed except for Ht, thrombocytes, alpha 2 and albumin. All haematological and biochemical parameters were tested for correlation using Spearman's correlation test. The results are listed in table 16. No significant relationships were found.

Table 16 Rho and P-values of DAT titre and haematological and biochemical parameters systems in dogs with DAT titres 1:40 – 1:10240 as highest serial dilution (n = 39)

Parameter (units)	Spearman's correlation coefficient (r_s)	*P*-value
Ht (L/L)	-0,104	0,529
Leukocytes (x10^9/L)	-0,074	0,656
Neutrophils (x10^9/L)	-0,071	0,673
Thrombocytes (x10^9/L)	-0,309	0,085
Alpha 1 (g/L)	0,329	0,082
Alpha 2 (g/L)	-0,138	0,475
Beta 1 (g/L)	0,283	0,137
Beta 2 (g/L)	0,216	0,261
Gamma globulin (g/L)	0,256	0,181
Total Protein (serum) (g/L)	-0,063	0,754
Albumin (spectre) (g/L)	-0,190	0,323
Creatinine (blood) (µmol/L)	0,065	0,753
UPC Ratio	-0,026	0,915

Discussion

The aim of the second part of the study was to determine the relation between the level of the DAT antibody titre and several different staging systems used to classify a patient based on severity of clinical signs and blood results of Canine Leishmaniasis. Besides the significant relation between the height of the DAT titre and the staging system of CLWG, no significant relations were found. As for the relation between haematological and biochemical alterations, no significant relations were found either.

Since most of the dogs used in this study were provided by the UUCCA, it is possible that this cohort had a selection bias as well. Most dogs visit the UUCCA because they are difficult cases with either unspecific or very severe clinical signs, which the local practices could not solve. Dogs with more specific clinical signs of CanL might be more easy to diagnose at the local practices. This might explain why unspecific signs, such as decreased endurance, weight loss and fever were found more often in this study than the more specific signs as the cutaneous forms of CanL, considering height of the DAT titre as well as comparing the positive and the negative titres. Considering that 26 dogs already received treatment and 12 of these dogs received this treatment for more over than a year, some clinical signs of these dogs might have been suppressed.

When considering the difference between positive and negative DAT titres, the most commonly found signs were lymphadenopathy (respectively 57,5% and 66,3%) and decreased endurance (respectively 61,5% and 61,4%). Less commonly found clinical signs were weight loss and dry exfoliative dermatitis (both 23,1%) in negative titres and epistaxis (6,0%) and atrophic masticatory myositis (4,8%) in positive titres. When considering the height of the titre, the most common clinical signs found were decreased endurance (66,7%), lymphadenopathy (59,0%) and fever (41,0%) and less commonly found signs were dry exfoliative dermatitis (23,1%), epistaxis (7,7%) and onychopathy (5,1%). In both ways, the more unspecific signs are found in high percentages. Meléndez-Lazo *et al*. (2018) found in CanL-infected dogs 78,4% cutaneous lesions. Dogs in their study were recruited from the *Fundació Hospital Clinic Veterinari* of the *Universitat Autónoma de Barcelona* and the *Hospital Ars Veterinaria* in Barcelona, Spain (16). Proverbio *et al.* (2016) found exfoliative dermatitis in 66% of the dogs, recruited from the Faculty of Veterinary Medicine University of Milan, Italy (32). Both studies recruited dogs from a University clinic as well. However, both Spain and Italy are endemic countries, which might support the high percentages in skin lesions and cutaneous forms of CanL found in these studies. In comparison with Slappendel et al. (1988) that reported 80 untreated but diagnosed cases of CanL in the Netherlands, diagnosed at the UUCCA as well, the most common clinical sign found were lymphadenopathy (90%) and skin involvement (89%). Since Slappendel et al. (1988) did not differentiate in form of skin involvement it is hard to compare this percentage with the result in this study (33). One might say that over the years, probably since more information and guidelines on diagnosis, treatment and prevention are provided, cases with more specific signs of CanL (as the skin involvement) are easier to diagnose at the local veterinary practices in the Netherlands.

As for the haematological and biochemical parameters, most commonly found alterations in dogs with a negative DAT titre were anaemia (69,2%) and leucocytosis (34,6%). In dogs with a positive DAT titre most commonly found alterations were hypergammaglobulinemia (73,5%), followed by anaemia and hypoalbuminemia (respectively 67,5% and 42,2%). Meléndez-Lazo *et al. (*2018) reported hypergammaglobulinemia in 72,5%, which is comparable with the result found in this study. All dogs in the study of Meléndez-Lazo had confirmed CanL using a positive result on ELISA or by presence of Leishmania amastigotes in lymph nodes, cutaneous lesions, bone marrow or peripheral blood smears. As for anaemia and hypoalbuminemia, they found other percentages (respectively 58,8% and 54,9%) (16) . Proverbio *et al.* (2016) found anaemia in 68% of the dogs, comparable to the result of this study. Hypergammaglobulinemia and hypoalbuminemia were found in high percentages (100% and 95%). All dogs had confirmed CanL established by clinicopathological abnormalities, positive serology using IFAT and cytological identification of Leishmania amastigotes (34). All three of those alterations are commonly found haematological abnormalities in dogs infected with CanL (25).

In this study, 26,5% of the dogs with a positive titre had a UPC ratio >1,00, which was considered as proteinuria. According to Maia *et al.* (2018) common findings in dogs with CanL considering urinalysis are proteinuria and renal azotaemia which is agreement with the result in this study (25).

Considering the relation between height of antibody titre and biochemical alterations, urinary sediment was determined in 19 dogs, of which 9 dogs suffered from an increased UPC ratio. In case of CanL, important components in the urinary sediment, according to Ibba *et al.* (2016), are granular casts and epithelial cells since these components are a sign of tubular damage caused by the CanL infection (35). Kidney epithelium was not found in any of the dogs. Granular casts were found in 5 dogs. Three of these dogs also suffered from increased UPC, which might support the presence of the granular casts as well.

The mean of Ht and alpha 1 was below laboratory references and the mean of beta 2, gamma globulin and UPC ratio was above laboratory references. The low Ht might be caused by a decreased erythropoiesis due to disorders in the bone marrow or a decreased erythropoietin production caused by chronic renal failure (1)(25). Slappendel *et al*. (1988) reported an increase of the total serum protein in 91% of the dogs, hypoalbuminemia in 94% of the dogs and hyperglobulinemia in 100% of the dogs (33). The hyperglobulinemia was based on increases in beta- and gamma globulins, alpha-globulins contributed a little to this. The result in this study show that the mean of beta 2 and gamma globulins are increased. These findings conform with previous data (28) (1). The high UPC ratio might be caused by several individual dogs with high values of UPC ratios. As discussed before, Maia *et al.* (2018) reported that proteinuria is a common finding in CanL dogs, which makes it plausible to say that this finding is not an abnormality (25).

Although different studies show a correlation between the number of antibodies and clinical signs, not all authors agree on the usefulness of antibody titres as a means of assessing the activity of the Leishmania infection at the time of diagnosis, during treatment, or flare-ups of the disease (11) (19) (22). In this study, five different clinical staging systems are used to classify 39 dogs. A difficulty during this study was to classify the dogs retrospectively. Important to keep in mind, is the fact that all these dogs are retrospectively classified by one person, which makes it possible that this cohort had a form of observer bias. Since these dogs visited the UUCCA in a period between 2008 and 2019, various different students have reported the dogs used in this study. Due to this, it might be possible that not every report was as complete or as detailed as the others. Also, dogs with negative DAT titres (<1:40) were included when anamnesis and clinical signs in the medical file were suggestive for CanL. Since all dogs with titre 1:5120 must be excluded due to the fact that none of these dogs had a titre that was serially diluted to a maximum of 1:81920, the group of dogs with a high antibody titre was smaller than the group of dogs with a titre which is considered low or dubious (23). Besides, most signs were quite unspecific, which made it more difficult to classify these dogs.

Clinical staging systems can be helpful on deciding on the therapy most suitable for each patient, to evaluate the efficacy of different therapies and to consider a prognosis (16). Not all used clinical staging systems were considered as applicable to this cohort as others, since not all dogs could be classified in all systems. Besides, different criteria for staging was used in the different systems. Meléndez-Lazo *et al.* (2018) compared different clinical staging systems and described that the stages should be based on clinical and laboratory test results more than just in clinical signs, as the one from Mancianti *et al.* (1988) (16). This is in agreement with the results found in this study, since 57,4% of the dogs were staged as 'symptomatic'. Meléndez *et al.* stated that clinical signs used for assignment to the categories should be easily detected, avoiding the use of subjective observations and that laboratory results should not be overlapped between categories since it could cause difficulties when choosing a category to include the dog in. They also proposed that a more desirable method to include quantitative serological techniques like IFAT or ELISA would be to group serologic results in low, medium and high level, over the concrete antibody titres since they may not be comparable among different laboratories (16), to which the author of this study agrees.

When reviewing the percentages 'unclassified dogs', the system of Ciaramella *et al.* (1997) had the highest percentage (35,9%) followed by the system of LeishVet (30,8%). An explanation for this

might be that both systems do not include asymptomatic dogs. Other reasons might be that Ciaramella *et al.* (1997) stated that dogs in the category 'mild signs' must have a mild form of lymphadenomegaly (involving one or two lymphnodes). Not all dogs, even with a high DAT titre and other more specific CanL signs, had a form of lymphadenomegaly or -pathy.

Proverbio *et al.* (2016) compared that LeishVet system with the CLWG system and asserted that the correlation between the antibody titre and clinical symptoms or clinicopathological abnormalities for each stage in the LeishVet system make it more difficult to classify the dogs. Dogs without a direct correlation between IFAT titre and clinical signs were not allowed in the classification (20). Proverbio *et al.* (2014) proposed their own clinical scoring system to compare with the height of the titre. Meléndez-Lazo *et al.* (2018) stated that the establishment of a clinical score model should especially consider selected clinical signs and laboratory parameters that seem to have a role in disease progression. Each parameter should be studied for relative relevance to make a valid score (16). This might be an easier system to compare the height of the titre with the severity of the clinical signs, but further research would be desirable.

Aside from the difficulties experienced during classifying the dogs, no correlations were detected between clinical stages and the DAT titres by all 5 methods in this study, except for the system of CLWG ($p = 0{,}0302$). A higher DAT titre is related to more severe clinical signs according to the CLWG by Paltrinieri *et al.* (2016) system, but since this is the only correlation found in this study, one might say that this correlation is not very solid. According to the writer's knowledge, there are no studies on the relation between antibody titres and the system of Ciaramella *et al.,* Solano-Gallego *et al.* and Paltrinieri *et al.*

Amusategui *et al.* (2003) themselves did a research on the relation between IFAT titres and severity of clinical signs, using their own system and did not find a relationship. However, they also compared the IFAT titre with the different stages and found that animals in the initial stage displayed the lowest antibody titres. Also, the IFAT was significantly associated with the established stage, but not with the advanced stage. Moreover, they observed a relationship between IFAT titres and the presence of clinical signs, similar to the results obtained by Reis *et al.* (2004)(29)(36), which was not investigated in the present study. Another study by Abranches *et al.* (1991) found that 53,8% of the dogs with a significant IFAT titre showed no symptoms. Dogs were divided in two groups, symptomatic dogs who displayed moderate symptoms of CanL and dogs who were showing evident symptoms that included 3 or more signs of the disease. They suggested the prolonged asymptomatic period of CanL as a reason for not finding a relation between antibody titre and clinical signs, which could support the findings in this study (37). Amusategui *et al*. suggests three other studies describing no correlation between severity of disease and anti-Leishmania titre, however these articles were not available in English. Two studies did a research on the relation between height of titre and clinical signs. Ferrer *et al*. (1995) found no relation between serological titres and severity of clinical signs, using a Dot-ELISA technique on dogs with CanL that were treated with N-methylglucamine and allopurinol. Mateo *et al.* (2009) found that decrease of the IFAT titre did not correlate with clinical improvements after the dogs received milteforan or glucantime (22). However, the relationship between height of the titre and clinical improvement after treatment are beyond the point of this study.

In contrast to the results of this study, Da Costa-Val *et al.* (2007) used the Mancianti *et al.* (1988) system and did find a significant relation between high ELISA values and severity of disease, using a similar sized cohort (38).

With regard to haematological and biochemical parameters, no significant relations were found between height of DAT titre and the parameters in this study. This is in contrast with other studies like Solano-Gallego *et al*. (2016), who found a negative correlation between antibody levels and Ht (11). Amusategui *et al.* (2003) did find a correlation between antibody titre and, albumin, haemoglobin, PCV and RBC and also, reported a high correlation between antibody titre and total protein and gamma globulins (29). Proverbio *et al. (*2014) and Solano-Gallego *et al.* (2016) found similar results: higher total protein and gamma globulins were recorded in dogs with higher IFAT titres. A reason for the differences in results could be the small cohort used in this study ($n = 39$).

Since in this study only one dog had further diluted DAT titres than the regular 1:5120, it is it difficult to say whether or not higher diluted titres point to more severe clinical signs. The dog in this cohort with the 1:10240 DAT titres was not staged in the highest stages in all the systems. Therefore, it would also be desirable test if further diluted DAT titres are related to severity of clinical signs. As an extra for this study, the relationships between clinical stages, haematological and biochemical parameters were analysed in the larger cohort (n = 109), including the dogs with a 1:5120 DAT titre, to investigate if a relation could be found when using a larger cohort (attachment 6). In all systems, a positive correlation was found, meaning that severe clinical signs were related to higher DAT titres. Also, significant correlations were found between DAT titre and Ht, leukocytes, neutrophils, gamma globulin, total protein and creatinine. These results show that when using a larger cohort, with higher diluted DAT titres (>1:5120), could indicate a certain relationship between height of the titre and clinical signs, haematological and biochemical alterations. Therefore, further investigation with more higher serially diluted samples is desired.

Conclusion

No major differences were found between positive and negative DAT titres in clinical signs and haematological or biochemical parameters.

The height of the DAT antibody titre is not significantly related to clinical, haematological and biochemical manifestation. However, the results in this study show a positive significant relationship between the height of the DAT titre and severity of clinical signs according to the CLWG system, but since this is the only correlation found in this study, one might say that this correlation is not very solid. All parameters were analysed in a larger cohort than the original study in the unpublished data (attachment 6) and significant correlations were found between DAT titre and clinical stages in all systems, and between DAT titre and Ht, leukocytes, neutrophils, gamma globulin, total protein and creatinine. Further investigation with a bigger cohort, including more higher serially diluted samples, might be necessary to confirm or reject this study's hypothesis.

Attachment

1. Informed consent for dog owners (seroprevalence)

Informatiebrief en toestemmingsformulier

Onderzoek naar Leishmania (*en andere vector-overgedragen aandoeningen*) bij geïmporteerde zwerfhonden

Graag vragen wij u middels dit schrijven toestemming voor deelname van uw hond aan een onderzoek van het departement Geneeskunde van Gezelschapsdieren van de faculteit Diergeneeskunde van de Universiteit Utrecht. Het is voor deelname aan een wetenschappelijk onderzoek vereist dat u een schriftelijke verklaring geeft dat u volledig bent ingelicht over het onderzoek en dat u bereid bent om mee te werken. Dit wordt '*informed consent*'; ofwel geïnformeerde toestemming genoemd.
U zult door middel van dit document uitgelegd krijgen wat de opzet is van het onderzoek, wat uw medewerking precies zal inhouden, en wat de voordelen, nadelen en mogelijke risico's zijn. Ook zal u worden uitgelegd hoe er met de resultaten van het onderzoek wordt omgegaan zodat uw privacy gewaarborgd is.

Het doel van dit project
Leishmania is een ziekte die in een vroeg stadium goed te behandelen is, daarom is het van belang dat het zo vroeg mogelijk wordt opgespoord. Dat geldt ook voor andere vector overgedragen aandoeningen. Er is helaas in het verleden weinig onderzoek gedaan naar de beste richtlijnen voor de snelste opsporing. Wij willen onderzoeken hoe vaak Leishmania bij geïmporteerde zwerfhonden voorkomt, zodat wij betere richtlijnen voor het stellen van de diagnose en behandelmethodes kunnen ontwikkelen. Hiermee willen wij bijdragen aan een betere gezondheid en welzijn van *zwerf*honden.

Algemene informatie
Uw hond is afkomstig uit Zuid-Europa of heeft er een deel van zijn leven doorgebracht. Een van de hondenziektes die in Zuid-Europa veel voorkomt is Leishmaniasis. Dit is een ziekte die door een zandvlieg wordt overgebracht. Dergelijke ziektes, waarbij een insect zorgt voor besmetting, zijn vector-overdraagbare aandoeningen. Omdat de zandvlieg die Leishmania overdraagt in Nederland niet voorkomt kunnen honden in Nederland de ziekte niet oplopen. Een hond die in Zuid-Europa geïnfecteerd is geraakt, heeft daar in het algemeen een zomer doorgebracht. Onderzoek naar besmetting en ontwikkeling van de ziekte is van belang om tijdig met medicatie in het ziekteproces te kunnen ingrijpen. Dit is de reden dat er een leeftijdscriterium aan gebonden is.

Waarom is mijn hond hiervoor geselecteerd?
Uw hond is recent geïmporteerd uit het buitenland door een zwerfhonden stichting. Via deze stichting is er contact met u gelegd. Ook voldoet uw hond aan de leeftijdscriteria.

Waar wordt mijn hond op getest?
Uw hond wordt lichamelijk onderzocht en er wordt bloed afgenomen. Het bloed wordt getest op antilichamen tegen Leishmania. Deze uitslag komt ongeveer 14 dagen na de bloedafname beschikbaar. In een later stadium van het onderzoek wordt het bloed onderzocht op de aanwezigheid van hartworm larven en antilichamen tegen hartworm en Ehrlichia. Deze uitslagen komen na afronden van het onderzoek beschikbaar.

Wie voert het onderzoek uit?

Het onderzoek staat onder leiding van dr. C.J. Piek, Internist voor Gezelschapsdieren, hoofd van de afdeling Hematologie. Studenten van de laatste fase van de opleiding Diergeneeskunde hebben een ondersteunende functie, als onderdeel van de studie.

Hoe ziet de procedure eruit?
Eerst worden contactgegevens van u en uw hond vastgelegd om verslag te doen van de bevindingen van het onderzoek van uw hond.
Daarna wordt uw hond lichamelijk onderzocht om vast te of er afwijkingen zijn die wijzen op Leishmania of een van de andere infecties. Daarna wordt bloed afgenomen voor onderzoek naar Leishmania antilichamen, en opslag voor onderzoek in later stadium.
Het lichamelijk onderzoek en de bloedafname zullen twee keer plaatsvinden. De eerste keer zal zijn als uw hond ongeveer een maand in Nederland is. De tweede keer zal zijn als uw hond ongeveer 9 maanden in Nederland is.
Als er ziekteverschijnselen van Leishmania worden gevonden kunt u zonder verwijzing een afspraak bij een van de specialisten op de hematologie poli van de Universiteitskliniek voor Gezelschapsdieren. Mochten er afwijkingen worden gezien die waarschijnlijk niets te maken hebben met Leishmania, zal in overleg met u, uw eigen dierenarts geïnformeerd worden.

Wat zijn negatieve/positieve gevolgen van meewerken aan dit onderzoek?
Bloedafname is een kleine ingreep. Het zou kunnen gebeuren dat er als gevolg van het bloedprikken een onderhuidse bloeding optreedt, dit komt echter zeer zelden voor. Een dergelijke bloeding is meestal niet ernstig en voorbijgaande aard.
Nadat u uw hond heeft laten onderzoeken en bloed heeft laten afnemen ontvang u een rapport met de bevindingen van het lichamelijk onderzoek. Na 10 dagen krijgt u de uitslag van de test met aanvullende informatie. Dezelfde procedure zal volgen bij het tweede onderzoek moment.
Verder wordt uw hond onderzocht onder leiding van een vooraanstaand specialist op het gebied van Leishmania.

Wat gebeurt er met de gegevens/informatie?
De gegevens die tijdens dit project zijn verzameld worden gebruikt voor een publicatie in een wetenschappelijk tijdschrift. De gegevens zullen niet te herleiden zijn tot het individuele dier.
Het bloed wat tijdens dit onderzoek wordt na uw toestemming afgenomen wordt bewaard voor vervolgonderzoek.

Vertrouwelijk- wie heeft er toegang tot de data?
Persoonsgegevens die worden verzameld tijdens deze studie worden opgenomen in het dossier van uw hond in het beveiligde elektronische patiënt-informatiesysteem Vetware. Persoonsgegevens zullen nooit worden vermeld in publicaties of presentaties. In publicaties of presentaties naar aanleiding van het onderzoek worden, naast de uitkomsten van het onderzoek, uitsluitend de uiterlijke kenmerken van uw hond, zoals ras, geslacht, leeftijd en de relevante delen van de ziektegeschiedenis en medische gegevens anoniem gepubliceerd. De onderzoekers hebben wel toegang tot de persoonlijke gegevens, zodat er wel correspondentie kan plaatsvinden. Dit is vooral van belang om de uitslag van het onderzoek van uw hond aan u kenbaar te maken. De gegevens worden bewaard gedurende het onderzoek, indien u toestemming geeft wordt het bewaard voor vervolgonderzoek.

Kan ik mij terugtrekken uit dit onderzoek?
U kunt zich terugtrekken uit dit onderzoek op elk moment zonder daarvoor een reden te geven. Dit kunt u doen door een email te sturen naar: C.J.Piek@uu.nl

Wie kunt u benaderen bij vragen over dit onderzoek?
Email: ProjectLeishmania@uu.nl

Toestemmingsverklaring

Ik bevestig dat ik het informatieformulier heb gelezen. Ik begrijp de informatie. Ik heb de mogelijkheid gehad om aanvullende vragen te stellen en deze zijn naar tevredenheid beantwoord. Ik heb voldoende tijd gehad om over deelname na te denken.
Ik weet dat mijn deelname geheel vrijwillig is en dat ik mijn toestemming op ieder moment kan intrekken zonder dat ik daarvoor een reden moet geven.

Ik geef toestemming voor het uitvoeren van de procedure (Het uitvoeren van lichamelijk onderzoek en bloedafname) zoals in de informatiebrief is beschreven.

Ik geef toestemming om de gegevens te verwerken voor de doeleinden zoals beschreven in de informatiebrief.

Ik geef **WEL/NIET*** toestemming om lichaamsmateriaal (bloed) na afloop van de studie te bewaren en dit in de toekomst te gebruiken voor onderzoek.
Ik geef toestemming voor deelname aan dit onderzoek.

Naam eigenaar: _____

Datum: _____

Handtekening: _____

Naam onderzoeker: _____

Datum: _____

Handtekening: _____

2. Information letter for dog owners

Universitair Diergeneeskundig Centrum Utrecht
Universiteitskliniek voor Gezelschapsdieren

Leishmania bij geïmporteerde zwerfhonden

Afdeling Hematologie, Universiteitskliniek Gezelschapsdieren, Universiteit Utrecht
Dr. C. J. Piek, specialist interne ziekten gezelschapsdieren
A.E. Bakker, BSc en A.E.H. Albers, BSc, studenten Diergeneeskunde

U bent recent eigenaar geworden van een hond die geïmporteerd is uit Zuid-Europa. In sommige gevallen zijn honden uit Zuid-Europese landen besmet zijn met chronisch verlopende ziekten als gevolg van Leishmania, Ehrlichia en hartworm. Het is gebruikelijk bij deze honden voorafgaand aan de adoptie te testen op de aanwezigheid van deze ziektes. Helaas zijn het alledrie lastige ziektes om te diagnosticeren met een eenmalige test in het land van herkomst. Dit komt omdat er een groot individueel verschil is tussen het tijdstip waarop honden na de infectie antilichamen aanmaken. Het gevolg is dat er sporadisch toch nog een hond, ondanks een eerdere negatieve test in het land van herkomst, ziek wordt in Nederland.

Wij willen onderzoeken hoe vaak een hond die afkomstig is uit Zuid-Europa in Nederland alsnog positief test op antistoffen tegen deze ziektes. Een beter inzicht hierin maakt dat we betere richtlijnen voor het stellen van een tijdige diagnose kunnen ontwikkelen. Een tijdige diagnose is de sleutel tot een succesvolle behandeling. Hiermee willen wij bijdragen aan een betere gezondheid en welzijn van deze honden.

Wat is de opzet van het project?
De honden zullen in dit project de honden tweemaal onderzocht worden. De eerste keer binnen enkele weken na de aankomst in Nederland, en de tweede keer negen maanden later. Het onderzoek van de honden bestaat uit een vragenlijst, een lichamelijk onderzoek, en een bloedonderzoek. Het onderzoek staat onder leiding van dr. Christine J. Piek, specialist interne ziekten bij gezelschapsdieren en internationaal expert op dit gebied. Het onderzoek wordt mede mogelijk gemaakt door bijdragen van verschillende vermogensfondsen, die het welzijn en de gezondheid van dieren willen beschermen en verbeteren.

Wat levert het onderzoek op?
Met dit onderzoek wordt het meest zinvolle moment bepaald om de test op antilichamen van Leishmania in Nederland uit te voeren. Dit is het moment waarop een hond negatief verklaard kan worden voor deze ziektes en hertesten niet meer nodig is. Hoewel het percentage besmette honden in Zuid-Europa hoog is, is de verwachting dat door de selectieprocedure die toegepast wordt door de stichtingen het percentage van besmetting met een of meer van deze ziektes onder de in Nederland geïmporteerde honden lager ligt.

Wat vragen we van u als eigenaar?
Wij willen u vragen om met uw hond deel te nemen aan dit project over Leishmania. Het onderzoek van uw hond vindt plaats op de Universiteitskliniek voor Gezelschapsdieren. Uw hond wordt twee keer uitgebreid onderzocht, en beide keren wordt bloed afgenomen.
Het bloed wordt direct onderzocht op antistoffen tegen Leishmania en hartwormlarven. Hiervan ontvangt u na ongeveer 10 dagen de uitslag. Als tijdens het bezoek blijkt dat uw hond ziek is of uw hond wordt tijdens de onderzoeksperiode ziek, dan verwijzen we u in overleg naar ofwel uw eigen dierenarts. U bent als deelnemer aan het onderzoek ook van harte welkom voor onderzoek op de Universiteitskliniek. De kosten die hieraan verbonden zijn voor uw eigen rekening.

Als u deelneemt vragen we u ook toestemming om de bloedmonsters op te slaan. In een latere fase onderzoek vindt onderzoek plaats naar andere ziektes, in ieder geval naar Ehrlichia en hartworm antistoffen.

Contactgegevens:

ProjectLeishmania@uu.nl

Dr Christine J. Piek
Specialist Interne ziekten Gezelschapsdieren
Hoofd afdeling Hematologie, Immunologie, Vector overgedragen aandoeningen
Universiteitskliniek Gezelschapsdieren, Universiteit Utrecht

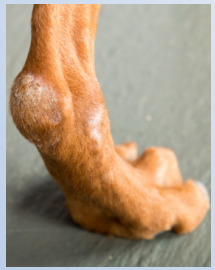
Symptomen

Leishmania is een ziekte die veel verschillende verschijnselen kan veroorzaken. Voorbeelden van verschijnselen zijn hieronder beschreven.

Een opvallend verschijnsel bij honden met Leishmania is de huid op de kop van de hond. Vaak komen er schilferige plekken rondom de ogen, neus en op de oren. Deze schilferige plekken zijn soms ook te zien op de hakken en ellebogen.

Ook kan het voorkomen dat de hond stram en stijf wordt. Leishmania kan ervoor zorgen dat de gewrichten gaan ontsteken, wat pijnlijk is. Dit vindt bij meerdere gewrichten plaats en uit zich daarom in stram bewegen.

Verder treedt er vaak vermagering op. Een infectie doormaken kost veel energie voor het lichaam, hierdoor verliezen de honden gewicht.



Schilferige plekken op de hak

Heeft u vragen naar aanleiding van deze folder?

EMAIL: ProjectLeishmania@uu.nl

Informatiefolder

Project Leishmania

FACULTEIT DIERGENEESKUNDE
UNIVERSITEITSKLINIEK VOOR GEZELSCHAPSDIEREN

Algemene informatie

In deze folder wordt er beschreven wat er bij uw hond wordt onderzocht en hoe bloedafname in zijn werk gaat. Verder worden in deze folder de belangrijkste symptomen beschreven.

De tijd tussen de besmetting en de verschijnselen van Leishmania varieert tussen een aantal maanden en jaren. De tijd waarin antilichamen worden gemaakt is ook variabel. Hierdoor is het lastig te bepalen wanneer de juiste tijd is om een hond hierop te testen. Dit is de reden dat het onderzoek en de test twee keer wordt uitgevoerd. Op deze manier kan er worden bepaald wanneer het beste tijdstip is dat een hond op Leishmania wordt getest.

Een ander doel van dit onderzoeksproject is om uit te vinden welk percentage geïmporteerde honden uit Spanje besmet is met Leishmania. Hierdoor is er betrouwbare informatie beschikbaar voor dierenartsen.

Lichamelijk onderzoek

In het onderzoek wat er wordt uitgevoerd wordt er gekeken naar de voedingstoestand van de hond. Daarvoor wordt de 'Body Condition Score' gebruikt. Dat is een schaalverdeling van 1 tot 9, waarbij 1 heel mager is en 9 ernstig overgewicht.

Verder wordt de ademhaling, de hartslag en de temperatuur van de hond onderzocht. Ook wordt er gekeken naar de slijmvliezen, zo kan een idee worden gevormd over de doorbloeding van kleine bloedvaten.

Dan komen de lymfeknopen aan bod. Lymfeknopen zijn een belangrijk onderdeel van het immuunsysteem. Er wordt onder andere bepaald of deze vergroot zijn. In de afbeelding is te zien welke lymfeknopen worden beoordeeld.

Een ander onderdeel is de huid en beharing van de hond. Bij Leishmania is dat extra belangrijk omdat er vaak karakteristieke schilferige plekken rondom ogen en neus worden gezien.

Bloedafname

Het bloed wordt afgenomen uit de halsader, dit is te zien in onderstaande afbeelding. Het bloed wordt bewaard in bloedbuisjes. In het laboratorium wordt er een antilichamen test op Leishmania uitgevoerd. De uitslag van deze test is na 14 dagen beschikbaar en wordt direct aan de eigenaar gecommuniceerd.

Bloedafname bij een hond

4. Protocol Knott Test

Protocol KNOTT test

Project Buitenlandziekten

1. Meng 1 ml EDTA bloed met 9 ml Formaldehyde 2%

    - Ht boven 0.45? Dan de vloeistof met Formaldehyde +- 2 minuten langer laten staan.*

2. 5 minuten afdraaien bij 1000 – 1500 rpm kamertemperatuur (21 graden Celsius)
3. Afpippeteren tot 1 ml vloeistof
4. Breng druppelsgewijs 1 druppel vloeistof aan op het voorwerpglaasje
5. Afdekken met dekglaasje
6. De resterende ml mengen met enkele druppels methyleenblauw
7. Breng druppelsgewijs 1 druppel vloeistof aan op het voorwerpglaasje
8. Afdekken met een dekglaasje
9. Bekijken onder microscoop, 10x en/of 40x

(* Bij een hond met een Ht van 0.56 werden de ery's niet allemaal gelyseerd door de formaldehyde, door langer de formaldehyde te laten inwerken werden de ery's gelyseerd. Als de erythrocyten te weinig gelyseerd waren onder de microscoop grote plakken van verschillende lagen intacte erythrocyten zichtbaar. Het beeld werd daardoor minder betrouwbaar.

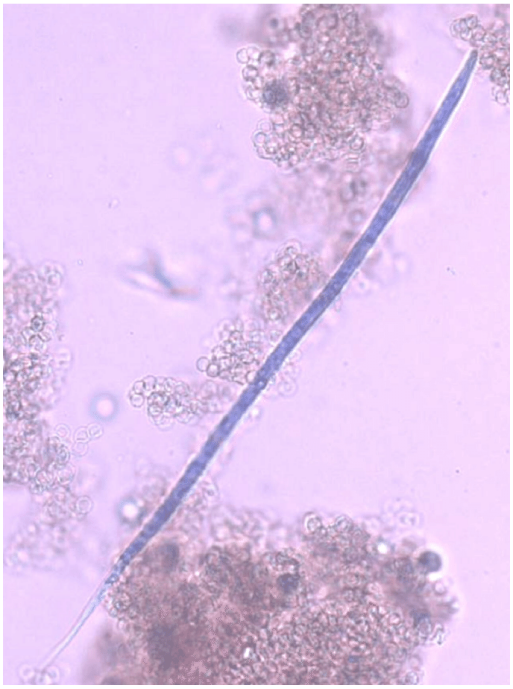


Figure 1: Knott test, Dirofilaria Immitis
Derived from: Traversa, D., Di Cesare, A., & Conboy, G. (2010). Canine and feline cardiopulmonary parasitic nematodes in Europe: emerging and underestimated. *Parasites & vectors*, *3*(1), 6

5. Informed consent dog owners (antibody titre)

Informatiebrief en toestemmingsformulier

Onderzoek naar Leishmania (*en andere vector-overgedragen aandoeningen*) bij geïmporteerde zwerfhonden

Graag vragen wij u middels dit schrijven toestemming voor deelname van uw hond aan een onderzoek van het departement Geneeskunde van Gezelschapsdieren van de faculteit Diergeneeskunde van de Universiteit Utrecht. Het is voor deelname aan een wetenschappelijk onderzoek vereist dat u een schriftelijke verklaring geeft dat u volledig bent ingelicht over het onderzoek en dat u bereid bent om mee te werken. Dit wordt '*informed consent*'; ofwel geïnformeerde toestemming genoemd.

U zult door middel van dit document uitgelegd krijgen wat de opzet is van het onderzoek, wat uw medewerking precies zal inhouden, en wat de voordelen, nadelen en mogelijke risico's zijn. Ook zal u worden uitgelegd hoe er met de resultaten van het onderzoek wordt omgegaan zodat uw privacy gewaarborgd is.

Het doel van dit project
Door middel van dit onderzoek willen wij uitzoeken of er een relatie is tussen de hoeveelheid antilichamen in het bloed en de klinische verschijnselen een hond met Leishmania.

Algemene informatie
Uw hond is afkomstig uit Zuid-Europa of heeft er een deel van zijn leven doorgebracht. Een van de hondenziektes die in Zuid-Europa veel voorkomt is Leishmaniasis. Dit is een ziekte die door een zandvlieg wordt overgebracht. Dergelijke ziektes, waarbij een insect zorgt voor besmetting, zijn vector-overdraagbare aandoeningen. Omdat de zandvlieg die Leishmania overdraagt in Nederland niet voorkomt kunnen honden in Nederland de ziekte niet oplopen. Een hond die in Zuid-Europa geïnfecteerd geraakt is heeft daar in het algemeen een zomer doorgebracht. Onderzoek naar besmetting en ontwikkeling van de ziekte is van belang om tijdig met medicatie in het ziekteproces te kunnen ingrijpen.

Waarom is mijn hond hiervoor geselecteerd?
Uw hond is voor dit onderzoek geselecteerd omdat hij Leishmaniasis heeft of op dit moment sterk verdacht wordt van deze ziekte.

Waar wordt mijn hond op getest?
Het bloed van uw hond wordt getest op de hoeveelheid antilichamen tegen Leishmania.

Wie voert het onderzoek uit?
Het onderzoek staat onder leiding van dr. C.J. Piek, Internist voor Gezelschapsdieren, hoofd van de afdeling hematologie. Studenten van de laatste fase van de opleiding Diergeneeskunde hebben een ondersteunende functie als onderdeel van de studie.

Wat zijn negatieve/positieve gevolgen van meewerken aan dit onderzoek?
Bloedafname is een kleine ingreep. Het zou kunnen gebeuren dat er als gevolg van het bloedprikken een infectie of bloeding optreedt, dit komt echter zeer zelden voor en is van voorbijgaande aard.

Wat gebeurt er met de gegevens/informatie?
De gegevens die tijdens dit project zijn verzameld worden (anoniem) gebruikt voor een publicatie in een wetenschappelijk tijdschrift. De gegevens zullen niet te herleiden zijn tot het individuele dier. Het bloed wat tijdens dit onderzoek wordt afgenomen wordt bewaard voor vervolgonderzoek.

Vertrouwelijk- wie heeft er toegang tot de data?
Persoonsgegevens die worden verzameld tijdens deze studie worden opgenomen in het dossier van uit hond in het beveiligde elektronische patiëntinformatiesysteem Vetware. Persoonsgegevens zullen nooit worden vermeld in publicaties of presentaties. In publicaties of presentaties naar aanleiding van het onderzoek worden, naast de uitkomsten van het onderzoek, uitsluitend de uiterlijke kenmerken van uw hond, zoals ras, geslacht, leeftijd en de relevante delen van de ziektegeschiedenis en medische gegevens anoniem gepubliceerd.

Kan ik mij terugtrekken uit dit onderzoek?
U kunt zich terugtrekken uit dit onderzoek op elk moment zonder daarvoor een reden te geven. Dit kunt u doen door een email te sturen naar: C.J.Piek@uu.nl

Wie kunt u benaderen voor dit onderzoek?

Email: ProjectLeishmania@uu.nl

Toestemmingsverklaring

Ik bevestig dat ik het informatieformulier heb gelezen. Ik begrijp de informatie. Ik heb de mogelijkheid gehad om aanvullende vragen te stellen en deze zijn naar tevredenheid beantwoord. Ik heb voldoende tijd gehad om over deelname na te denken.

Ik weet dat mijn deelname geheel vrijwillig is en dat ik mijn toestemming op ieder moment kan intrekken zonder dat ik daarvoor een reden moet geven.

Ik geef toestemming voor het uitvoeren van de procedure (Het uitvoeren van lichamelijk onderzoek en bloedafname) zoals in de informatiebrief is beschreven.

Ik geef toestemming om de gegevens te verwerken voor de doeleinden zoals beschreven in de informatiebrief.

Ik geef WEL/NIET toestemming om lichaamsmateriaal(bloed) na afloop van de studie te bewaren en dit in de toekomst te gebruiken voor onderzoek.

Ik geef toestemming voor deelname aan dit onderzoek.

Naam: _____

Datum: _____

Handtekening: _____

Naam onderzoeker: _____

Datum: _____

Handtekening: _____

6. DAT titre height compared to clinical stages, haematological and biochemical alternation in 109 dogs with (potentially) CanL infection.

Significant relations between DAT titre and classification systems were found in all systems using Spearman's coefficient of rank correlation test (table 17).

Table 17 Rho and P-values of DAT titres and classification systems

Classification system	Spearman's correlation coefficient (r_s)	*P*-value
Mancianti et al.	0,271	0,004*
Ciaramella et al.	0,339	0,003*
Amusategui et al.	0,483	<0,0001*
LeishVet	0,330	0,005*
CLWG	0,461	<0,0001*

In all systems, a positive correlation was found, meaning that severe clinical signs were related to higher DAT titres. The boxplots show the significant relation between the DAT titre and the system.

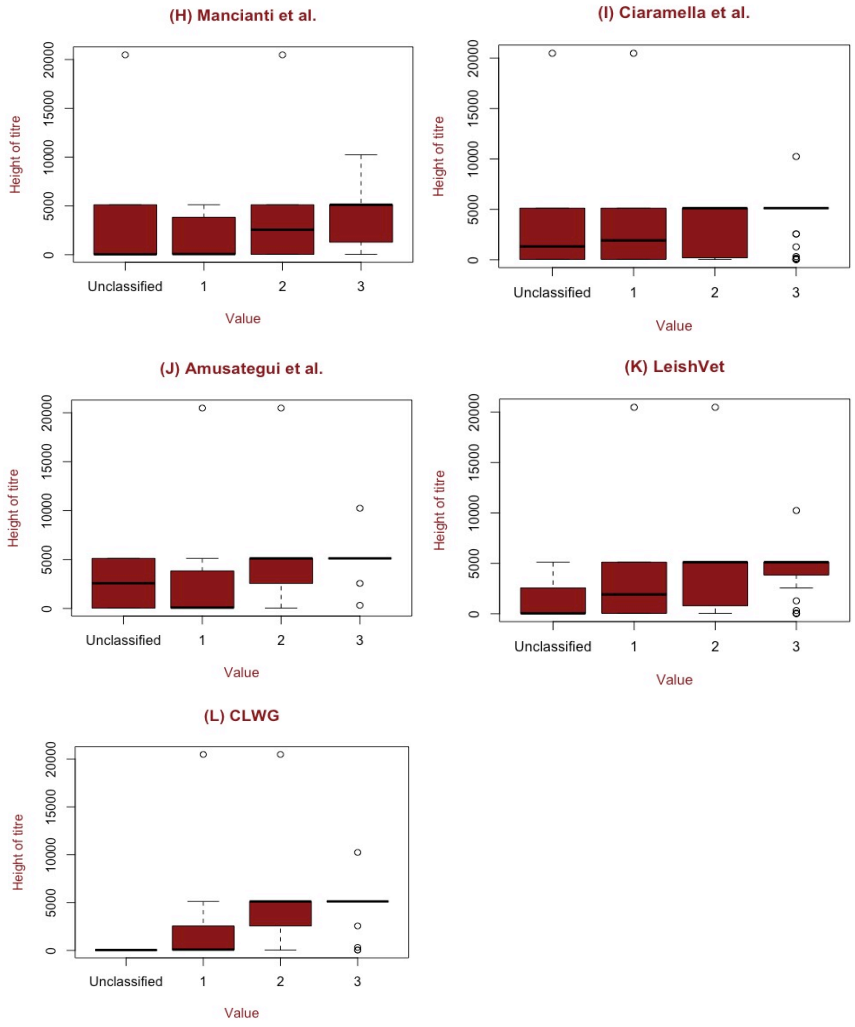


Figure 1 Boxplots of significant relations between DAT titres and classification systems. The black line in the middle of the red boxes represent the mean of the parameter and dots represent the outliers.

None of the data was normally distributed except for thrombocytes and albumin. All haematological and biochemical parameters were tested for correlation using Spearman's correlation test. The

significance and the degree of association between DAT titre and the haematological and biochemical parameters are listed in table 9. A significant correlation was found between DAT titre and Ht, leukocytes, neutrophils, gamma globulin, total protein and creatinine.

Table 9 Rho and P-values of DAT titre and haematological and biochemical parameters

Parameter (units)	Spearman's correlation coefficient (r_s)	*P*-value
Ht (L/L)	-0,223	0,021*
Leukocytes (x10ˆ9/L)	-0,274	0,004*
Neutrophils (x10ˆ9/L)	-0,280	0,004*
Thrombocytes (x10ˆ9/L)	-0,166	0,294
Alpha 1 (g/L)	0,142	0,178
Alpha 2 (g/L)	-0,054	0,609
Beta 1 (g/L)	0,186	0,078
Beta 2 (g/L)	0,117	0,270
Gamma globulin (g/L)	0,394	<0,0001*
Total Protein (serum) (g/L)	0,267	0,012*
Albumin (spectre) (g/L)	-0,204	0,052
Creatinine (blood) (µmol/L)	0,241	0,024*
UPC Ratio	0,164	0,200

In case of a significant relation between the DAT titre and a parameter, a plot was made. DAT titre values were transformed into logs.

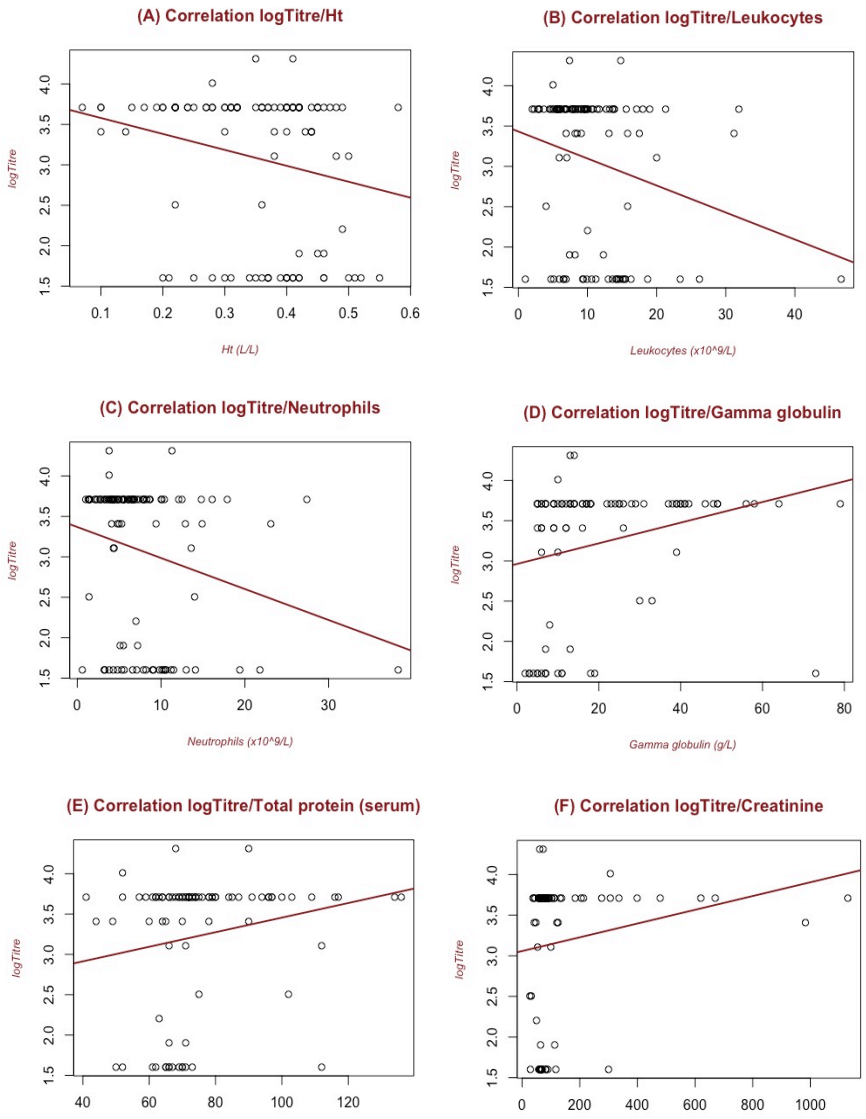


Figure 2 Correlation between antibody titre and Ht, leukocytes, neutrophils, gamma globulin, total protein and creatinine. The red line represents the correlation between the antibody titre and the parameter.

Plot A, B and C show the negative correlation between antibody titre and respectively Ht, leukocytes and neutrophils. Higher titres are correlated with lower values of Ht, leukocytes and neutrophils. Plot D, E and F show the positive correlation between antibody titre and respectively gamma globulin, total protein and creatinine. Higher titres are correlated with higher values of gamma globulin, total protein and creatinine.

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