Leishmaniasis: response to Allopurinol treatment and seroprevalence in imported dogs from endemic areas

Research Project Veterinary Medicine Utrecht University

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Abstract 1

An increasing number of dogs is imported from endemic countries every year. However, the seroprevalence of Leishmaniasis in the Netherlands is still unknown. The aim of this study is to make an estimate of the seroprevalence for *Leishmania* and *Dirofilaria immitis* in dogs imported from endemic geographical areas to the Netherlands. Dogs from endemic areas were tested on Leishmania antibodies maximum six weeks after import to the Netherlands. This study found two out of nine imported dog were infected with Leishmania. However, due to the small number of dogs further research should be conducted to determine the seroprevalence in the Netherlands.

Abstract 2

The aim of this study is to study the changes in clinical pathological parameters of leishmania infected dogs over time during treatment with Allopurinol within one year. Therefore, this retrospective cohort study assessed the clinicopathological parameters of 21 dogs with leishmaniasis at the start of the treatment and 3, 6 and 12 months after the start of the treatment. This study shows that haematocrit, thrombocyte and albumin values decrease significantly 2.5 to 4.5 months after the start of Allopurinol monotherapy. Furthermore, total protein, β-2 globulin and γ-globulin decrease significantly 2.5-4.5 months after treatment is provided. Also, Xanthine crystals were found in the urine sediment of 62.5 % of the dogs, 2.5-4.5 months after the start of the therapy. Thus, it may be concluded that allopurinol monotherapy normalises most of the involved parameters in Leishmania infected dogs.

Introduction

Leishmaniasis in general

Leishmaniasis has serious consequences on the quality of life of dogs. It is caused by the protozoan parasite *Leishmania*. In the 'Old World' *Leishmania donovani* and *infantum* cause visceral Leishmaniasis. Dogs are the main reservoir species of *Leishmania infantum.* Leishmania is transmitted through a vector. Female sand flies of the genus *Phlebotomus* are responsible for the transmission in Southern Europe (Bates, 2007).

Flagellated protozoans (promastigotes) develop in the sand fly. When the sand fly feeds, the promastigotes are injected in the host species. Macrophages accumulate the promastigotes and distribute through the body. The amastigote form is intracellular and replicates in the host cell. When the sand fly feeds this infected dog, it takes in amastigotes. This completes the life cycle of *Leishmania*. Other ways of transmission of Leishmania are canine blood donors and contact through wounds or bites (Solano-Gallego et al., 2011).

Clinical manifestations develop 1 month to 7 years after infection. Clinical signs are indefinite and various. Most dogs develop visceral leishmaniasis, multiple organs can be affected. Without a treatment, some dogs develop chronic progressive disease, which includes chronic kidney disease and eventually die (Nelson & Couto, 2014).

Diagnosis is based on clinical signs and basic laboratory test, in combination with a positive Leishmania test or cytological confirmation of Leishmania amastigotes in lymph nodes. Multiple tests can be used to detect Leishmaniasis antibodies, namely: Immunofluorescence antibody test(IFAT), Direct Agglutination Test(DAT) and PCR (Paltrinieri et al., 2010).

Treatment

Koutinas et al. (2001), a randomised clinical trial, found a significant improvement compared to the control group in clinical signs, mainly body condition score, peripheral lymfadenopathy and epistaxis while Allopurinol treatment was provided. Also, clinicopathological abnormalities, mainly anemia, lymphopenia and hyperglobulinemia normalised after 4 months of treatment. 37 dog received Allopurinol therapy (10mg/ kg B.W. twice daily), the control group consisted of eight dogs.

In contrast to the effectivity of the Allopurinol treatment, allopurinol may cause some side effects. Torres et al. (2016) studied urinary adverse effects of allopurinol treatment. The most important adverse effect is xanthinuria. All of the dogs treated with allopurinol showed xanthinuria with urinalysis. 21 out of 42 dogs showed urolithiasis with abdominal ultrasound. Allopurinol is responsible for an increased concentration of xanthine in urine, due to a decreased formation of uric acid. The metabolite xanthine is little soluble in urine. It is unknown in which period of time after treatment the xanthine crystals develop.

Torres et al. (2011) found that a combination of meglumine antimoniate and allopurinol therapy results in a long term survival and improvement of the clinical conditions. The meglumine antimoniate injections may cause severe pain and phlebotoxicity and thrombophlebitis. Furthermore, it may have nephrotoxic side effects, such as vomiting. However, another study found improvement of the renal functions after treatment (Reguera et al., 2016).

Immunological response

The immunological response differs between different breeds of dogs. Solano-Gallego et al. (2000) performed a delayed-type hypersensitivity(DTH) test and an ELISA on 31 Ibizian hounds and 25 other breeds in an endemic area. This DTH test causes a type 1 hypersensitivity response, a cellular response to the leishmania antigen. In this study, 81% of the Ibizian Hounds had a positive DTH test in comparison to 48% of other breeds. The percentage of infection was 81% for Ibizian hound and 72% for other breeds. Thus, this study found that Ibizian hounds are more likely to develop a cellular immune response against Leishmania. This may result in less clinical signs.

Seroprevalence of Leishmania

Leishmaniasis is endemic in Southern- Europe and distributes through Northern-Europe with imported dogs. Teske et al. (2002) found that the estimated risk of infection with Leishmania for dogs due to traveling to endemic areas in Southern Europe was between 0.027% and 0.23%. Thus, traveling between countries may not be the main cause of distribution. Furthermore, it was reported that 12608 dogs were imported from other countries to the Netherlands in 2016. The number of imported dogs is increasing over the years (Radstake., 2017). A large part of these dogs is imported from Southern- Europe.

It is known that the seroprevalence in endemic countries varies between different areas. Muniesa et al. (2016) found, based on previous studies, that the seroprevalence of Leishmania varies in different counties of Spain. The highest prevalence was found in Mallorca (67%), the lowest was found in Granada (5,3%). This could have an effect on the number of infected dogs imported to the Netherlands, depending on the geographical origin.

Besides, de dog's purpose is associated with a different seroprevalence. In Muniesa et al. (2016) 311 dogs were serologically tested. The highest seroprevalence was found in breeder dogs, namely 33.3%. Companion (16.1%), guard (10.6%) and hunter (6.2%) dogs had a lower seroprevalence (Muniesa et al., 2016). This study also compared the seroprevalence of urban and rural areas. In urban areas the seroprevalence was 17.9% compared to 8.2 % in rural areas. The reason for the high seroprevalence in urban areas is, according to the study, that most of the breeder dogs lived in urban areas.

The association between age and Leishmaniasis was not significant in a meta-analysis of dogs in endemic areas of Brazil. However, the prevalence of dogs younger than 1 year or 2 years of age was lower compared to older dogs (Belo et al., 2013). Transmission of humoral and cellular immunity is possible from the mother to offspring (Andrade et al.,2001). Maternal antibodies in young dogs could conflict with the tests and result in a higher seroprevalence.

In Germany, a non-endemic area, Menn et al. (2010) studied the seroprevalence of Leishmania. They studied 4681 blood samples of dogs from mostly animal welfare organisations and private persons, 4226 of these dogs were imported dogs, 68.7 % of the imported dogs were from the Mediterranean region. This study found a seroprevalence of 12.2% for Leishmania (Menn et al., 2010). Also, They detected microfilaria from Dirofilaria immitis in 7.7 % of the dogs with a Knott test. Little is known about the seroprevalence of canine Leishmania and Dirofilaria Immitis in the Netherlands. Thus, the first aim of this study is to investigate the prevalence of leishmaniasis, a non-endemic vector borne disease, and Dirofilaria Immitis in dogs imported from endemic areas to the Netherlands.

Clinical and bio-pathological parameters

Clinical and bio-pathological parameters indicate different stages of leishmaniasis and a degree of organic involvement. Amusategui et al. (2002) found that dogs with both visceral and cutaneous signs had higher total serum protein and γ-globulin levels in comparison with asymptomatic dogs. Furthermore, the A-G ratio, mean albumin, haemoglobin, packed cell volume (PVC) and red blood cell (RBC) values were lower than asymptomatic dogs.

The total protein and γ-globulin rate decreases significantly within the first 90 days after the start of treatment (Torres et al., 2011). The treatment consisted of a combination therapy of meglumine antimoniate and allopurinol. This decrease was found in a group of 23 dogs. Also, a correlation was found between the normalisation of serum proteins and improvement clinical signs.

Besides, Provebio et al. (2016) found a significant decrease of the Urine Protein Creatinine (UP/C) ratio in 20 dogs with leishmaniasis, while treatment of Allopurinol and miltefosine was provided. The parameters were recorded at the start of the therapy and 28 days later. Both of these studies investigated a combination therapy, one with allopurinol and meglumine antimoniate and one with miltefosine and allopurinol. Little is known about the change of parameters with allopurinol monotherapy.

Furthermore, little is known about the period of time in which the parameters may change. Therefore, the second aim of this research is to study the alteration of clinical and bio-pathological parameters in Leishmania infected dogs whilst treatment is provided within one year.

Research goal

The goal of this study is

1) to make an estimate of the seroprevalence for Leishmania and *Dirofilaria immitis* in dogs imported from endemic geographical areas to the Netherlands

2) to study the changes in clinical pathological parameters of leishmania infected dogs over time during treatment with Allopurinol within one year.

Hypothesis

Aim 1

H0 = The prevalence of Leishmaniasis in imported dogs is equal to reported prevalence in Spain.

Aim 2
H0 = there is no difference in the response of the clinicopathological variables at T = 1 compared to T = 0 in dogs between respective periods of therapy.

Material and methods *aim 1*

Material

The first part of the prospective cohort study was done. Several students will contribute to the next part of this study. Infection for the purposes of this study was defined in seroconversion and/or clinical disease. The cohort was selected from the dogs that are imported from endemic Mediterranean countries by several animal Welfare organisations. These dogs were examined for clinical or laboratory evidence of these diseases within 6 weeks after import, and will be examined 9 months to 1 year after the first examination.

Inclusion criteria

Dogs were born in an endemic area in a southern European Country. They were minimal 7-8 months of age, and resided in the Netherlands for less than 6 weeks.

Ethical Approval

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

Methods

Data collection

The Stray Animal Foundation Platform (SAFP) selected a list of Animal Welfare Organisations, that imported dogs from endemic areas to the Netherlands. These organisations were contacted per email or phone call from December 2018 to January 2019 and were asked to inform new potential owners about participation in this study.

The entire examination took place at the Department of Clinical Sciences of Companion Animals of the Faculty of Veterinary Medicine of Utrecht University in the period between December 2018 up to February 2019.

The following data was sampled:

Signalment

Sex, breed, age, weight, chip number were obtained.

History

The history of the dog was retrieved by questionnaire. Additional questions were asked about environmental history, time the dog resided in the Netherlands, the results of earlier performed laboratory tests and clinical signs according to the owner.

Physical exam

The physical exam included general appearance, respiratory rate and character, heart rate, rectal temperature, coat and skin, palpation of lymph nodes, mucous membranes and CRT. A dermatological examination was performed: morphology, configuration, distribution of lesions and

coat and nails were recorded. Some dogs had an orthopaedic, ophthalmologic or dermatologic exam performed by a European specialist.

Samples

The first blood sample was taken within 6 weeks after import. The second samples will be taken 9- 12 months after import. This will be performed by other students. The blood sample of 10 ml was taken from the jugular vein. The blood was divided over 3 EDTA 1 ml tubes and 3 Z Serum Sep Clot Activator 2 ml tubes.

Laboratory Tests

The University Veterinary Diagnostic Laboratory (UVDL), of the faculty of Veterinary Medicine, Utrecht University, performed testing on de blood. The haematocrit, leukocyte count, neutrophil count, thrombocyte count, reticulocyte count and reticulocyte hemoglobin content(CHr) were determined. For these tests a Siemens Advia 2120i system with veterinary software was used. Furthermore, the UVDL performed Leishmania serology by a Direct Agglutination Test(DAT). The test can be used for both L. donovani and L. infantum (Harith et al., 1989). Stained killed promastigotes were added to blood or serum. If positive, agglutination occurred, this was visible with the naked eye. The DAT was performed accordant El Harith et al. (1989).

Within 24 hours a Knott Test was executed by research students. Firstly, 1 ml EDTA blood was mixed with 9 ml formaldehyde 2% solution. This was centrifuged for 5 minutes at 1250 rpms, at 21°C. The supernatant was discarded (up to 1 ml). A plastic pipette was used to add a drop to the centre of the slide, a cover slip was placed on top of the drop. A plastic pipette was used to add a drop of methylene blue to the remaining sediment. This was mixed with a plastic pipette. A drop was added to the centre of the slide, a cover slip was placed on top of the drop. The samples were examined on the microscope under 10x power.

Dirofilaria immitis antigen was detected with a commercial test kit (FASTest® HW Antigen, MegaCor Diagnostik GmbH, Hörbranz, Austria).
Unused samples were stored for further research on Ehrlichia antibodies.

Statistical Analysis

Commercial software (Rstudio, version 1.1.463 – © 2009-2018 RStudio, Inc.) was used to perform statistical analysis. 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% from the number of imported dogs (n=11.300, 2015) to the Netherlands. For this, an online available calculator was used (“Samplesize”, 2019).

Material and methods *aim 2*

Material

This study was set up as a retrospective cohort study. It assessed the changes in clinical pathological parameters in this cohort before and after treatment up to one year after the start of therapy. In addition, this study assessed if the clinical pathological parameters are significantly different in dogs that experienced a treatment change after 3 months after the start of therapy. A previous study (de Jong et al., 2018) was performed on partly, the same data as the current study. For this, patient records (2008-2017) were retrieved from the patient database system of the Department of Clinical Sciences of Companion Animals of the Faculty of Veterinary Medicine of Utrecht University (CSCA) (Vetware, v. 1.6.135-rc01, Canada).

Inclusion criteria and Clinical Procedures

Included were dogs with the diagnosis Leishmaniasis, based on abnormalities in clinical exam (e.g. skin lesions, lymphadenopathy, polyuria/polydipsia) and/or clinicopathological abnormalities (e.g. anaemia, azotaemia, elevated γ-globulin) in combination with cytological confirmation of Leishmania amastigotes or a positive Leishmania antibody titre. Only dogs that received their initial therapy at the CSCA were included.

The treatment was executed in accordance with the protocol used at the CSCA. This protocol consisted of initial therapy with allopurinol (A) monotherapy. If the response to the therapy was inadequate according to the attending veterinarian during the control visit, 3 months after the start of the therapy, N-Methylglucamine-antimonate(G) or miltefosin (M) was added to the therapy. If the owner noticed clinical signs prior to the control visit, a similar protocol was executed. The choice between G and M depended on renal function of the dog, practical reasons and owner preference. For the follow-up period, information was collected on treatment during diseased period and date/cause of death if deceased. If this information was not available from the patient records owners and/or referring veterinarians were contacted. Also, the dog had to attend to at least one of the control visits the protocol includes at 3, 6 or 12 months after the start of the therapy. Dogs that had an incomplete file were excluded.

Methods

The University Veterinary Diagnostic Laboratory (UVDL), of the Faculty of Veterinary Medicine, Utrecht University, performed all laboratory tests. The Leishmania titre was determined with a Direct Agglutination Test (DAT) accordant El Harith et al. (1989). The titres ≤1:160 were considered negative, 1:320 doubtful and >1:640 positive (limit 1:5120). For the determination of blood parameters a Siemens Advia 2120i or Advia 120 haematology system with veterinary software was used. The urine analysis sediment and Urine-Protein-Creatinine-Ratio (UPC) was determined on Olympus AU 680 (Beckman Coulter, Woerden, The Netherlands). The protein spectrum was determined by protein electrophoresis (hydrogel β-1, β-2, Hydriasis, Sebia, Surrey, The United Kingdom).

The following parameters were obtained from the patient database system at each control visit: Haematocrit, leukocyte count, neutrophil count, thrombocyte count, plasma creatinine concentration, total protein and the serum protein spectrum. From the urine was the specific gravity, Urine Protein to Creatinine ratio(UPC) and the result from the microscopic evaluation of the urine sediment such as the presence of renal epithelium in urine and number of xanthine crystals in urine recorded.

To evaluate the response to treatment over time the following time ranges were composed:

T0 = Diagnosis/start therapy
T1 = 75-135 days after T0 (2.5-4.5 months)
T2 = 165-225 days after T0 (5.5-7.5 months)
T3 = 345-400 days after T0 (11.5-13.8 months)

For the comparison of allopurinol monotherapy with allopurinol and miltefosin (*A+M*) combination therapy and allopurinol and N-Methylglucamine-antimonate (*A+G*) combination therapy.

Statistical Analysis

Commercial software (Rstudio, version 1.1.463 – © 2009-2018 RStudio, Inc.) was used to perform statistical analysis. The Shapiro-Wilk normality test was used to assess the distribution of the data. Parameters at T0, T1, T2, T3 were compared using a paired t-test when the data were normally distributed. A non-parametric two sample-Wilcoxon test was used when the data were not normally

distributed. The level of significance was set at $p<0.05$. To assess the difference in parameters between two therapies, the same statistical analysis was performed on T0 and T1.

Results

Aim 1

Informed consent and information for owners were manufactured (Supplementary materials, Part 1-Part 2). Also, a protocol for students to perform the Knott test was developed (Supplementary materials, Part 3)

Nine dogs were included based on the inclusion criteria. The mean age was 3 years and varied from 8 months old to 4,6 years old. Six females and three males. Most of the dogs were crossbreeds, four dogs were from Greece, three dogs from Spain and two dogs were from Portugal.

Of nine dogs, six dogs were negative for both Leishmania antibodies and Dirofilaria antigen. Of the other three dogs, two dogs had a positive Leishmania antibody titre (1:5120). One dog had a vague treatment history in the country from origin and diarrhoea, anaemia, thrombocytopenia, leukopenia, neutropenia. The second dog had skin lesions around the eye and nose and had received meglumine antimoniate (glucantime) and allopurinol treatment after import in the Netherlands. Furthermore, both of the dogs had lymphadenopathy and skin lesions at the ears, elbows and tarsi.

And finally, the third dog had a positive *Dirofilaria immitis* antigen test, the Knott test was negative. This dog didn't have notable clinical signs.

Aim 2

45 dogs were reviewed based on positive titre, suggestive clinical signs for Leishmaniasis or positive cytology of lymph nodes. 24 out of 45 dogs were excluded because too little data was available. The lack of data in 24 dogs was due to death by Leishmaniasis (4), loss of contact before first control visit (13) or the time of the control visits was not within the time ranges used for this study (8). Thus, 21 dogs were included in this study based on inclusion criteria. The mean age of the dogs was 4.6 years. The dogs were between 11 months to 11 years of age at the time of diagnosis. In the group of dogs were 14 males and 7 females, most dogs were crossbreeds. The country of origin was mainly Spain.

Two dogs were excluded from urine parameters as a result of too little data, the first dog was also excluded from protein spectrum due to lack of data.

A non-significant difference between T0 and T1, T2 and T3 for the results of the CBC (complete blood count) was found in leukocytes, neutrophils, creatinine, α-2 globulin, β-1 globulin, UPC Ratio values (*Table 1*). In neutrophils, most values, except outliers, were within the reference values. In β-1 globulin, a large part of the values were below the reference values. At T0, a large distribution of the UPC ratio values can be seen, this reduced at T1, T2 and T3.

A significant difference between the time ranges was found in haematocrit, thrombocytes, total protein, albumin, α-1 globulin, β-2 globulin and γ-globulin (*Table 1*).

In haematocrit and albumin, a significant increase was found both between T0 and T1, T2 and T3. Furthermore, in *Figure 2* is shown that the variation of haematocrit values is higher at T0 and T3 in comparison to T1 and T2. In thrombocytes a significant increase was found between T0 and T1. In *Figure 2* is showed that the distribution is wider at T3 compared to T0, T1 and T2. In total protein, β-2 globulin and γ-globulin was a significant decrease found between T0 and T1 and T0 and T2. Also, in γ-globulin, the decrease between T1 and T2 was significant. In α-1 globulin an significant increase was

found between T0 and T3. Furthermore, in α-1 globulin the median is below the reference value at all time ranges.

The patient files from the outliers in the results were checked to detect abnormalities. It was found that most of the outliers in the boxplots were caused by five dogs, three of these dogs were not under control with allopurinol monotherapy and started later with either M or G. These dogs with values under or above the reference value at T3 may had a relapse of Leishmaniasis.

	p-value T0-T1	p-value T0-T2	p-value T0-T3
Haematocrit	0.0008*(I)	0.0011*(I)	0.04232*(I)
Leukocytes	0.4648	0.5635	0.1415
Neutrophils	0.5771	0.6565	0.3008
Thrombocytes	0.0084*(I)	0.2260	0.9009
Creatinine	1	0.3757	0.9593
Total protein	0.0068*(D)	0.0033*(D)	0.1921
Albumin	0.0059*(I)	0.0032*(I)	0.0061*(I)
α-1 globulin	0.4374	0.2449	0.0411*
α-2 globulin	0.0531	0.0765	0.1823
β-1 globulin	0.3902	1	1
β-2 globulin	0.0080*(D)	0.0080*(D)	0.0569
γ globulin	0.0058*(D)	0.0007*(D)	0.0751
UPC ratio	0.5	0.0625	0.1250

*Table 1: P-values of the comparison between time ranges (T0-T1, T0-T2, T0- T3) after Allopurinol treatment of blood and urine parameters in dogs with leishmanisasis performed with Wilcoxon test or t-test. Significant increase (p<0.05) is showed with *(I), significant decrease (p<0.05) Is showed with *(D) .*

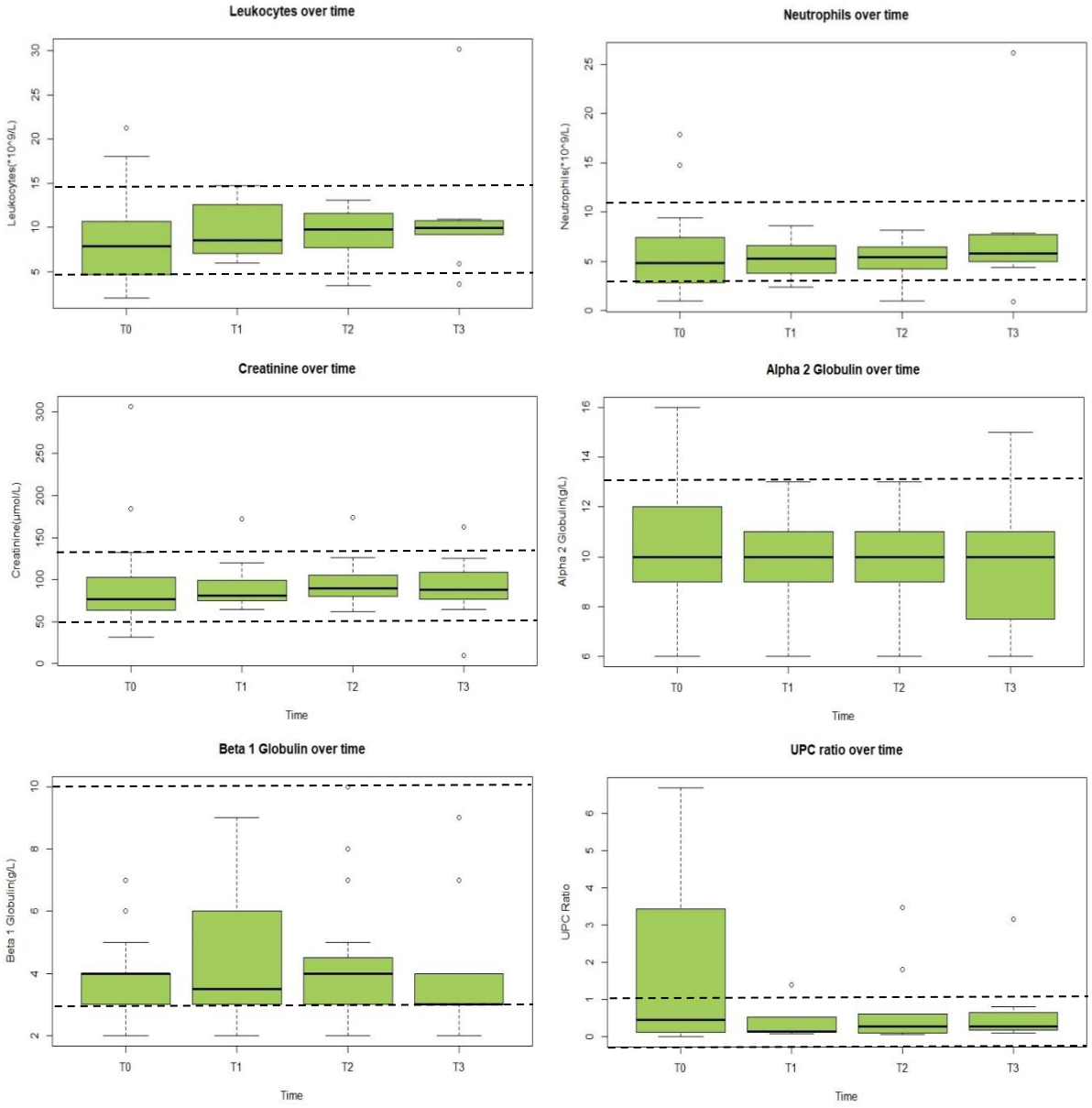

*Figure 1: Dogs (n = 21) with leishmaniasis, treated with Allopurinol monotherapy. Sampled at T0, T1 (75-135 days after T0), T2 (165-225 days after T0), T3 (345-400 days after T0). Evolution over time of Leukocytes(Ref: 4.5-14.6(*10^9/L)), Neutrophils(Ref: 2.9-11.0 (*10^9/L)),, Creatinine(Ref: 50-129 (µmol/L)), α-2 Globulin (Ref: 4-13(g/L)) , β-1 Globulin(Ref: 3-10(g/L)), UPC Ratio(Ref: <1). Vertical black dotted lines represent the reference values. Horizontal bold black lines in the boxplot represent the median and dots represent outliers.*

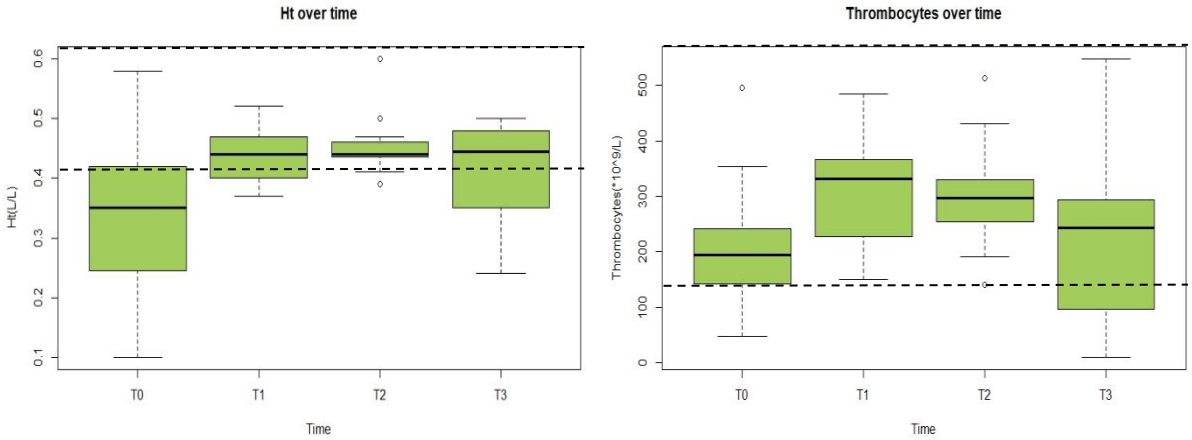

*Figure 2: Dogs (n = 21) with leishmaniasis, treated with Allopurinol monotherapy. Sampled at T0 (start of the therapy), T1 (75-135 days after T0), T2 (165-225 days after T0), T3 (345-400 days after T0). Evolution over time of Haematocrit(Ref: 0.42-0.61(L/L)) and Thrombocytes(Ref: 144-603(*10^9/L)). Vertical black dotted lines represent the reference values. Horizontal bold black lines in the boxplot represent the median and dots represent outliers.*

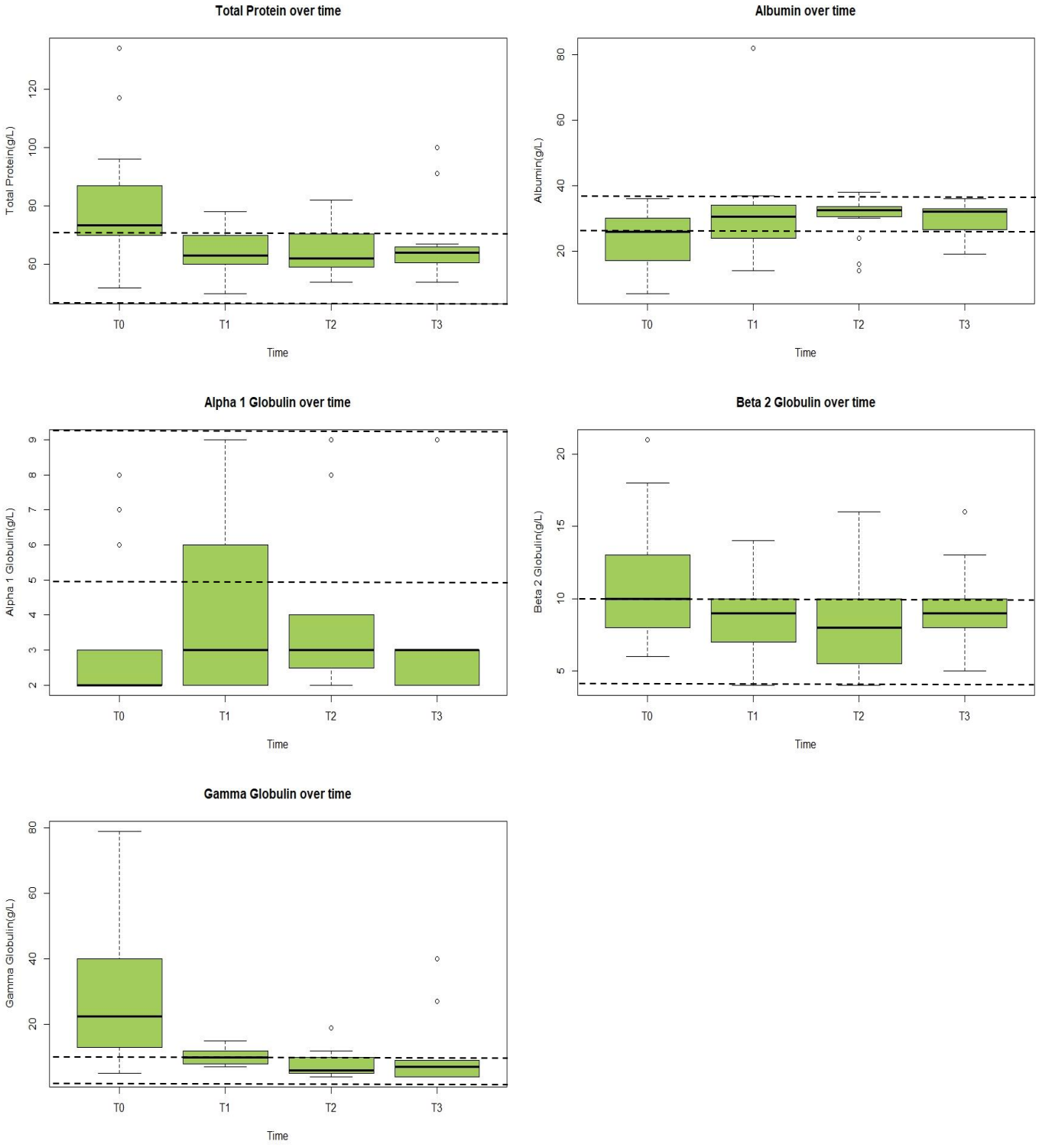

Figure 3: Dogs (n = 21) with leishmaniasis, treated with Allopurinol monotherapy. Sampled at T0 (start of the therapy), T1 (75-135 days after T0), T2 (165-225 days after T0), T3 (345-400 days after T0). Evolution over time of Total Protein(Ref: 55-72(g/L)), Albumin(Ref: 26-37(g/L)), α-1 Globulin(Ref: 5-10(g/L)), β-2 Globulin(Ref: 4-10(g/L)), γ-Globulin(Ref: 3-9(g/L)). Horizontal bold black lines in the boxplot represent the median and dots represent outliers.

Urine sediment

Much data is unavailable due to lack of urine collection in the dogs. Therefore xanthinuria was the only parameter of the urine exam that has been assessed in the urine samples. In at least 50% of the tested dogs xanthine crystals were present in urine sediment at T1, T2 and T3 compared to T0, the start of the therapy (*Table 2*).
Furthermore, renal epithelium in urine sediment was studied. The amount of positive urine samples was very low: 1,2%. Therefore, statistics could not be performed and this data was not included in results.

Urine sediment: Xanthine Crystals	**T0**	**T1**	**T2**	**T3**
Positive	0	5	4	4
Negative	12	3	3	2
Not Available	7	11	12	13

Table 2: (n=19) Number of times Xanthine crystals were present in urine.

Treatment comparison

Nine of 46 dogs received both allopurinol(*A*) and allopurinol and N-Methylglucamine-antimonate (*A+G*) treatment. The reason for this is that *A* monotherapy was insufficient. Four dogs were excluded based on limited data within the specified time ranges. One dog directly started the therapy with both *A+G*. The course of the therapies and the time between the start of the first and the second treatment is shown in *Figure 4*. The *A+G* treatment showed no significant difference with *A* treatment on haematocrit, leukocyte count, neutrophil count, creatinine concentration and protein spectrum at T1. Ranges T2 and T3 were not comparable due to limited data and were therefore excluded.

Six of 46 dogs received both allopurinol(*A*) and allopurinol and miltefosine (*A+M*) treatment. The reason for this is that *A* monotherapy was insufficient. Two dogs were excluded based on lack of data within the time range. The UPC ratio was not tested because too little data was available. The course of the therapies and the time between the start of the first and the second treatment is shown in *Figure 4*. At T1, *A+M* treatment showed a significant decrease compared with *A* treatment in γ-Globulin values (*Figure 5*). At T0 there was no significant difference found between *A* and *A+M in* γ-globulin values. (*Figure 5*). The difference between these therapies was not significant in parameters haematocrit, leukocyte count, neutrophil count, thrombocyte count, creatinine concentration, albumin and the rest of the protein spectrum at T0 and T1 . Ranges T2 and T3 were not comparable due to limited data and were therefore excluded.

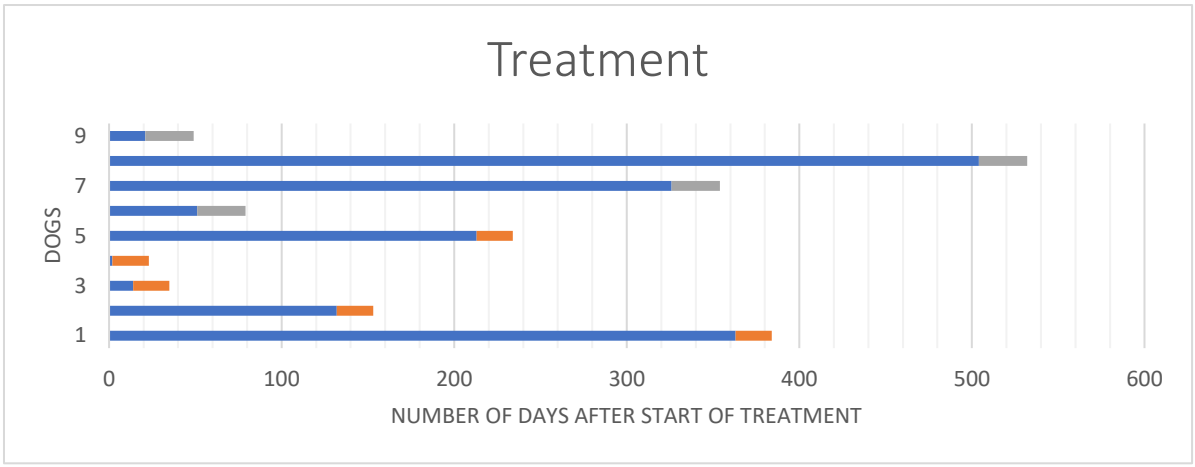

Figure 4: Leishmaniasis treatment over time. The blue line represents the number of days the dog (n=9) received Allopurinol monotherapy. The grey line represents the number of days the dog received Allopurinol and Miltefosin. The orange line represents the number of days the patient was treated with both Allopurinol and N-Methylglucamine-antimonate.

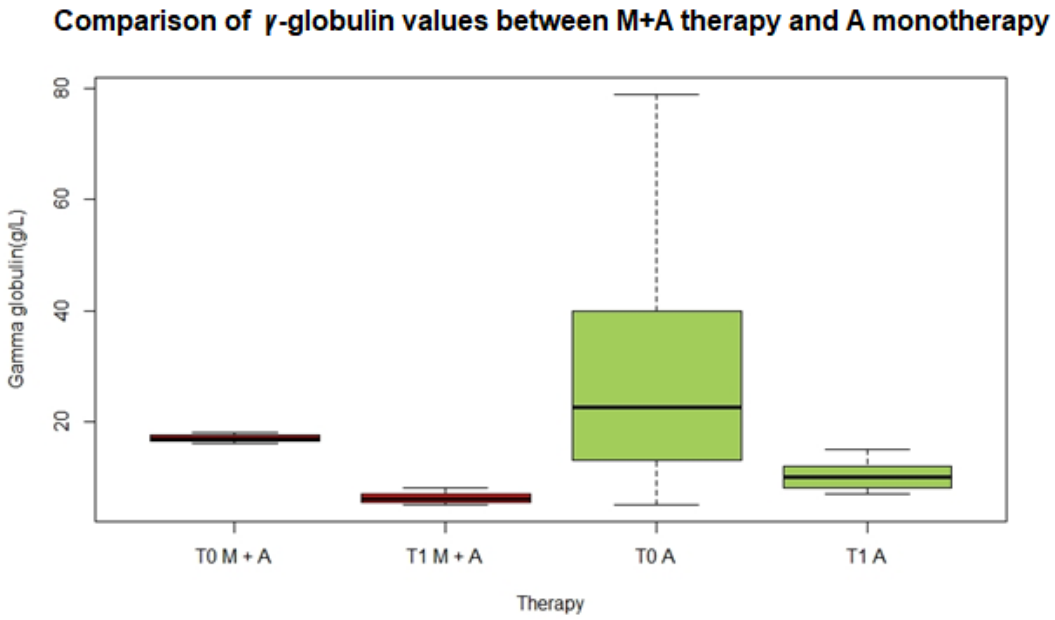

Figure 5: Comparison of γ-Globulin values between M+A therapy (n=4) on the right(red) and A monotherapy (n=21) on the left(green) in dogs with Leishmaniasis. The horizontal bold black lines represent the mean.

Discussion

Aim 1

The aim of this study was to make an estimate of the seroprevalence for Leishmania and *Dirofilaria immitis* in dogs imported from endemic geographical areas to the Netherlands. In this study the percentage of Leishmania positive dogs in this study is 22%. The number of dogs in this study is nine, so this percentage may be unreliable (95%CI: 0.028 0.600). And additionally, It may be that owners who suspected their dog was sick were more motivated to participate in this study. Thus, the percentage of leishmania antibody positive dogs could be lower than 20 % in the total population of imported dogs from a endemic area.

A study in a non-endemic country (Germany) found a percentage of 12.2% of positive antibody titres in 4681 dogs after import (Menn et al., 2010). However, 31.2% of the dogs used in this study were imported from non-endemic countries. In the current study, only dogs from an endemic area were included. The percentage of positive dogs may be higher if all the dogs were from endemic areas. In Menn et al. (2010), the blood samples were obtained from welfare organisations and veterinary practitioners. The dogs that attended to a veterinarian could be the dogs that had certain clinical signs, this may have resulted in a higher percentage of Leishmania antibody positive dogs in comparison to the total population of imported dogs. However, compared to the current study, the seroprevalence is lower.

Leschnik et al., 2008 found that 55 of 119 imported dogs in Austria, a non-endemic country, were infected with *Leishmania* and 6 of 174 dogs were positive on *Dirofiliaria Immitis*. However, the dogs used for this study were presented at the Veterinary Clinic of Vienna University with suspicious symptoms. The actual prevalence in the country is most certainly lower than this study implies.

In conclusion, further research should be done to determine the seroprevalence of Leishmania in the Netherlands.

Tests

A faster way to detect *Leishmania infantum* is a immunochromatographic dipstick rk39 test. Mohebali et al. (2004) compared this immunochromatographic dipstick test to direct agglutination (DAT). This study found that the immunochromatographic dipstick test has a sensitivity of 70.9% and a specificity of 84.9% compared to the DAT. Thus, it may be concluded that the DAT is more reliable for this research.

Aim 2

The aim of this study was to investigate the alteration of clinical and bio-pathological parameters in Leishmania infected dogs while treatment is provided within one year. A significant decrease of the total protein and γ-globulin values after 2.5 to 4.5 months was found in this study. This is similar to Torres et al. (2011), they found that the total protein and γ-globulin decreased at 90 days and in the current study was found that these parameters decreased significantly at 75-135 days(T1) after the start of the therapy.

In the current results, most of α-1 globulin and β-2 globulin values were below the reference values and α-1 globulin increased significantly at T3 in comparison to T0. This was most likely due to of the measurement of the protein electrophoresis. Due to the ratio of the percentages of the protein

spectre, if some values are high, in this case the γ-globulins, the other values of the protein spectre will be lower than expected at T0.

Furthermore, In the current study was a significant increase over time found in haematocrit, thrombocytes and albumin after treatment. β-2 Globulin decreased significantly after 2.5 to 4.5 months after the start of Allopurinol treatment. This is similar to Torres et al. (2011), they found that the protein electrophoresis normalises after at least 3-4 months. Furthermore, they performed a complete blood count. However, they did not mention any alterations in blood parameters other than the protein electrophoresis mentioned before. In Paltrinieri et al. (2016) was described that dogs with leishmaniasis develop anaemia, thrombocytopenia and azotaemia. Yet, this study did not mention how these parameters change over time after administration of treatment.

The significant change in some parameters may be as a result of treatment. However, no control group was used in this study because of ethical reasons. Hence, to draw definite conclusions on the success of the treatment is challenging.

Urine

In the present study was found that five of twenty-one dogs had xanthine crystals in the urine sediment at 2.5-4.5 months after the start of the therapy. Out of twenty-one dogs eleven dogs had no urine sample, Thus, in eleven dogs, the number of dogs with xanthine crystal formation could not be determined. It seems likely that dogs without a urine sample had the same chance to develop xanthine crystal formation. This could implicate that 62.5 % of the dogs develop had crystals in urine sediment at T1. At T2 and T3 the percentage was respectively 50% and 66%. It should be considered that the number of available urine samples was lower at T3. Urine sediment analysis was performed at T0, T1, T2 and T3 on respectively 12, 8, 7 and 6 dogs. These results suggest that the percentage of dogs developing urolithiasis as a result of A therapy is higher than reported so far.

The current results confirmed the previous results concerning the side effects of A therapy. In Torres et al. (2010) was found that a side effect of A therapy is that after a longer period of time(24 months) xanthine urolithiasis developed. They found that three of twenty-three dogs (13%) developed xanthine calculi in the urinary tract.

In contrast to Torres et al. (2010) and the current study, Manna et al. (2015) did not find xanthine crystals or urolithiasis in dogs treated with A during 72 months. In comparison with the current study, in Manna et al. (2015) received nine dogs A (10mg/kg/per day) treatment in combination with meglumine antimoniate. Urine sediment analysis and echo graphic scanning were performed. In the current study, the therapy consisted of A monotherapy.

Unfortunately, the UPC ratio and urine analysis were determined in a small number of dogs. The reason for this is that urine was not obtained in at all the control visits. The current study did not found a significant difference in UPC ratio between T0 and T1. In Figure 1 in boxplot '*UPC ratio over time*' is shown that most of the values normalise at T1 and stay similar at T2 and T3. The outliers represented two dogs. One of them had very high UPC values at the start of the therapy. Both of the dogs were suspected to have renal damage. This may have an effect on the reliability of the results.

In contrast to the current results, Pierantozzi et al. (2013) found that proteinuria decreases 4-8 weeks after treatment with *A* and meglumine antimoniate. Unless severe renal damage was present. Furthermore, Provebio et al. (2016) found a significant decrease of the UPC ratio in 20 dogs with leishmaniasis, while treatment of *A* and *M* was provided. The parameters were recorded at the start of the therapy and 28 days later. The contrast between the current results and these studies may be due to the lack of data.

Comparison of therapy

A significant difference in M and A combination therapy in comparison to A monotherapy with regard to γ-globulin values at T1 at 3.5-4,5 months after the start of the treatment was found. M+A combination therapy showed a greater decrease at 3.5- 4.5 months after the start of the therapy. This was not reported in previous studies. Most studies compared the effectivity of the treatment based on clinical improvement. Manna et al. (2015) found that dogs treated with A and M in comparison to meglumine antimoniate(G) and A, develop clinical improvements slower. Only nine dogs were used in this study and no data was available about γ-globulins over time.

Conclusion

Thus, it may be concluded that allopurinol monotherapy normalises most of the involved parameters in Leishmania infected dogs. This study also showed that most of the normalisation of the parameters occurred in 2.5-4.5 months after treatment. Hereafter, the values of the parameters remain similar. Furthermore, miltefosine treatment may reduce γ-globulins more compared to A monotherapy at 2.5-4.5 months after the start of the therapy.

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Supplementary materials

Part 1

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 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 alle drie 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 selectie procedure 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 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

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Universiteitskliniek Gezelschapsdieren, Universiteit Utrecht

Part 2

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: _____

Part 3

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. Af-pipetteren 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.