Specific Antibody Detection in Dogs with Filarial Infections

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Abstract

Dirofilariosis is a mosquito-transmitted disease of wild and domestic carnivores. Etiological diagnosis on canine *Dirofilaria*-infections is generally either based on the morphological or molecular characterization of microfilariae (L1), or in case of *D. immitis*-infection on the detection of circulating antigens shed by mature female worms. However, these tests do not detect infections during the long prepatent period of 182–238 days. We hereby present a monoclonal antibody based sandwich-ELISA used for on-plate purification of somatic antigen of adult *D. immitis* stages for the detection of antibodies against *D. immitis* and *D. repens* in dog sera. Sensitivity of the assay for *D. immitis* patent infections was calculated to be 93.8% (95% CI: 79.2–99.2%), and for patent *D. repens*-infections 100% (95% CI: 81.9–100%). Specificity was determined to be 98.6% (95% CI: 92.2–100%) with sera of 69 dogs from a non-endemic area. Cross-reactions against other nematodes such as *Acanthocheilonema* and *Dipetalonema* spp. (50% and 66.7%, respectively), *Crenosoma vulpis* (16.7%), *Capillaria aerophila* (14.3%) and naturally, but not experimentally infected dogs with *Angiostrongylus vasorum* (14.3%) were present. However, in all positive dogs a residence in a filarial endemic area cannot be excluded. No positive reactions could be shown in experimentally infected dogs with *Toxocara canis*, *Ancylostoma caninum* and *Trichuris vulpis*. Dogs experimentally infected with *D. repens* showed seroconversion between 24–80 days post inoculation (dpi), far earlier than beginning of patency (189–259 dpi). Accordingly, the presented ELISA could be a supplementary or alternative tool for

the diagnosis of *Dirofilaria*-infected dogs in low or non-endemic areas to document the contact rate and infection pressure.

Introduction

The causative agents of dirofilariosis are nematodes of the genus *Dirofilaria,* parasitizing predominantly wild carnivores of different species but also affecting companion animals such as dogs and rarely cats and ferrets. Dirofilariosis is a zoonotic, vector-borne disease and its transmission is dependent on the occurrence of competent vector species of the genera *Culex* spp., *Aedes* spp. and *Anopheles* spp. (Ludlam et al. 1970, Cancrini et al. 2007). Apart from Switzerland, Germany, Austria and Scandinavian countries, the often apathogenic agent *D. repens* is (low-)endemic in Europe by now. The clinically more relevant species *D. immitis*, commonly known as "heartworm", occurred sporadically in Central and Western Europe, whereas southernmost countries are assumed to be (highly) endemic (Baneth et al. 2016). Increasing European animal transfers of infected dogs from the Mediterranean as well as travelling habits of people including their dog companions to endemic areas are common practice and therefore problematic in the context of disease distribution (Genchi et al. 2011). Global trade is furthermore of increasing concern when vectors are accidentally trafficked, leading to new vector habitats or the possibility of parasite transmission and distribution via invasive mosquito species to populations of susceptible hosts in hitherto non-endemic regions (Silaghi et al. 2017).

There are several approaches to diagnose *D. immitis*-infection *intra vitam* in affected dogs, whereas no test is currently able to detect larval infections. Case history combined with radiography is used in veterinary practice for presumptive evidence of heartworm disease in dogs due to (pathognomonic) clinical and pathological signs. However, diagnosis based on these criteria only is assumed to be insensitive, since clinical signs vary from being absent, mild, moderate or severe (reviewed by Bowman and Atkins 2009). Beside molecular proof of DNA from microfilariae in a blood sample by PCR (Rishniw et al. 2006, Latrofa et al. 2012, Magnis et al. 2013), microfilariae can be detected from EDTA-blood after enrichment via Knott- (Knott 1939) or filter-method (Bell 1967) and can be differentiated by well described morphological criteria such as body length and shapes of front end and tail (Magnis et al. 2013). Furthermore, the distribution of acid phosphatase activity is used to distinguish between microfilariae of the genus *Dirofilaria* and from other filarial nematode genera such as *Acanthocheilonema* or *Dipetalonema* (Chalifoux et al. 1971, Peribáñez et al. 2001). It has to be pointed out that all diagnostic approaches mentioned above require a patent infection and since microfilarial periodicity was demonstrated, samples collected in the evenings (between 06.00–10.00 p.m.) usually contain higher values of microfilariae per milliliter blood (Di Cesare et al. 2013). However, occult (amicrofilaremic) infections lacking circulating microfilariae due to prepatency, unigender infection, anthelmintic treatment-induced adult sterility and infection in which microfilariae are killed by anthelmintics or by immune response are common (Rawlings et al. 1982, Bowman and Mannella 2011). The potential absence of microfilariae in heartworm-infected dogs led to serious diagnostic uncertainties and inaccuracies.

A substantial scientific achievement and the most common *D. immitis* diagnosis nowadays is based on the detection of antigens produced by mature females in the definitive host's blood (Weil 1987). Antigenemia during *D. immitis* infection generally occurs at the conclusion of the prepatent period approximately 5–7 months post inoculation with L3 larvae and persists for months-years during patency (Goodwin 1998). Various commercially available ELISAs detect infections with at least one adult female worm and are assumed to be highly sensitive (increasing with higher female heartworm burden and age of worms) and almost

100 % specific (Atkins 2003, Lee et al. 2011). However, another study revealed cross-reactions with sera from dogs infected with *Angiostrongylus vasorum* in 3/6 commercially available *D. immitis* test kits (Schnyder and Deplazes 2012). Furthermore, antibody-complexes due to e.g., chemotherapy (Drake et al. 2015) or other reasons leading to false-negative results in current antigen-diagnosis were present in 7 % of samples in a study from the United States of America (Velasquez et al. 2014). This phenomenon may be reverted by pre-treatment of sera with heat (Little et al. 2014, Ciucă et al. 2016). The aim of this study was the development of an ELISA to detect antibodies against *Dirofilaria* spp. infections as a supplementary or alternative tool to diagnose (larval, immature and patent) infections in dogs for e.g., epidemiological and surveillance studies in hitherto low- or non-endemic regions.

Materials and Methods

Mice experiments were conducted according to the Swiss guidelines for animal experimentation and approved by the Cantonal Veterinary Office of Zurich prior to the study (permission number 139/2015).

Dog sera

Sera from dogs proven positive for patent *D. immitis* (n = 32) or *D. repens* (n = 15) infection by evidence of microfilariae or necropsy results were included in this study. Furthermore, sera from three experimentally infected *D. repens* dogs (kindly provided by Bayer Animal Health GmbH, Leverkusen, Germany) that were tested positive for cutaneous adult worms at necropsy were used to determine the course of antibody development during infection (sampling days cf. Fig. 1) (Petry et al. 2015). Thirty-four sera from healthy blood donor dogs originated from a known *Dirofilaria* non-endemic area in Switzerland were used to determine the cut-off value (mean value plus 3 standard deviations). Specificity was calculated with randomly selected sera of 69 dogs presented at the Clinic for Small Animals and tested at the Clinical Laboratory of the Vetsuisse Faculty, University of Zurich, Switzerland, for various reasons excluding suspected parasitological infections (Schucan et al. 2012). Potential cross-reactions were tested with sera from dogs, experimentally infected with *Toxocara canis* (n = 5), *Ancylostoma caninum* (n = 4) and *Trichuris vulpis* (n = 2) (last two mentioned were kindly provided by the Institute of Parasitology, University of Veterinary Medicine, Hannover); from experimentally (n = 10) and naturally (n = 7) infected dogs with *Angiostrongylus vasorum* (Schucan et al. 2012); from naturally infected dogs with *Crenosoma vulpis* (n = 6) diagnosed by the presence of L1 in faeces (Barutzki and Schaper 2009); from naturally infected Italian dogs tested positive for the presence of *Capillaria aerophila* eggs (n = 7) by PCR-coupled sequencing (Traversa et al. 2011); and from naturally infected dogs with *Acanthocheilonema reconditum* (n = 10) and *Dipetalonema dracunculoides* (n = 9) (Magnis et al. 2013).

Antigens

D. immitis adult E/S antigen was obtained from cultured worms (Deplazes et al. 1995), *D. immitis*/*D. repens* adult somatic antigen was prepared as described by Schucan et al. (2012). Protein concentrations were assessed by absorbance assay at 280 nm using a spectrometer (NanoDrop® ND-1000; Witec AG, Littau, Switzerland).

Monoclonal antibodies

Production of murine monoclonal antibodies (mAbs) against *D. immitis* adult male E/S antigen was based on a protocol already described by Schnyder et al. (2011). Antibody production of the fused cells was checked by ELISA (cf. Screening of mAbs (primary screening) in the next paragraph). Supernatants positive in the primary screening were subsequently checked for cross-reactivity by ELISA with *A. vasorum* somatic antigen (10 µg/ml). Selected (*A. vasorum*-negative) clones were subcloned to assure that antibody production
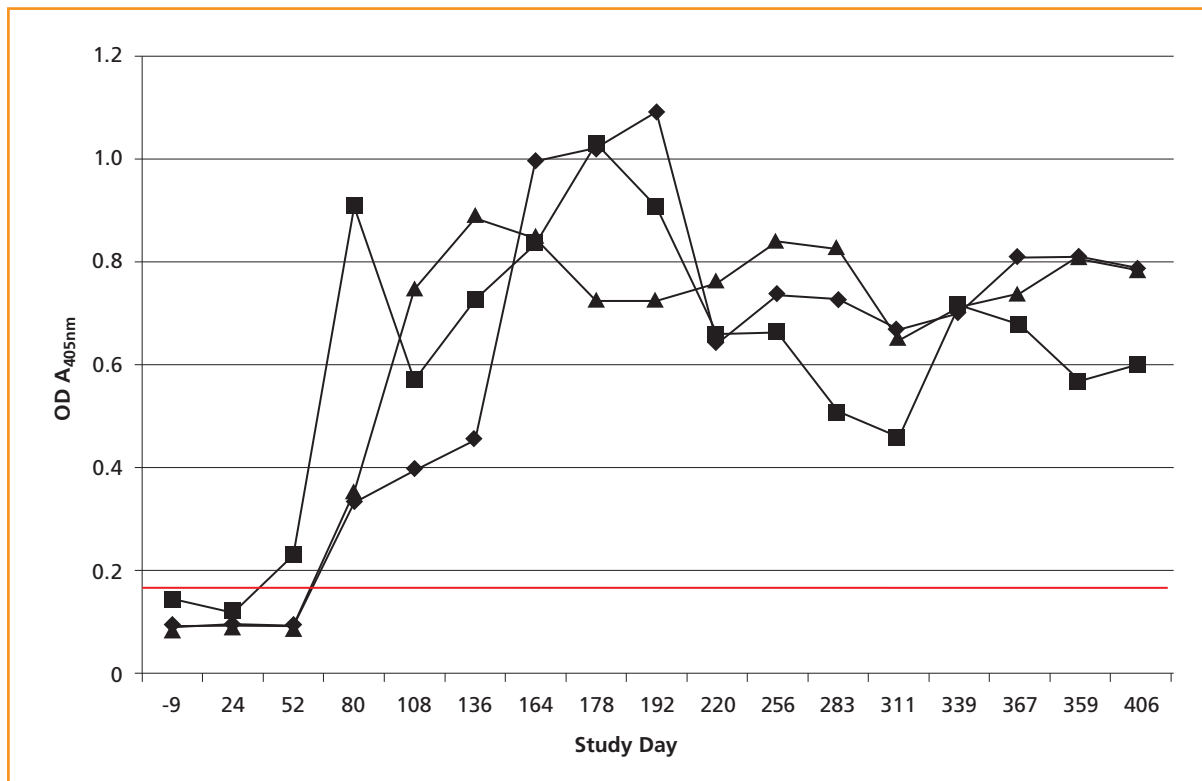
Fig. 1 Detection of serum antibodies (IgG) by ELISA in three dogs (IDs 108: hash, 109: square, 113: triangle) experimentally inoculated at study day 0 with 75 *D. repens* L3 larvae (Petry et al. 2015). The cut-off value (red horizontal line) was calculated as mean plus 3 standard deviations of the ELISA OD A_{405nm} values of sera from 34 healthy dogs from a known filarial non-endemic region.

Table 1 Evaluation of sensitivity and specificity of the ELISA in defined canine sera. Cut-off determination: mean value of optical density (A_{405nm}) plus 3 standard deviations of 34 healthy dogs from a known *Dirofilaria* spp. non-endemic region.

Sensitivity % (95 % CI)		Specificity % (95 % CI)
*D. immitis**	*D. repens**	Random samples**
(n = 32)	(n = 15)	(n = 69)
93.8 % (79.20 – 99.20)	100 % (81.90 – 100)	98.6 % (92.20 – 100)

CI: Confidence interval
*Patent *Dirofilaria*-infections confirmed by the presence of microfilariae in EDTA-blood, or adult worms at necropsy.
**Sera of randomly selected dogs without suspicion of a parasitological disease tested at the Clinical Laboratory of the Vetsuisse Faculty of the University of Zurich.

was monoclonal. A clone mAbDi36/1 positive for *D. immitis and D. repens* adult somatic and E/S antigen was cultivated and supernatants were concentrated using Amicon® Ultra-15 Centrifugal Filters Ultracel®-30K (Merck Millipore Ltd., Ireland) and the mAbs were subsequently purified by Magne^TM Protein G Beads according to manufacturer's instructions (Promega, USA). Isotyping of mAbDi36/1 was performed using a commercial Kit (Sigma Isotyping kit ISO2, Sigma Aldrich, Switzerland) according to manufacturer's instructions and resulted in IgG1 subclass.

ELISAs

Screening of mAbs (primary screening)

ELISAs have been performed in principle as described by Schucan et al. (2012). Briefly, plates were coated with ♂/♀ *D. immitis* E/S (1:200 dilution in coating buffer: 0.1 M carbonate/bicarbonate, pH 9.6) and *D. immitis* or *D. repens* adult somatic antigen (10 µg/ml coating buffer) at 4 °C overnight in a humid chamber. If not stated otherwise, all following incubations were performed for one hour at 37 °C, followed by a four times washing step with ELISA wash buffer (physiological NaCl + 0.3 % Tween -20). After blocking (300 µl/well) for 30 minutes with PBS-HT (containing PBS (pH 7.2) with 0.02 % NaN3, 0.05 % bovine haemoglobin (Fluka/Sigma Aldrich, Switzerland) and 0.3 % Tween-20), mAb containing supernatants in a 1:2 dilution in PBS-HT were added. Alkaline phosphatase labelled goat anti-mouse IgG (Sigma-Aldrich, Switzerland) in a 1:10´000 dilution in PBS-HT was used as conjugate. For enzymatic reaction, 100 µl/well of a 1 mg/ml solution of 4-nitrophenylphosphate (Sigma-Aldrich, Switzerland) in 0.05 M carbonate/bicarbonate buffer (pH 9.8) containing 1 mM MgCl were added and incubated for 10 min at 37 °C. Absorbance values were read at 405 nm in a Multiscan RC ELISA reader (Thermo Labsystems, Finland).

Specific antibody detection in dogs by sandwich-ELISA

ELISAs were performed using optimal concentrations of antigen, antibody and conjugate according to previous titration results. Test runs included a background and conjugate control. ELISA plates were coated with 5 µg mAbDi36/1 per ml coating buffer and stored at 4 °C in a humid chamber overnight. After washing the plates four times with ELISA wash buffer, the plates were blocked (300 µl/well) with PBS-HT for 30 minutes at 37 °C. All following incubations were performed for one hour at 37 °C, followed by a four times washing step with ELISA wash buffer. *D. immitis* female somatic antigen (5 µg/ml in PBS-HT; 100 µl/well) was added. Sera were used in a standard dilution of 1:200 in PBS-HT + 10 % fetal calf serum (100 µl/well). Subsequently, ReserveAP^{TM} Phosphatase Labeled Goat anti-Dog IgG(γ) (Kirkegaard and Perry Lab. Inc., Gaithersburg, USA) in a 1:1250 dilution in PBS-HT was added (100 µl/well). Visualization of the reaction and OD-measurement was performed as stated above. Positive control samples from animals with proven infections and negative control samples from healthy dogs were included in all tests to adjust for plate-to-plate variations.

Statistical and data analysis

Calculation of sensitivities was performed by dividing the number of seropositive animals by the total amount of infected animals, while specificity was calculated dividing the number of seronegative animals by the total number of uninfected animals tested. Exact binominal 95 % confidence intervals (CI) for means of binominal variables were calculated with unweighted data according to Clopper and Pearson (1934). The cut-off value was calculated as mean plus 3 standard deviations of the ELISA OD A_{405nm} values of sera from 34 healthy dogs from a known filarial non-endemic region. Graphics were generated using Excel 2010 (Microsoft Corporation, Redmond, US).

Results

The mAb against an epitope of crude adult *D. immitis* antigen, designated 'mAbDi36/1' (IgG1 isotype), allowed the detection of filarial infection in dog serum samples.

Sensitivity and specificity

Sera of dogs naturally infected with *Dirofilaria* spp. or other parasites as well as sera from healthy dogs were used to determine the diagnostic values of the ELISA. Values on sensitivity and specificity are provided in Table 1. Sensitivity of the assay for

Table 2 Cross-reactivity of the ELISA in sera of dogs infected with nematodes other than *Dirofilaria* spp. (Cut-off determination see Table 1).

Parasite species	number of dogs (n)	Positive dogs (n)	Positive dogs (%) (95 % CI)
*Acanthocheilonema reconditum**	10	5	50.0 % (18.7–81.3)
*Dipetalonema drunculoides**	9	6	66.7 % (29.9–92.5)
*Capillaria aerophilia**	7	1	14.3 % (0.4–57.9)
Trichuris vulpis[#]	2	0	0 % (0.0–77.6)
Ancylostoma caninum[#]	4	0	0 % (0.0–52.7)
*Crenosoma vulpis**	6	1	16.7 % (0.4–64.1)
Toxocara canis[#]	5	0	0 % (0.0–45.1)
Angiostrongylus vasorum[#]	10	0	0 % (0.0–25.9)
*Angiostrongylus vasorum**	7	1	14.3 % (0.4–57.9)

*naturally infected dogs;   [#]experimentally infected dogs;   CI: Confidence interval

D. immitis patent infections was calculated to be 93.8 % (95 % CI: 79.2–99.2 %), whereas sensitivity on patent *D. repens*-infections reached 100 % (95 % CI: 81.9–100 %). Specificity was determined to be 98.6 % (95 % CI: 92.2–100 %) with sera of 69 dogs from a non-endemic area.

Sera of experimentally and naturally helminth-infected dogs were analyzed by the presented ELISA to determine potential cross-reactions. Results are provided in Table 2. No positive ELISA results were obtained with sera of dogs with patent infections of *T. vulpis, A. caninum* and *T. canis*. Cross-reactions were evidenced in 50 % and 66.7 % of dogs naturally infected with *A. reconditum* and *D. dracunculoides*, respectively. Sera of dogs naturally infected with *C. aerophila* (syn. *Eucoleus aerophilius*) cross-reacted in 14.3 % of cases, sera of *C. vulpis* naturally infected dogs in 16.7 % and sera of *A. vasorum* naturally infected dogs in 14.3 %, whereas sera of *A. vasorum* experimentally infected dogs resulted negative in ELISA.

Detection of antibodies during experimental *D. repens* infections

Results are illustrated in Figure 1. Seroconversion of experimentally infected dogs was demonstrated between 24 and 80 dpi. The earliest antibody detection took place between 24 and 52 dpi in one infected dog (ID 108). Antibody levels increased until 164–178 dpi, before they reached an almost constant plateau around 220 dpi until necropsy at 406 dpi.

Discussion

The development of several ELISAs for serological antibody diagnosis of heartworm disease in dogs was initiated in the early 1980th (Grieve et al. 1981, Sisson et al. 1985, Grieve and Knight 1985). ELISAs were designed to detect antibodies against purified somatic antigen of adult *D. immitis* and infected dogs became antibody positive as early as 4 months after single or repeated inoculations with L3. Furthermore, the antibody response persisted for at least 6 months following adulticidal treatment or natural death of the adult heartworms (Grieve et al. 1981). In the present study, antibodies in experimentally *D. repens* infected dogs could be detected earlier between 24 and 80 days post inoculation (dpi) and remained on a high level during the course of the study until necropsy at

406 dpi. These results suggest that the present test detects antibodies directed against larval and adult stages of *D. repens* in dogs. It is highly likely that similar courses of antibody development also occur in *D. immitis*-infected dogs. However, further studies with sera of experimentally *D. immitis*-infected dogs during course of infection need to confirm this assumption.

Considerable problems with previous antibody tests include their poor specificity due to homologous helminth interspecies epitopes. Previous assays based on the use of adult somatic *D. immitis* antigens have therefore provided false-positive results (cross-reactions), when dogs are infected with *Toxocara canis* (Grieve et al. 1981, Ott et al. 1985, Thilsted et al. 1987), *Angiostrongylus vasorum* or *Acanthocheilonema* spp. and *Dipetalonema* spp. (Maier and Deplazes, personal communications). In the presented ELISA, cross-reactions were observed in 50 % and 66.7 % of sera from dogs naturally infected with *A. reconditum* and *D. dracunculoides*, respectively. Sera of these dogs were originated from *Dirofilaria* endemic regions such as Italy, Spain and Portugal and exposure of these dogs with *Dirofilaria* spp. cannot be ruled out. However, due to the close taxonomic relation of the genus *Acanthocheilonema / Dipetalonema* and *Dirofilaria,* cross-reactions between these genera are most probable. Nonetheless, *Acanthocheilonema / Dipetalonema* spp. are not endemic in hitherto *Dirofilaria* spp. non-endemic regions, where the present test is recommended to screen large dog populations. Cross-reactions were not observed with sera of dogs experimentally infected with *T. canis*, *T. vulpis, A. vasorum* and *A. caninum*, whereas one dog of each group naturally infected with *A. vasorum* (14.3 %)*, C. vulpis* (16.7 %) and *C. aerophila* (14.3 %) showed a positive serological reaction. Nevertheless, all the last mentioned positive dogs from this study were naturally infected with helminths and particularly in case of *C. aerophila* when dogs were originated from Italy, potential multi-infections with various filariae leading to positive results in the presented

ELISA cannot completely be excluded. To increase the specificity of the presented ELISA, somatic *D. immitis* antigen was on-plate purified with a filarial-specific mAb resulting in a highly specific sandwich-ELISA. The use of native antigens of adult *D. immitis* represents an easy way of antigen preparation and the (on-plate) antigen purification step insures a high reproducibility of the ELISA as experienced with a similar test by Schucan et al. (2012).

Poor Sensitivity around 85 % was observed in earlier serological ELISA approaches for the detection of antibodies against *D. immitis* somatic antigen (Glickmann et al. 1984, Thilsted et al. 1987). In contrast, sensitivities of the presented ELISA were very high in *Dirofilaria*-infected dogs (*D. immitis* 93.8 %; *D. repens* 100 %). However, for the confirmation of these data, more field studies have to be performed with naturally infected hosts including occult infected animals and critically analyzed in different endemic areas. Furthermore, a positive ELISA indicates possible parasite-exposure, but not necessarily active heartworm or skin worm infection.

The here presented ELISA demonstrated its potential for use on a large scale initial screening in epidemiological or surveillance studies, best in combination with other serological tests for nematode infections for the documentation of filarial transmission in low- and non-endemic regions.

Conflict of interest

The authors declare that they have no competing interests.

Ethical standards

All applicable international and institutional guidelines for the care and use of animals were followed. Mice experiments were conducted according to the Swiss guidelines for animal experimentation

and approved by the Cantonal Veterinary Office of Zurich prior to the study (permission number 139/2015).

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