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An ELISA for sensitive and specific detection of circulating antigen of *Angiostrongylus vasorum* in serum samples of naturally and experimentally infected dogs

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Abstract

Canine angiostrongylosis is an emerging cardiopulmonary disease in Europe which can be fatal if left untreated. We developed a sandwich-ELISA based on a monoclonal antibody (mAb Av 56/1/2) and on polyclonal rabbit antibodies directed against *Angiostrongylus vasorum* adult excretory/secretory – antigen for the detection of circulating serum antigen of *A. vasorum*. The sensitivity of the test was 95.7% (78.1-99.9, 95% CI) as determined with sera of 23 dogs naturally infected with *A. vasorum*. The specificity was 94.0% (83.5-98.7, 95% CI) using 50 dog sera (control group) submitted for reasons other than parasitic infections. Potential cross-reactions were investigated with sera of a group of totally 61 dogs with proven infections with *Dirofilaria immitis* (n = 23), *Crenosoma vulpis* (n = 14), *Ancylostoma caninum* (n = 4) or *Toxocara canis* (n = 20). No significant difference was observed concerning the proportion of positive reactions between the control group and the group with proven helminth infections other than *A. vasorum*. In experimentally inoculated dogs with proven worm burdens of *A. vasorum*, the proportion of seropositive dogs increased over the first 3 months of infection, starting from 35 days post inoculation (dpi) which was before the onset of larval excretion. Ten weeks post inoculation, 98.6% of the dogs were seropositive, and circulating antigen persisted in two dogs with long-term follow-up over 286 and 356 days, respectively. In contrast, in dogs with a single treatment with imidacloprid/moxidectin at four or 32 dpi, no circulating antigen was observed, while in dogs treated at 88-92 dpi, OD values decreased within 13-34 days. The specific detection of circulating *A. vasorum* antigen by ELISA represents a valid alternative for reliable
diagnosis and for follow-up investigations after anthelmintic treatment. Moreover, the test can be used for mass screening in large epidemiological investigations.

Key words: Angiostrongylus vasorum; canine angiostrongylosis; circulating antigen; serology; ELISA; dog.

1. Introduction

Angiostrongylus vasorum is a metastrongylid nematode living in the pulmonary arteries and the right atrium and ventricle of wild and domestic carnivores like dogs and wild foxes. Definitive hosts are infected by the ingestion of snails and slugs containing third-stage larvae (Guilhon, 1963). After a prepatent period of 38-57 days, the adult female worms shed eggs which develop into first-stage larvae (L1) that are coughed to the pharynx, swallowed and excreted in the faeces (Bolt et al., 1994; Schnyder et al., 2010). Dogs affected by angiostrongylosis primarily show respiratory signs due to verminous pneumonia but neurological deficiencies and coagulopathies are also described (Chapman et al., 2004; Koch and Willesen, 2009). If left untreated, the infection can be fatal (Koch and Willesen, 2009; Staebler et al., 2005).

The current diagnosis of A. vasorum infections is based on the detection of L1 in faecal samples. Simple faecal smears can be used for diagnosis in general practice (Humm and Adamantos, 2010) but larval migration techniques such as the Baermann-Wetzel technique (Eckert et al., 2008) are recommended. Both methods may allow morphological differentiation from further lung worm larvae such as Crenosoma vulpis and Filaroides spp. (Eckert et al., 2008; McGarry and Morgan, 2009). However, the sensitivity of these methods is reduced in cases of low worm burdens and when analysing posted faecal samples that arrive with delay at the laboratory and therefore containing deformed or dead larvae. Also, dogs with prepatent infections are not detected.

Therefore, alternative diagnostic tools are needed. An alternative may be represented by the employment of novel molecular tools such as PCR (Traversa and Guglielmini, 2008). Real-time PCR with blood or faeces spiked with L1 had low detection limits, but when samples of suspected clinical animals were compared with single faecal examination performed with the Baermann’s method, in both subsets PCR negative/Baermann’s positive and PCR positive/Baermann’s negative results were observed (Jefferies et al., 2009). Furthermore, a ‘sieve-PCR’ method developed to facilitate the processing of larger faecal volumes was described as a non-invasive tool for wildlife surveillance and for confirmative diagnosis in dogs or foxes (Al-Sabi et al., 2010). Molecular techniques are particularly recommended for prevalence studies in intermediate mollusc hosts (Ferdushy et al., 2009). The serologic detection of A. vasorum – infections in dogs could represent a valid alternative for diagnosis both, in individuals and in population studies. The detection of specific antibodies against A. vasorum was first described by Guilhon et al. (1971) and later applied in follow-up studies in experimentally infected dogs (Costa et al., 1996; Cury et al., 1996). Specific antibody reactions were analysed by Western blot, revealing immunogenic antigens of different molecular weight in crude extracts of adult female worms (Cury et al., 2002). Later, stage-specific antigens
were identified using sera of dogs experimentally infected with *A. vasorum* (De Oliveira Vasconcelos et al., 2008). A sandwich-ELISA using polyclonal antibodies of rabbits immunized with adult worm antigens illustrated the potential of the detection of circulating antigen in dogs naturally infected with *A. vasorum* (Verzberger-Epshtein et al., 2008). The aim of this investigation was to evaluate the diagnostic features (sensitivity, specificity) of an ELISA for the detection of *A. vasorum* circulating antigen in experimentally and naturally infected dogs.

2. Materials and Methods

2.1 *Angiostrongylus vasorum* antigens

Excretory/secretory (E/S) antigen was obtained from living adult parasites isolated by a reverse lung perfusion technique from foxes and dogs during previous experiments (Schnyder et al., 2010; Webster et al., 2007). Parasites were thoroughly washed with RPMI 1640 L-glutamine with 200 IU penicillin/ml and 200 μg streptomycin/ml and incubated in the same medium at +37° C and with 5% CO₂ in a concentration of 20 worms/30ml. The medium was replaced every day during the first five days and discarded in order to reduce potential contaminations with host antigens. The viability of the worms was judged by their motility, and inactive or dead worms were removed before fresh medium was added. Afterwards, the medium was harvested twice a week and supernatant stored at -20° C after centrifugation (2000 g, 10 minutes) until further processing. After sterile filtration through a 0.22 μm sieve (Millipore Corporation, Billerica, Massachusetts, USA), the collected media were concentrated using YM-10 membranes (discs of 10 kDa NMWL) (Millipore) in a pressurized (3.5 bar) Amicon ultrafiltration unit.

2.2. Polyclonal antibodies

Female New Zealand White rabbits were subcutaneously immunized with 150 μg of *A. vasorum* adult E/S-antigen diluted in PBS (phosphate buffered saline) and Freund’s complete adjuvant. A booster was performed four weeks later with Freund’s incomplete adjuvant, followed by a terminal bleed four weeks later. Serum was collected and stored at -20° C.

2.3. Monoclonal antibodies

Monoclonal antibodies (mAb) were produced with a modified protocol (de StGroth and Scheidegger, 1980) as follows. NMRI (Naval Medical Research Institute) mice (Janvier, Le Genest St. Isle, France) were subcutaneously immunized with 20 μg of adult *A. vasorum* E/S antigen in a volume of 50 μl PBS (28.25 μl antigen and 21.75 μl sterile PBS) mixed with 50 μl Freund’s complete adjuvant. This was repeated twice in an interval of 3 weeks using the same amount of antigen in Freund’s incomplete adjuvant, followed by subcutaneous boosters of antigen with PBS for three days directly before decapitation under anaesthesia.
Spleen cells of the immunized mice were then removed aseptically, washed and lymphocytes were mixed with the same amount of AG8 myeloma cells. The mixed cells were washed and incubated for 90 seconds at 37°C in polyethylene glycol (PEG) which was carefully diluted stepwise with pre-warmed PBS. Fused myeloma cells were initially cultivated in 96-well plates (Techno Plastic Products, Trasadingen, Switzerland) at 37°C and 5% CO₂ in conditioned Iscoves mod. Dulbecco medium (Sigma, St. Louis, Missouri USA), with HAT (Sigma), 5% fetal calf serum, 1% L-glutamine (Sigma), 0.5% L-Anyl-L-Glutamine 200mM (Sigma), 2% IL-6 and 0.1% Gentamicin (Sigma). This culture medium was gradually substituted by non-conditioned medium and later by a culture medium with HT (Sigma) instead of HAT. Finally, the cells were cultivated in culture medium without any further additives.

Antibody production of the cells was checked by an ELISA using *A. vasorum* adult E/S antigen. Supernatants positive in the primary screening were subsequently tested for specificity in ELISA with *Dirofilaria immitis* adult E/S antigen (Deplazes et al., 1995). Sera from known naturally infected and from negative dogs served as controls. Selected clones were subcloned twice to assure that the antibody production was monoclonal. Clones positive for *A. vasorum* adult E/S and negative for *D. immitis* adult E/S antigens were transferred into cell culture flasks (Techno Plastic Products, Trasadingen, Switzerland), the supernatant containing the antibodies was collected and stored at -20°C. Supernatants were concentrated with an Amicon filtration unit and YM-30 membranes (both Millipore). The mAbs were purified on protein G columns (Protein G Sepharose 4 Fast Flow, GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s instructions, and dialyzed against PBS with a pleated dialysis tubing (Snake Skin®, Pierce, Thermo Fisher Scientific, Waltham, MA, USA). Isotypes were determined by a monoclonal antibody isotyping kit (Ap/PNPP, Pierce). All protein concentrations were assessed by a Bio-Rad protein assay and with a protein standard of 1.37μg/ml.

### 2.4. Sandwich-ELISA

Tests were performed in 96-well microtiter plates (Maxisorp, Nunc Roskilde, Denmark). Optimal concentrations of monoclonal antibodies, serum dilutions, polyclonal rabbit serum and goat anti-rabbit IgG (F(ab’)_2 fragment) conjugated to alkaline phosphatase (Sigma) were determined by titration experiments. Plates were coated overnight at 4°C with 2.5 μg/ml of a selected mAb (mAb Av 56/1/2), diluted in 0.1 M carbonate/bicarbonate buffer (pH 9.6) containing 0.02% NaN₃. Plates were washed four times with physiological NaCl/0.3% Tween-20 (NaCl-T) before saturation for 30 min at 37°C with PBS (pH 7.2) containing 0.02% NaN₃, 0.05% bovine haemoglobin (Fluka) and 0.2% (v/v) Tween-20 (PBS-T). Serum dilutions (100 μl/well, 1:2 in PBS-T) were incubated for 1 h at +30°C and the plates were subsequently washed four times with NaCl-T. Afterwards, hyperimmune polyclonal rabbit serum (100 μl/well) was added in a concentration of 1:500, incubated for 1 h at 30°C, and plates again washed four times with NaCl-T before adding 100 μl/well goat anti-rabbit IgG conjugated to alkaline phosphatase and diluted in PBS-T (1:2’500). After the repetition of the incubation and washing steps, 100 μl/well of a 1 mg/ml solution of p-
nitrophenyl phosphate (Sigma) in 0.05 M carbonate/bicarbonate buffer (pH 9.8) containing 1 mM MgCl₂ were added. Absorbance values were read at 405 nm (OD₄₀₅) with a Multiscan RC ELISA reader (Thermo Labsystems, Helsinki, Finland). A panel of positive (from experimentally inoculated dogs) and negative control sera (from laboratory dogs, known to be free of *A. vasorum*) were included in all test runs. In particular a strong positive serum (serum P) diluted 1:200 was run twice in every plate. The mean OD value was compared with the values of a reference plate and used for the adjustment between plates applying the following correction factor: (mean OD value of serum P diluted 1:200 from the reference plate) / (mean OD value of serum P diluted 1:200 from the test plate).

2.5. Source of dog sera

The sera used for analytical and comparative investigations were obtained from the following sources:

(a) A total 40 dogs were experimentally inoculated with third stage larvae (L3) harvested from experimentally infected *Biomphalaria snails* and regularly (mostly every 1-2 weeks) bled during previously performed experimental trials. Three dogs were inoculated with 50 and further 3 dogs with 500 L3, while the remaining dogs received 200 L3 (Schnyder et al., 2009; Schnyder et al., 2010). Twenty-three dogs were subject to anthelmintic treatment: two groups of eight dogs each were treated with imidacloprid 10 mg/kg body weight (BW)/moxidectin 2.5 mg/kg BW spot-on (Advocate®, Bayer Animal Health) at 4 or 32 days post inoculation (dpi), respectively, while a third group (n=5) was treated at 88-92 days dpi. Further two dogs were treated at the same moment with an experimental compound (Schnyder et al., 2010). The remaining seventeen dogs were left untreated and the worm burden was determined at necropsy at different time points in all dogs (two dogs: long term follow-up). The larval excretion was followed up daily starting from 40 dpi by the Baermann-Wetzel technique (Eckert et al., 2008). Patency in untreated dogs was detected at 47-53 dpi, and larval excretion in dogs treated at 88-92 dpi stopped within 21 days after treatment.

(b) Twenty-three sera were collected from naturally infected dogs presented at the Veterinary Hospital of the Vetsuisse Faculty of the University of Zurich and from German dogs with clinical symptoms of respiratory and circulatory disease, bleeding disorder and/or neurological signs (Barutzki and Schaper, 2009). Infection was confirmed by isolation of L1 in faecal samples using the Baermann-Wetzel technique.

(c) A further 14 sera from the above mentioned sources were collected from dogs excreting L1 larvae of *Crenosoma vulpis*.

(d) Additionally, the following sera diagnosed at the diagnostic unit of the Institute of Parasitology, University of Zurich, in Zurich, were used for specificity evaluations: 20 sera from dogs experimentally infected with *Toxocara canis* eggs and tested positive for egg excretion, 4 from dogs experimentally infected with *Ancylostoma caninum* (kindly provided from the Institute of Parasitology, University of Veterinary Medicine, Hannover), and 23 sera tested positive for the
presence of *Dirofilaria immitis* antigen (DiroCHEK®, Synbiotics, San Diego, USA) and/or microfilariae characterized with the acid phosphatase stain.

(e) A total of 30 randomly chosen samples submitted to the Veterinary Laboratory of the Veterinary Hospital of the Vetsuisse Faculty of the University of Zurich for various reasons not correlated with suspected parasitological infections were used for the determination of the cut-off value. The cut-off was determined by the mean optical density (OD) value adding 3 standard deviations (SD). Further 50 randomly chosen sera were used to calculate the specificity (control group).

2.7. Statistical analysis

Statistical analysis was performed using Excel 2007 for Windows (Microsoft Corporation, Redmond, USA) and GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

2.8. Animal permissions

The experimental foxes were reared and purchased from a fur farm and housed individually at the LIFE faculty’s animal experimental facilities under the Danish experimental animal licence no. 2005/561-1060 and treated according to the animal ethics laws of Denmark. Experiments with dogs, mice and rabbits were conducted according to the Swiss guidelines for animal experimentation and approved by the Cantonal Veterinary Office of Zurich prior to study start. They were carried out with facility-born animals at the experimental units of the Vetsuisse Faculty in Zurich (permission numbers 25/2006, 26/2007, 13/2008, 185/2008).

3. Results

The use of a selected mAb, designated ‘mAb Av 56/1/2’ (IgG1 isotype), and rabbit polyclonal antibodies in a sandwich-ELISA allowed the detection of circulating *A. vasorum* antigen. Titration of *A. vasorum* adult E/S antigen revealed a detection level of 0.06 µg/ml in an *A. vasorum* negative dog serum that was diluted 1:1 with PBS-T.

3.1. Determination of the diagnostic sensitivity and specificity

In 22 out of 23 dogs naturally infected with *A. vasorum* (diagnosed by Baermann-Wetzel technique), ELISA values above the diagnostic cut-off were determined (Fig. 1), indicating a sensitivity of 95.7% (95% Confidence Interval – CI: 78.1-99.9).
The specificity of the ELISA calculated with 50 control samples submitted for various reasons that were not correlated with suspected parasitological infections was 94.0 % (95% CI: 83.5-98.7). Cross-reactivity was tested with a total of 61 samples from dogs with proven parasitic infection and is shown in Fig. 1. One of 14 sera from dogs excreting L1 of *C. vulpis* showed strong positive reactions. A single serum positive for *D. immitis* – antigen had a low positive reaction in the *A. vasorum* ELISA. No significant difference was found concerning positive reactions between the control group and dogs with proven other parasitic infections.

3.2. Circulating *A. vasorum* serum antigens during experimental *A. vasorum* infections

In dogs experimentally inoculated with *A. vasorum*, seropositivity increased over the first 3 months of infection (Tab. 1). The results of serological follow-ups of 17 dogs confirmed to be infected with *A. vasorum* at necropsy and corresponding worm burdens are shown in Fig. 2 and 3. Dogs became positive for *A. vasorum* circulating serum antigen starting from 35 dpi and latest at 77 dpi. One dog harbouring a high worm burden (165) at 55 dpi was seropositive only at 35 dpi, while all samples of the other dogs had increasing optical densities during the progressive course of infection. During a long-term follow-up of two dogs, circulating antigen was regularly detected at high levels (Fig. 3).

In contrast, OD values of four out of five infected dogs that were treated with the spot-on solution containing imidacloprid/moxidectin decreased after the drug administration. No worms could be found in these dogs at necropsy (Fig. 4). Three out of 5 dogs became negative within 16-34 days after treatment, while the one dog harbouring still one worm at necropsy remained positive for *A. vasorum* antigen detection. In sera of dogs treated at 4 (n=8) or 32 (n=8) dpi, no antigen could be detected by the sandwich-ELISA during the whole follow-up until 55 dpi.

4. Discussion

A valid and affordable diagnostic method for the detection of *A. vasorum* - infected animals before the appearance of clinical signs could avoid the onset of severe pathological changes in early anthelmintic-treated animals. Diagnosis of infections with this potentially fatal parasite is mainly achieved by larval detection in faecal samples, and the sensitivity of these methods can be increased by multiple faecal examinations, as reported from recent prevalence studies (Barutzki and Schaper, 2009; Taubert et al., 2008). However, due to low compliance of animal owners for multiple faecal collections and difficulties to always guarantee proper storage of faecal samples, alternative methods are needed.

The identification of stage-specific antigens of *A. vasorum* suggested that they are potential candidates for future use in diagnostics (De Oliveira Vasconcelos et al., 2008). Verzberger-Epshtein et al. (2008) developed a sandwich-ELISA for the detection of circulating worm antigen using rabbit polyclonal antiserum directed against whole adult worm antigen. The test was evaluated with sera from dogs from endemic and non-endemic regions from Canada showing high specificity and sensitivity. Cross-reactions against several
helminths were evaluated, and in particular no reactions were found with sera of animals infected with C. vulpis, a lung worm known to be widely distributed (Conboy, 2004). However, no samples of animals infected with D. immitis, which, like A. vasorum, is residing in the right heart of definitive hosts as an adult stage, and known to produce circulating antigens, were investigated.

The present study aimed to develop a reliable diagnostic tool for the serological diagnosis of an A. vasorum infection in dogs. Our first results using A. vasorum adult E/S antigen for the production of rabbit polyclonal antibodies resulted in a prototype ELISA with low specificity when sera of dogs infected with D. immitis were tested (data not shown). These trials showed that A. vasorum and D. immitis share common epitopes inducing strong cross-reactions. For this reason, mAbs produced in this study were evaluated with E/S antigen of both adult A. vasorum and D. immitis, and several mAbs strongly reacting with D. immitis were excluded. Consequently, the use of the mAb Av 56/1/2 which did not react with D. immitis E/S antigen resulted in a highly specific sandwich-ELISA. Employing this species-specific mAb together with polyclonal rabbit antibodies directed against A. vasorum adult E/S antigen, the sandwich-ELISA was able to detect circulating antigens in the sera of patent naturally infected dogs with a high specificity. Therefore, the few positive reactions observed with sera of the control group or the group with proven other helminth infections (one of 23 D. immitis and one of 14 C. vulpis infected dogs) are unlikely to be caused by cross-reactions. Rather, occult A. vasorum infections or coinfections which could not be excluded, might the reasons for the seemingly false-positives. As a matter of fact, expertise is needed for larval differentiation from faecal samples, and a coinfection can be easily missed particularly if one larval type is dominating.

The test sensitivity of the sandwich-ELISA increased over time of infection, reaching a sensitivity of 98.6% in experimentally inoculated dogs infected for 10 and more weeks. However, also during late prepatency 4-5 weeks after inoculation, 20% of the dogs were seropositive, indicating that the employed mAb Av 56/1/2 is directed against epitopes on parasitic stages that are present before larval excretion starts. The stage-specificity of the monoclonal used will be analysed in detail in further studies. No evident correlation between worm burden detected at necropsy and OD values in ELISAs was observed. The reason why one experimentally infected dog with a high worm burden at necropsy at 55 dpi had only one seropositive sample (at day 35) remains enigmatic. Possibly, this dog would have become seropositive again later. The long term follow-up of two experimentally inoculated dogs showed that they remained seropositive up to a year. Serodiagnosis for circulating antigen is therefore a helpful tool even for dogs with chronic angiostrongylosis tending to excrete low numbers of larvae.

Antigen detection was negative in experimentally inoculated dogs treated before patency, at 4 or 32 dpi, confirming the efficacy of the prophylactic use of imidacloprid/moxidectin (Advocate®) (Schnyder et al., 2009). In four out of five dogs treated later (at 88-92 dpi), the therapeutic efficacy (Schnyder et al., 2010; Willesen et al., 2007) was verified by the absence of worms at necropsy, corresponding with decreasing ODs in the ELISA. One dog positive at necropsy but negative with the Baermann-Wetzel technique (data not shown) was still seropositive, as well as one dog necropsied 30 days after successful treatment. Thus, an effective treatment was followed by decreasing level of detected circulatory antigens, mainly until
seronegativity was reached. In contrast, in dogs that remain seropositive for more than 6 weeks after treatment, it can be assumed that the treatment was not successful and should therefore be repeated. Also, post-treatment faecal examinations performed with the Baermann-Wetzel technique are not recommended to be done before 4 weeks after treatment, since larval excretion can be observed up to 3 weeks after successful treatment (Schnyder et al., 2010).

Alternatively, the serological detection of antibodies directed against adult *A. vasorum* antigen could potentially represent a valid method for the diagnosis of canine angiostrongylosis. In early attempts for the serological detection of antibodies directed against adult *A. vasorum* antigen (Guilhon et al., 1971; Mishra and Benex, 1972), antibody detection started from 14 dpi, but potential cross-reactions against other nematodes such as *C. vulpis* or *D. immitis* were not analysed. Furthermore, it has to be considered that antibodies might persist over months after elimination of the worm burden.

As shown by experimental infections, clinical signs and also echocardiographic changes in *A. vasorum* – infected animals may be subtle, even if the pathological changes of the lungs are considerable (Kranjc et al., 2010; Schnyder et al., 2010) and not completely reversible by anthelmintic treatment. In known endemic regions, prophylactic anthelmintic treatment (Conboy, 2004; Koch and Willeisen, 2009; Schnyder et al., 2009) or routine screening of dogs for *A. vasorum* infection (Verzberger-Epshtein et al., 2008) are recommended. The presented ELISA for the detection of circulating antigen represents a valid method for the reliable diagnosis of *A. vasorum* infection in dogs and for the control of the success of anthelmintic treatments. Moreover, the test can easily be used for mass-screening (i.e. with automated procedures) of dog populations, intended for a better understanding of epidemiological aspects of this probably underestimated disease.

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References


Table 1: Detection of circulating antigen of *Angiostrongylus vasorum* in serum by ELISA in dogs experimentally inoculated with *Angiostrongylus vasorum* (CI: Confidence Interval).

<table>
<thead>
<tr>
<th>Weeks post inoculation</th>
<th>No. dogs</th>
<th>positive / tested sera (%; 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td>22</td>
<td>0/22 (0; 0.0-12.7)</td>
</tr>
<tr>
<td>1-3</td>
<td>22</td>
<td>0/52(^b) (0; 0.0-5.6)</td>
</tr>
<tr>
<td>4-5</td>
<td>22</td>
<td>6/30(^c) (20.0; 7.7-38.6)</td>
</tr>
<tr>
<td>6-7</td>
<td>22</td>
<td>10/30(^d) (33.3; 17.3-52.8)</td>
</tr>
<tr>
<td>8-9</td>
<td>22</td>
<td>19/30(^e) (63.3; 43.9-80.1)</td>
</tr>
<tr>
<td>10-51</td>
<td>14</td>
<td>70/71(^f) (98.6; 92.4-100)</td>
</tr>
</tbody>
</table>

\(^a\) The study included 17 untreated dogs necropsied at different time points and 5 dogs with anthelmintic treatment at 88-92 days post inoculation (dpi).

\(^b\) Blood sampling frequency: all dogs on 7 and 21 dpi, additionally 8 dogs on 13 dpi.

\(^c\) Blood sampling frequency: all dogs on 35 dpi, additionally 8 dogs on 27 dpi.

\(^d\) Blood sampling frequency: all dogs on 49 dpi, additionally 8 dogs on 41 dpi.

\(^e\) Blood sampling frequency: 16 dogs on 55 dpi, 14 dogs on 63 dpi.

\(^f\) Blood sampling frequency: 14 dogs every week or every second week up to 92 dpi; afterwards 3 dogs every second week up to 188 dpi, followed by bleeding on 230 (3 dogs), 286 (2 dogs), 314, 342, 356 (one dog) dpi.
Figure 1:
Detection of circulating *Angiostrongylus vasorum* antigen in sera of dogs excreting *Angiostrongylus vasorum* or *Crenosoma vulpis* larvae, positive for circulating *Dirofilaria immitis* antigen in serum and/or microfilariae, or experimentally infected with *Ancylostoma caninum* or *Toxocara canis*. The cut-off value for the discrimination between seropositive and seronegative samples was determined by the mean plus 3 standard deviation of the OD values of 30 control sera.

Figure 2:
Serological follow up of circulating *Angiostrongylus vasorum* antigen in sera of 15 dogs experimentally infected with *Angiostrongylus vasorum* (the number represents the worm burden of adult stages at necropsy; cut-off determination see Fig. 1). Bleeding intervals: 1-2 weeks.

Figure 3:
Serological long-term follow up of circulating *Angiostrongylus vasorum* serum antigen in 2 dogs experimentally infected with *Angiostrongylus vasorum* (the number represents the worm burden of adult stages at necropsy; cut-off determination see Fig. 1). Bleeding intervals: 1-6 weeks.

Figure 4:
Serological follow up of circulating *Angiostrongylus vasorum* antigen in sera of 5 dogs experimentally inoculated with *Angiostrongylus vasorum* and treated with imidacloprid/moxidectin (Advocate®) at 88 or 92 (↓) days post inoculation (the number represents the worm burden of adult stages at necropsy; cut-off determination see Fig. 1). Bleeding intervals: 1-2 weeks.
Fig. 1

ELISA values $A_{405\text{nm}}$

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample Size</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. vasorum</td>
<td>(n = 23)</td>
<td>23</td>
</tr>
<tr>
<td>C. vulpis</td>
<td>(n=14)</td>
<td>14</td>
</tr>
<tr>
<td>D. immitis</td>
<td>(n=23)</td>
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<tr>
<td>A. caninum</td>
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</tr>
<tr>
<td>T. canis</td>
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Fig. 4