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Title:**Comparison of three methods for the detection of *Angiostrongylus vasorum* in the final host**

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Highlights

- Angiostrongylosis can be difficult to diagnose
- Plucks from foxes, reservoir hosts for *A. vasorum* are analysed using 3 methods
- Sensitivity of direct inspection, nested PCR and the Angio Detect kit were 84.1%, 69.5% and 76.8% respectively
- Agreement between tests ranged from 45.6 to 79.7%
- A novel nested PCR-RFLP for the detection of dog lungworm spp is described

Abstract: Angiostrongylosis is potentially fatal parasitic nematode infection affecting dogs which can be difficult to diagnose. In recent years several microscopical, serological and molecular detection methods have been developed, however there are few studies that have compared the relative performance of these methods. Screening necropsy material from an opportunistic sample of 140 foxes (82 of which were

considered to be infected with *Angiostrongylus vasorum*), indicated sensitivities of 84.1% for dissection and visual examination of plucks, 69.5% for nested PCR of an 18S rRNA fragment and 76.8% for a canine *A. vasorum* antigen detection test (IDEXX Angio Detect) of tissue fluid samples respectively. Agreement between the tests ranged from 45.6 to 79.7%. A novel nested PCR-RFLP for the detection and identification of canid lungworm spp is described.

Keywords

Angiostrongylus vasorum, *Crenosoma vulpis*, *Vulpes vulpes*, diagnosis, post-mortem examination, IDEXX Angio Detect kit, nested PCR-RFLP

1. Introduction

Angiostrongylus vasorum, also known as French heartworm, is a parasitic nematode that infects wild and domestic canids. Since it was first described in the south west of France in the 19th century, it has been reported from many parts of Europe and in Africa, South America and Canada (Gallagher et al., 2012). While it was previously known to have a patchy distribution within this wide range, chiefly confined to endemic foci, its distribution is now thought to be expanding with clinical cases being reported in new geographic areas (Jefferies et al., 2010).

Within the final canid host, the worms reside in the right atrium, ventricle and pulmonary artery where, following sexual reproduction, they release eggs into the pulmonary circulation (Taylor et al., 2007). These first stage larvae hatch almost immediately, emerge into the alveolar space and migrate up the bronchial tree to the larynx. They are then swallowed and passed with the faeces of the host.

The presence of the parasites in the heart and lungs and the inflammatory response they elicit, result in a broad spectrum of clinical signs including exercise intolerance and respiratory distress, weight loss, vomiting, neurological signs and bleeding diathesis (Morgan et al., 2005; Gallagher et al., 2012). This broad disease spectrum, long prodromol period and the fact that infections may be mild and asymptomatic in some individuals, and severe, or even fatal in others, means that angiostrongylosis is difficult to diagnose and indeed may go undetected in areas outside its known distribution.

The most commonly used diagnostic method is the detection of first stage larvae in faecal samples using Baermann's modified larval migration assay, variations thereof or, less commonly, faecal flotation methods (Schnyder et al., 2010). Diagnosis based on the direct detection of larvae and the cytological profile in bronchoalveolar lavage fluid (BALF) has also been described (Barcante et al., 2008). In addition, several conventional and real-time PCR assays have been developed (Jefferies et al., 2009; Jefferies et al., 2011) along with a number of ELISA assays (Verzberger-Epshtein et al., 2008; Jefferies et al., 2011; Schnyder et al., 2011) including a pet-side antigen detection kit (Schnyder et al., 2014).

We recently carried out a national survey of lungworm in Irish foxes which are considered important reservoir hosts for the parasite. For this study plucks consisting of the trachea, lung and heart, from 542 foxes caught all over Ireland were assessed for the presence of nematodes by dissection, visual examination and microscopic identification of adult worms (McCarthy et al., *in press*). In the present study, a subset of these samples was screened by nested PCR analysis of BALF and analysis of tissue fluid using the antigen detection kit. The purpose of our study was to compare the three detection methods and to assess their level of agreement.

2. Materials and methods

2.1. Source of fox plucks

Plucks analysed during this study represent a subsample of a larger set collected as part of a nationwide study on the prevalence of *A. vasorum* in foxes in Ireland (McCarthy et al., *in press*). As described in that publication, all foxes were killed for pest and predator control reasons or were caught in traps set for other wildlife and were sourced through an Irish Government screening programme for the presence of *Echinococcus multilocularis*. Plucks had been bagged individually and stored at -20°C until further analysis.

2.2. Dissection of plucks, visual examination and microscopic identification of extracted worms

Following removal of the heart from the pericardium, an incision was made through the right atrium and ventricle, the pulmonary arterial trunk opened by cutting along its length towards the lungs and the arterial supply to each of the seven lung lobes opened in turn. The cardiac chambers and arteries were inspected visually for the

presence of parasites. The pluck was then immersed in a white, shallow bowl of water and washed thoroughly. This process was repeated twice. Subsequently all blood clots were manually disrupted and examined for the presence of worms. Recovered parasites were collected and stored in 10% buffered formalin. Prior to microscopic identification at 40x magnification, worms were clarified in lactophenol (48 hours at room temperature). Identification to species level was based on published morphological descriptions (Dunne, 1978; Urquhart et al., 1996; Traversa et al., 2010) and confirmed by a European College Board certified parasitologist (TdW).

2.3. Molecular analysis of BALF

Prior to dissection of the heart and lungs, the trachea was cropped to a length of approximately 1 cm above the carina. It was then grasped with a tissue forceps and elevated to allow the heart and lungs to be dependent. Ten ml of tap water were instilled into the trachea and lungs using a disposable syringe and collected using a disposable plastic pipette. An aliquot of this recovered fluid was centrifuged (2600g, 5 min), the supernatant removed and the pellets stored at -20°C for further analysis.

Genomic DNA was extracted from BALF using the High Pure PCR Template Preparation Kit (Roche) following the manufacturer's instructions. The nested PCR protocol targeted the 18S rRNA region and was designed to amplify *Filaroides martis* (AY295807), *Crenosoma vulpis* (AJ920367), *Crenosoma* 'red panda' (GU475120), *Oslerus osleri* (AY295812), *Ancylostoma caninum* (AJ920347) as well as *A. vasorum* (AJ920365). The external primers (NC18SF1 5' AAA GAT TAA GCC ATG CA 3' and NC5BR 5' GCA GGT TCA CCT ACA GAT 3') were previously described by Chilton et al. (2003), while the internal primers (LWnested fw 5' CGG CTC ATT AGA GCA GAT GTC 3' and LW nestedrev 5' TCC TCT TTT ATT ATT CCA TGA TCG 3') were designed using Primer 3 Plus. Both assays were performed in a standard reaction mix containing 200nM of each primer, 0.2mM dNTP's, 1.5mM MgCl₂ and 1u Taq polymerase (GoTac Flexi, Promega) per 50µl total volume. In the first PCR 8µl DNA extract were added as template, in the nested, 3µl primary amplicon. PCR conditions for both reactions consisted of an initial heating step at 95°C for 10min, followed by 40 cycles of 95°C (30s), 55°C (30s) and 72°C (1min), and a final extension at 72°C for 5min. Positive and negative control samples, consisting of DNA extracted from whole adult worms and nuclease-free water

respectively, were included in each PCR reaction. Amplicons detected by gel electrophoresis (2%) were subjected to sequence analysis (GATC Biotech, Germany using the internal primers) and Restriction Fragment Length Polymorphism (RFLP) analysis. Digestion of the 700bp nested PCR product with the restriction enzyme MsII (restriction site: CAYNNNNRTG, New England BioLabs) resulted in fragments of 130, 186 and 384bp in the case of *A. vasorum* and 17, 299 and 384bp in the case of *C. vulpis* respectively (Figure 1). All RFLP results were confirmed by sequencing using the internal primers (GATC Biotech).

2.4. Collection of tissue fluid and analysis using the *A. vasorum* antigen detection kit

Tissue fluid consisting of haemolysed blood and fluid leaked from the cardiopulmonary tissue was collected from the bags in which individual plucks had been stored and frozen. Prior to analysis all fluid samples were centrifuged (4000g, 5 min). Analysis was carried out according to the manufacturer's instructions.

2.5. Statistical analysis

All samples that tested positive for *A. vasorum* using any one of the three methods were considered positive. The sensitivity of each test was calculated by dividing the number of 'true positives' (i.e. the number of samples that were positive according any one of the three tests and tested positive using a particular test) by the sum of 'true positives' and 'false negatives' (i.e. the number of samples that were positive according any one of the three tests but tested negative using a particular test). Specificity of both the dissection & visual examination method and the nested PCR-RFLP assay was assumed to be 100% (as one was confirmed by a positive ID, the other by sequencing data). As stated above all samples that tested positive using any one of the three methods were considered infected. Therefore we had no known 'false positives' and the specificity of the antigen detection kit could not be calculated. The results of the various tests were compared using Cohen's Kappa Analysis (Cohen 1960). Cohen's Kappa not only provides information as to whether two tests agree but also a measure of the level of agreement, with agreement due to chance being factored out.

3. Results

Dissection and visual examination of plucks revealed 69 lungworm infections (65 *A. vasorum* and 4 mixed *A. vasorum* and *C. vulpis* infections respectively) (Table 1). Nested PCR analysis resulted in 65 amplicons. According to restriction fragment analysis, 50 BAL samples were infected with *A. vasorum*, 8 with *C. vulpis*, and 7 with mixed infections of both lungworm species. All RFLP results were confirmed by sequencing. According to the antigen detection assay, 63 tissue fluid samples were positive for *A. vasorum* antigen. Assuming that each sample that tested positive using any of the three methods was indeed positive, dissection and visual examination of plucks had a sensitivity of 84.1%, nested PCR-RFLP a sensitivity of 69.5% and the antigen detection test a sensitivity of 76.8%.

Comparison of the dissection/visual examination and molecular methods revealed that the two tests agreed in the case of 97 samples (40 *A. vasorum*-positive and 57 *A. vasorum*-negative respectively), resulting in a Cohen's Kappa agreement rate of 45.6% (after subtraction of agreement due to chance). Table 2 shows that dissection/visual examination failed to detect lungworm infections in 14 plucks that were positive by PCR-RFLP, while the latter was negative in 18 samples that were positive by visual examination.

When the results of dissection/visual examination and PCR-RFLP were combined a total of 78 samples was positive for *A. vasorum*. This compared to 63 positive samples according to the antigen detection test (Table 3), with 19 samples positive by visual examination/PCR-RFLP negative by antigen detection and 4 samples positive by antigen detection negative by visual examination/PCR-RFLP. Overall there was agreement in 117 cases resulting in a Cohen's Kappa percentage of agreement of 79.7% (after subtracting out the agreement due to chance). Of the 5 plucks that, according to dissection/visual examination and PCR-RFLP, were infected with *C. vulpis* only, 4 were negative according to the antigen detection test while one gave a weak positive signal.

4. Discussion

While a range of microscopical, serological and molecular methods have been developed for the detection of *A. vasorum*, only a handful of studies have compared the sensitivity of different methods. All of these studies agreed that ELISA (both, serum antigen and antibody detection assays) was the most sensitive and consistent

method for detecting *A. vasorum* infections in experimentally and naturally infected (Baermann positive and negative) dogs (Verzberger-Epshtein et al., 2008; Jefferies et al., 2009, 2011; Schnyder et al., 2014, 2015). A sandwich ELISA in particular, which uses monoclonal capture antibodies and hyper-immune polyclonal rabbit antibodies for the detection of circulating *A. vasorum* antigen, was reported to have sensitivity and specificity levels exceeding 94% (Schnyder et al., 2014, 2015). By comparison, the antigen detection test was reported to detect antigen 3 to 4 weeks later than the sandwich ELISA (following experimental infection), with a sensitivity of 84.6% and a specificity of 100% (Schnyder et al., 2014). The sensitivity and reliability of a real-time PCR-protocol (targeted at the second ribosomal transcribed spacer) was chiefly dependent on the type of sample tested, with PCR analysis of blood being superior to analysis of faecal samples, pharyngeal or tracheal swabs (Jefferies et al., 2009, 2011; Schnyder et al., 2015). The most commonly used diagnostic method, the Baermann larval migration assay, was consistently found to lack sensitivity due to intermittent faecal larval shedding, as a result of which, it was estimated to miss up to 50% of chronic infections (Verzberger-Epshtein et al., 2008).

Our study differed from these previous ones, in that our samples derived from foxes rather than dogs, that all of the infections had been acquired naturally and that most if not all of them were probably chronic. When planning the study we hypothesised that the availability of necropsy samples for dissection would facilitate definitive identification of current infections. As it happened, the visual examination of plucks, though more sensitive than either of the other methods, still failed to detect a small number of infections. While we had no access to faecal samples (to compare our results to the Baermann method) or serum samples, we were able to collect and analyse BALF which in the case of infected animals, would presumably contain eggs and/or stage 1 larvae (L1). We expected that using this fluid instead of pharyngeal or tracheal swabs (as in Jefferies et al., 2011; Schnyder et al., 2015), together with the fact that analysis was carried out using a nested PCR protocol would improve the sensitivity of the molecular approach.

In the event the PCR-based method was still the least sensitive of all three methods used. As Jefferies et al. (2011) suggested this might be a reflection of the small sample volume (200µl) used for DNA extraction. Nevertheless we find this result surprising, not only because the BAL fluid had been concentrated by centrifugation but also because the adult worms present in the plucks and detected during dissection

would be expected to produce a steady supply of eggs and/or L1 larvae. While freeze-thawing of the plucks may have ruptured some of the eggs and larvae, causing a certain amount of DNA to leak into the supernatant, we do not believe that this significantly affected the PCR analysis, since several cycles of freeze-thawing would be required to break down multicellular parasites.

Our results indicate that even in the absence of treatment during chronic infection, egg production may be low and/or intermittent and that any other excretory/secretory material shed by adult worms (Jefferies et al., 2009) contains little, if any DNA.

When the results of the dissection/visual examination and the nested PCR analysis were combined to assess the validity of the antigen detection kit, a level of agreement of almost 80% and a sensitivity of 76.8% was calculated which was slightly lower than the 84.6% reported by Schnyder et al (2014) who analysed sera from naturally and experimentally infected dogs using the same test kit. There are several possible reasons for this discrepancy. Firstly, it has been shown that estimates of test accuracy are affected by infection rate in the study population with high prevalence rates (over 50%) resulting in higher test sensitivity values than when the same tests are used to screen populations with lower prevalence rates (Lachs et al 1992; Leeflang and Bossuyt, 2005). The infection rate in our study (58.6% when all samples that tested positive for *A. vasorum* using any one of the three methods are included) was indeed somewhat lower than that in the study described by Schnyder and colleagues (88.5%). Another possible reason for the reduced sensitivity may have been that we tested tissue fluid rather than serum. Since tissue fluid constitutes plasma filtrate it is reasonable to assume that it contains the same antigens as blood, but these may be present at lower or more variable concentrations. Finally the reduced level of observed sensitivity may have been a reflection of chronicity or low levels of infection. In our opinion the fact that the tissue fluid samples derived from foxes, not dogs, is not significant, because the kit is specific for parasite antigen the release of which would be expected to be independent of host species.

With regards to specificity, it was assumed that neither dissection followed by microscopic identification, nor nested PCR-RFLP resulted in false positive results, because the former was based on clearly defined morphological characteristics, while the latter was confirmed by unequivocal sequencing data. Of the small number of plucks in our sample set that were infected with *C. vulpis* only, the antigen detection kit tested negative for all but one, indicating a high level of specificity. Indeed the

single *C. vulpis* positive sample that reacted with the antigen detection test may have carried an undetected mixed infection. Although *C. vulpis* and *A. vasorum* are closely related phylogenetically and share antigens (Verzberger-Epshtein et al., 2008; Jefferies et al., 2011), both the sandwich ELISA for the detection of antigen and the Angio Detect assay are reported to be 100% specific for *A. vasorum* (Verzberger-Epshtein et al., 2008; Schnyder et al., 2014).

While the use of necropsy material placed obvious limitations on our study, it provided a unique opportunity for screening a substantial number of naturally infected host animals using three fundamentally different diagnostic approaches. To perform a similar study in dogs would entail a very considerable cost and would in any case be ethically unacceptable. We would also argue that our findings are, with some reservations, transferable to dogs. This is because all three detection methods targeted the parasite directly, either as whole specimen, DNA or antigen, and were therefore independent of host species. With regard to sample type, the use of tissue fluid in the antigen detection test may have affected the sensitivity of the assay somewhat. Our results indicated however, that it was a good substitute for serum.

In conclusion, our study provides valuable information on the usefulness and scope of the three methods. For epidemiological studies, where carcasses are accessible, the dissection of plucks followed by microscopic identification of adult worms is the most sensitive and cost effective method. Used in conjunction with nested PCR-RFLP of BALF this method is likely to detect over 95% of all canid lungworm infections. On the other hand, screening of carcass tissue fluid samples for antigen provides a quick and reasonably sensitive alternative for detecting *A. vasorum* infections. With regard to clinical diagnostic samples, our results confirm that the *A. vasorum* antigen detection kit has good sensitivity and high specificity, making it a useful auxiliary tool for diagnosing angiostrongylosis in a hospital setting where no necropsy material is available.

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Figure 1 MsII restriction profiles of *Angiostrongylus vasorum* (sample ID 117, 149, 159, 144, 47, 91), *Crenosoma vulpis* (sample ID 138, 95, 32) and mixed infections (sample ID 64). Lane 1: 100bp ladder

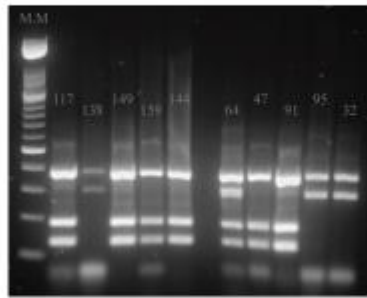


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Table 1 Infections rates (and numbers) of *Angiostrongylus vasorum* and *Crenosoma vulpis* in 140 foxes analysed using the 3 methods
(95% confidence intervals are presented in brackets)

	Prevalence of <i>A. vasorum</i> *(n)	Prevalence of <i>C. vulpis</i> *(n)
Dissection & visual examination of plucks	49.3% (n=69) (41.1-57.5)	2.9% (n=4) (0-7.6%)
Nested PCR- RFLP of BALF	40.7% (n=57) (32.6-48.8)	10.7% (n=15) (5.58-15.82)
<i>A. vasorum</i> antigen detection in tissue fluid	45% (n=63) (36.8-53.2)	n.a.

*including mixed infections

Table 2 Agreement of results according to dissection/visual examination of plucks and nested PCR-RFLP analysis of BALF for the presence of lungworm

Dissection & visual examination of plucks	Nested PCR-RFLP of BALF				
	Detection of <i>A. vasorum</i> (n)	Detection of <i>C. vulpis</i> (n)	Detection of mixed infections (n)	Uninfected (n)	Total (n)
Detection of <i>A. vasorum</i> (n)	40	2	6	17	65
Detection of <i>C. vulpis</i> (n)	0	0	0	0	0
Detection of mixed infections (n)	2	1	0	1	4
Uninfected (n)	8	5	1	57	71
Total (n)	50	8	7	75	140

Table 3 Agreement of combined dissection/visual examination and PCR-RFLP results and the *Angiostrongylus vasorum* antigen detection kit

	<i>A. vasorum</i> antigen detection assay		
Visual examination of pluck & Nested PCR-RFLP	Detection of <i>A. vasorum</i> (n)	Uninfected (n)	Total (n)
Detection of <i>A. vasorum</i> (n)	59	19	78
Uninfected (n)	4	58	62
Total (n)	63	77	140