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Genetic diversity and population structure of *Angiostrongylus vasorum* parasites within and between local urban foxes (*Vulpes vulpes*)

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Abstract

Angiostrongylus vasorum is a nematode parasite of the pulmonary arteries and heart that infects domestic and wild canids. Dogs (*Canis familiaris*) and red foxes (*Vulpes vulpes*) are the most commonly affected definitive hosts. Recent studies suggest that angiostrongylosis is an emerging disease, and that red foxes may play an important role in the epidemiology of the parasite. Genetic analyses of parasites collected from dogs and foxes throughout Europe have shown that the same parasite haplotypes are commonly shared between different host species. However, the extent of genetic diversity within local *A. vasorum* populations and individual hosts is unknown. The objective of the present study was to assess the occurrence of genetic diversity among *A. vasorum* (a) recovered from different foxes within the Greater London area (a localised population, single worm per fox dataset); and (b) hosted within single foxes (multiple worms per fox dataset). During 2016, *A. vasorum* worms were collected from foxes culled for other purposes in London. DNA was extracted from each parasite and a partial fragment of the mitochondrial cytochrome oxidase subunit 1 (mtCOI) gene was amplified and sequenced. Sequences from the single worm dataset were compared with those published elsewhere. Combined, 19 haplotypes were described of which 15 were identified from foxes found in London, indicating that considerable genetic diversity can be detected within a local geographic area. Analysis of the multiple worm dataset identified 22 haplotypes defining worms recovered from just six foxes, emphasising the relevance of wild canines as reservoirs of genetic diversity. This is the first study to explore the genetic complexity of individual fox-hosted *A. vasorum* populations.

Keywords: *Angiostrongylus vasorum*; mtCOI gene; dog; genetic variation; red fox; reservoir.

Highlights

- Foxes may act as wildlife reservoirs of *Angiostrongylus vasorum* for dogs in Europe
- Considerable genetic diversity was found among *A. vasorum* from foxes in Greater London
- First report highlighting genetic diversity of *A. vasorum* within individual foxes
- Foxes may act as reservoirs of genetic diversity of *A. vasorum* in the UK

1. Introduction

Angiostrongylus vasorum is a nematode from the family Metastrongylidae that affects the heart and pulmonary arteries of domestic and wild canids (Jefferies et al., 2010). The dog (*Canis familiaris*) and the red fox (*Vulpes vulpes*) are the main definitive hosts. However, other canid species such as the wolf (*Canis lupus*) and the coyote (*Canis latrans*) have also been described as definitive hosts (Segovia et al., 2001; Bourque et al., 2005), as well as some non-canid species such as the Eurasian badger (*Meles meles* L.) and otter (*Lutra lutra*) (Torres et al., 2001; Santoro et al., 2017). The life cycle of *A. vasorum* is indirect, with various species of gastropod molluscs acting as obligatory intermediate hosts (Morgan et al., 2008). In addition, other animals like frogs and birds can transmit the parasite as paratenic hosts (Bolt et al., 1993; Mozzer and Lima, 2015).

Clinical signs associated with *A. vasorum* infection in dogs can be unspecific and highly variable (Di Cesare et al., 2015). However, cardiorespiratory signs are most common , occurring alone or combined with bleeding and neurological disorders. This can eventually lead to death (Morgan et al., 2010; Helm and Morgan, 2017). In foxes, clinical signs of angiostrongylosis have been associated with the respiratory and cardiovascular systems (Jeffery et al., 2004; Morgan et al., 2008). Some studies have reported that infected foxes can present with right ventricular hypertrophy on post-mortem examination (Poli et al., 1984; Morgan et al., 2008), suggesting that the parasite might affect the health and fitness of these animals. In contrast, Jeffery et al. (2004) reported that infected foxes had a lower mean heart mass ratio compared with uninfected foxes. Disseminated cases of angiostrongylosis have recently been identified as cause of death in wild foxes from Italy (Eleni et al., 2014). However, foxes experimentally infected with *A. vasorum* did not show clinical signs during the time observed, other than elevated mean blood eosinophil counts (Webster et al., 2017).

Angiostrongylus vasorum is widely distributed and, to date, has been found in Europe, Africa, and some areas of North and South America (Jefferies et al., 2009b). The red fox is considered to be the main sylvatic host in Europe (Helm et al., 2010). The parasites' prevalence in foxes and clinical incidence in dogs has been reported to be restricted to endemic foci throughout Europe and North America, only occurring sporadically outside of these foci (Morgan et al., 2005). Despite this, recent studies show that *A. vasorum* is an emerging disease in dogs, since the parasite seems to be spreading within Europe to areas where it has not previously been identified (Helm et al., 2010; Kirk et al., 2014; Maksimov et al., 2017). Prevalence of *A. vasorum* in dogs appears to have increased in recent years, including examples such as Germany (Barutzki et al., 2017).

Several studies on *A. vasorum* prevalence and distribution have been conducted in dog and fox populations of Great Britain, with endemic foci recognised in the South of England and Wales for more than two decades (Simpson, 1996). Recently, a small number of cases have been reported in the North of England and Scotland (Helm et al., 2015), supporting the hypothesis that *A. vasorum* is an emerging disease (Helm et al., 2010; Kirk et al., 2014). Reasons for these recent increases are unclear, with dog transportation and the expansion of fox ranges suggested (Al-Sabi et al., 2013; Morgan et al., 2009; van Doorn et al., 2009). The distribution of *A. vasorum* is also thought to be influenced by climatic and environmental conditions that may modify parasite population dynamics and activity of its intermediate hosts, snails and slugs (Morgan et al., 2009). Given that the mean winter temperature throughout Great Britain commonly exceeds the limit reported by Jeffery et al. (2004), it can be assumed that transmission is possible in a much greater area than has been described to date (Morgan et al., 2009), suggesting that dog populations will be at greater risk in the future if the disease spreads to its potential (Morgan et al., 2010).

Wildlife, particularly red foxes, may play an important role in *A. vasorum* epidemiology since they have been identified as a reservoir for canine angiostrongylosis (Bolt et al., 1992; Taylor et al., 2015). Recent genetic analyses have shown that the same *A. vasorum* haplotypes can be found in different species of definitive hosts (Jefferies et al., 2009b, 2010), supporting the importance of wildlife as reservoir hosts. Genetic comparison of parasite populations across broad geographic ranges using markers such as mitochondrial cytochrome oxidase subunit 1 (mtCOI) have identified 24 different

haplotypes in parasites recovered from dogs and foxes in Europe (Jefferies et al., 2010), but the occurrence of diversity within local parasite populations, and even within individual hosts, remains unclear. For this reason, this study aimed to define the genetic diversity of *A. vasorum* in foxes from Greater London (1,569 km^2), as well as the diversity within multiple individual wild definitive hosts, in order to contribute to an assessment of the risk that foxes pose to dog health as parasite reservoirs. The objective of the present study was to assess the genetic diversity of *A. vasorum* (a) hosted in foxes within Greater London area; and (b) hosted within individual foxes. Our hypothesis was that foxes are likely to be reservoirs of genetic diversity for *A. vasorum* since their diet is likely to lead to (i) significant and repeated parasite exposure and, in the absence of routine de-worming (ii) accumulation of multiple adult worms. Thus, the opportunity for sex between genetically distinct worms and successful reproduction would be high in foxes.

2. Methods

2.1. Parasite isolation

Red foxes were culled as part of a routine pest control programme in the Greater London urban area throughout 2016 (Supplementary Fig. 1). All foxes were shot by a skilled marksman and sampling did not rely on trapping or targeting weaker or older animals. In total 175 adult foxes were admitted to the study, removing host age as a variable and maximising the opportunity to detect infected individuals. These animals were subsequently examined as part of an opportunistic surveillance scheme at the Royal Veterinary College (RVC). Each individual was sent in a sealed bag, which included the date and postcode of the area where it was killed. Post-mortem examinations were carried out within 48h of arrival at the RVC and a unique ID number was assigned to each carcass. No animals were culled specifically for this project and ethical review was not required.

Worms were recovered from lungs, heart and pulmonary arteries following the protocol detailed in Morgan et al. (2008) and identified microscopically as *A. vasorum* based on morphological description (Costa et al., 2003). Worms were counted and measured before being preserved in RNAlater as described by the manufacturer (ThermoFisher Scientific™; UK) and stored at -20°C for genetic analysis.

2.2. DNA extraction

DNA was extracted in two rounds. Initially, total genomic DNA was extracted from 83 worms, each representing a separate fox host (Dataset 1). Subsequently, DNA was extracted from a further 49 worms including between 7 and 10 worms from each of six foxes (Dataset 2, plus one sequence per fox from Dataset 1). Genomic DNA was extracted using a DNeasy Blood & Tissue Kit (QIAGEN, Germany) following the manufacturer's protocol for extraction from animal tissue (Spin-Column Protocol).

2.3. PCR amplification and sequencing

PCR was carried out targeting a partial region of the mtCOI locus (~710 bp) using the primers LCO (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') as described previously by Jefferies et al. (2010). The final reaction volume was 25 µL and contained 2 µL extracted DNA, 0.1 µL of each primer (100 µM stock; Sigma, Crawley, UK), 12.5 µL of MyTaq™ Mix (2x) (Bioline Reagents Ltd; UK) and 10.3 µL molecular grade water (Sigma). Genomic DNA extracted previously from *A. vasorum* and molecular grade water were used as positive and negative controls, respectively. The thermal cycling conditions were adapted from Jefferies et al. (2010), including an initial denaturation of 5 min at 95 ^{o}C, followed by 35 cycles of 45 sec at 95 ^{o}C, 1 min at 50 ^{o}C and 1 min at 72 ^{o}C, and a final extension step of 10 min at 72 ^{o}C.

PCR products were resolved by agarose gel electrophoresis using 1% (w/v) UltraPure™ Agarose (Invitrogen™; Paisley, UK) and TBE (Tris/Borate/EDTA) buffer (0.5x) with 0.005% (v/v) SafeView Nucleic Acid Stain (NBS Biologicals Ltd; Cambridgeshire, UK). PCR amplicons of the anticipated size were purified using the QIAquick PCR Purification kit (QIAGEN, Germany) following the manufacturer's protocol. DNA concentration was measured using a Nanodrop® ND-1000 (ThermoScientific™) and diluted using molecular grade water to obtain a final sample of 20 µL volume and 25 ng/µL concentration. Samples were subjected to Sanger chain-terminating dideoxynucleotide sequencing (GATC Biotech, Constance, Germany) using the primer LCO. Worms presenting unique polymorphisms were re-analysed (repeating both PCR and sequencing steps) to ensure the absence of false diversity as a consequence of PCR mutation. In recognition of the diploid, and thus potentially

heterozygous *A. vasorum* genomes sampled, sequence traces were manually annotated to identify the dominant haplotype from each worm.

2.4. Sequence alignment, phylogenetic and population analysis

Partial mtCOI sequences were curated using CLC Main Workbench (v6.0.2) and aligned with published sequences derived from dog, fox and coyote hosts (Jefferies et al., 2010; GenBank accession numbers GQ982734-GQ982876) using ClustalW with default parameters. The *Angiostrongylus costaricense* mtCOI sequence (GenBank accession number KX378965.1) was used as an outgroup.

MEGA version 6.0.6 was used to infer phylogeny in this study (Tamura et al., 2013). Using default parameters in MEGA, the TN93+G model was identified as optimal based on the Bayesian information criterion (BIC). Subsequently, a Maximum Likelihood (ML) tree was generated with 1,000 bootstrap iterations. The tree was left unrooted. Neighbor-joining (NJ) and Maximum Parsimony (MP) phylogenies were created for comparison, also using 1,000 bootstrap iterations. Sequence alignments were imported into the program NETWORK, version 5.0.0.1 (Bandelt *et al.* 1999) and haplotype networks were calculated using default parameters with parsimonious single nucleotide polymorphisms (SNPs) identified in the mtCOI sequences. Parameters defining genetic diversity were calculated using DnaSP version 5.10.

All sequences generated in this work have been made publically available under the accession numbers LT990053-LT990148.

3. Results

3.1. DNA extraction

A total of 175 foxes were sampled during 2016 in Greater London, including between one and seven foxes from each of 30 postcode districts. Of these foxes, 107 (61.1%) were found to contain *A. vasorum* worms (Martineau et al., manuscript in preparation). A panel of 83 foxes were used for parasite DNA extraction, including one worm per fox and excluding those worms which exhibited obvious signs of degradation (‘single worm per fox’, Dataset 1). Comparison of the foxes used in the

study revealed that 58% were female and 42% were male, and 45% were considered to be adult (skeletally mature) compared with 55% juveniles. Six individual foxes within this group that were found to contain at least seven worms were chosen for more detailed analysis, including a mix of sexes, locations and ages (Table 1). Up to ten worms were selected from each fox (as available) for use in the second 'multiple worms per fox' dataset (Dataset 2), providing a total of 55 worms in addition to one other worm per fox from Dataset 1.

3.2. PCR and sequence analysis

In total, 138 worms from 83 different foxes were analysed. Of 83 worms processed in Dataset 1, 59 (71.1%) produced full length amplicon sequences which passed quality control (CLC Main Workbench, default parameters). For Dataset 2, 37 quality sequences were derived from 55 worms (67.3%), supplemented by one additional sequence per fox extracted from Dataset 1 (n=43).

Sequences in Dataset 1 were aligned with the published *A. vasorum* mtCOI sequences GQ982734-GQ982876 in an alignment which comprised 588 bp. Topology of ML, NJ and MP trees derived using these data was comparable, although branch structure was unstable as a consequence of limited sequence diversity (see Supplementary Fig. 2 for an example). A summary of the genetic parameters calculated for each of the datasets is presented in Table 2. There were 15 parsimony informative nucleotide polymorphisms in total. Nineteen SNP haplotypes were identified (Table 2), of which 14 were detected in UK foxes (Fig. 1A). Eight SNP haplotypes were described in the UK for the first time, of which six had been described previously in canines from other countries. All haplotypes described previously from UK dogs with the exception of GQ982772 were detected here in foxes from the Greater London area with no evidence of spatial haplotype clustering detected. The four most common haplotypes described previously from Europe were all detected in London foxes, as was the haplotype identified from Canadian canines. Dataset 2 included 43 sequences after quality control, providing an alignment that included 574 nucleotides. There were 16 polymorphisms in total, only 10 of which were informative, and 22 different haplotypes were identified (Table 2). Despite the large number of haplotypes in dataset 2, overall nucleotide diversity was lower (Table 2). Non-parsimony informative SNPs were confirmed by repeat PCR and sequencing. A haplotype network constructed using the "multiple worms" dataset identified the occurrence of considerable genetic diversity within

individual foxes in London, with sequences obtained from separate worms collected from six different foxes presenting between two and nine haplotypes per fox (Table 1; Fig. 1B). Comparison of haplotype occurrence revealed two common examples, both of which were detected in half of the foxes analysed (F22, F46 and F94), despite these foxes coming from different postcode areas. However, these postcodes were relatively close to each other, all located in northern Greater London region (Supplementary Fig. 1). In contrast, fox F017, which had been culled in postcode W6 (west Greater London) and was the most isolated and western of the foxes sampled, presented a distinct series of haplotypes.

Alignment of the reference sequences and Datasets 1 and 2 revealed a total of 26 haplotypes, seven of which came from Dataset 2 and were new. A panel of 21 parsimonious SNPs were identified across the sequenced amplicon range, 19 featuring two variants and two featuring three variants (Table 3).

4. Discussion

This study provides detailed analysis of genetic diversity within *A. vasorum* collected from a restricted geographic area for the first time. It is also the first report to describe multiple haplotypes infecting individual foxes at a specific time, emphasising their relevance as a reservoir of genetic diversity with the potential for genetic exchange between *A. vasorum*.

The occurrence of *A. vasorum* among the foxes sampled in this study was notably high. One or more worms were detected in 61% of foxes sampled, higher than reported previously from British foxes (7.3%, varying from 0% to 23% by region; Morgan et al., 2008). A comparable sample set from domestic dogs was not available, but recent publications suggest a lower occurrence in this or equivalent populations across Europe (Helm et al., 2010; Kirk et al., 2014; Maksimov et al., 2017), likely a consequence of better controlled diets, administration of anthelmintic products and reduced access to intermediate/paratenic hosts of *A. vasorum*.

Several genomic loci have previously been investigated as genetic markers for *A. vasorum* including the second internal transcribed spacer (ITS-2) region and fragments of the mtCOI (Jefferies et al.,

2009a, 2010; Gasser et al., 2012), with the latter proving to be more informative (Blouin, 2002). The same region was used among others by Jefferies et al., (2010), who reported the presence of multiple haplotypes commonly shared between different host species in Europe and Canada. Here, a greater density of sampling was undertaken from a more spatially restricted local area, Greater London, to explore the occurrence of rarer haplotypes. Comparison of multiple mtCOI sequences permitted the detection of all but five mtCOI haplotypes described previously from Europe and North America. An explanation for this could be that London is a highly populated urban area with a large number of domestic dogs (PFMA, 2016). Following the introduction of the pet passport (European Union Regulation 998/2003) many of these dogs travel to/from Europe, creating opportunities to import different parasite strains and facilitating the spread of novel haplotypes throughout the UK. Further, new haplotypes have been described for the first time. The inclusion of additional markers is likely to have resulted in detection of even greater haplotype diversity. Published analyses of genetic diversity within closely related parasites such as *A. cantonensis* are not directly comparable, but work with loci such as mitochondrial cytochrome b and partial coding sequences of a 66 kDa protein have revealed considerable haplotype diversity, with detectable geographic structure (Eamsobhana et al., 2013; Peng et al., 2017). The work described here reinforces the geographic split between Europe and North America, but reveals no notable geographic structure within European *A. vasorum* populations (Jefferies et al., 2009b).

In agreement with previous reports (Jefferies et al., 2010), this study has confirmed that dogs and foxes can share common *A. vasorum* haplotypes, supporting the suggestion that foxes act as wild reservoirs of *A. vasorum* for domestic dog populations (Bolt et al., 1992). Comparison of diversity between worms from different local foxes identified notable levels of polymorphism, while sequencing multiple worms from individual hosts has demonstrated that foxes also act as reservoirs of genetic diversity for *A. vasorum*. A minimum of two *A. vasorum* haplotypes were found to infect an individual fox, with one fox hosting nine different haplotypes at the time of sampling. In the absence of comparable sampling from domestic dogs it is not possible to determine whether wild canines harbour more diverse parasite populations. *Angiostrongylus vasorum* infection of definitive hosts appears to be chronic and animals remain infected and shedding larvae for long periods (Al-Sabi et al., 2013; Webster et al., 2017). A study with experimentally infected dogs has reported that larval excretion

may occur for over three weeks, despite anthelmintic treatment, and shedding of larvae in untreated animals could last for at least 300 days (Oliveira-Júnior et al., 2006; Schnyder et al., 2010). This long shedding period offers the possibility of cross-fertilisation between different haplotypes if present. Thus, the suggestion that foxes harbour a greater number of more genetically diverse worms increases the chances of genetic segregation/recombination and emergence of new haplotypes, conferring *A. vasorum* the ability to evolve more rapidly.

Two of the foxes sampled in dataset 2 were culled in the same postcode area. The worms hosted within these foxes did not share the same haplotypes, suggesting a high haplotype diversity within a small urban area. Alternatively, some foxes culled from separated postcodes in datasets 1 and 2 were shown to host some shared haplotypes. The lack of information on the actual foraging range of these foxes does not allow us to infer whether they were infected by a similar source due to overlapping territories, or whether similar haplotypes were found in different locations. Sampling a higher number of foxes from single postcode areas would have allowed the detection of such geographic associations. Only six animals were selected to study genetic diversity within individual foxes. Despite this sample size being small, it was sufficient to confirm the genetic variation hosted within individual foxes, which had not previously been investigated.

5. Conclusions

In conclusion, this study emphasizes the importance of sequencing multiple worms within individual definitive hosts. Results showed that individual foxes were infected by genetically diverse *A. vasorum* parasites. As adult *A. vasorum* can persist within definitive hosts for extended periods when left untreated, it is reasonable to assume that genetically diverse worms harboured within wild foxes may be facilitating the emergence of new haplotypes through cross-fertilisation. Therefore, foxes are shown to be reservoirs not only of *A. vasorum* for domestic dogs, but also of parasite genetic diversity. More studies are needed to understand *A. vasorum* genetic diversity within individual foxes with appropriate comparisons from domestic dogs, and how this may influence the emergence of new haplotypes. Moreover, further studies sequencing multiple worms per animal from other definitive host species would be desirable to understand the role of other species as genetic reservoirs of the parasite.

315

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Tables

Table 1. Foxes included in the multiple worms per fox dataset (dataset 2). S = number of worm sequences included in the analysis (including one additional sequence from Dataset 1 per fox); H = number of haplotypes identified within each fox.

Fox code	Postcode culled	Age	Sex	Nº worms analysed	S	H
F17	W6	ADULT	Male	10	6	5
F20	NW3	ADULT	Male	7	6	4
F22	N16	JUVENILE	Male	10	10	5
F45	IG1	ADULT	Male	10	7	2
F46	IG1	ADULT	Female	10	11	9
F94	E7	JUVENILE	Female	8	7	3

Table 2. Summary of genetic parameters calculated for the single and multiple worm datasets

Alignment	Size (bp)	N	S	k	π Jukes Cantor	H	Hd
Single worm (Dataset 1)	588	202	15 (15)	1.989	0.156	19	0.826
Multiple worm (Dataset 2)	574	43	16 (10)	2.916	0.005	22	0.882

N = number of sequences tested; S () = number of variant sites detected, with the number of parsimony-informative variant sites shown in parentheses; k = average number of pairwise differences; π = nucleotide diversity calculated with the Jukes Cantor correction; H = number of haplotypes detected; Hd = haplotype diversity.

Table 3. Summary of parsimonious single nucleotide polymorphisms (SNPs) detected in sequenced mtCOI PCR amplicons.

	Alignment position (bp)																				
	4	47	131	179	293	308	326	332	337	368	**371**	395	413	419	**422**	428	478	501	520	528	531
Major	A	T	T	A	A	G	T	G	G	T	T	T	T	T	G	G	T	G	T	A	G
Minor	G	C	C	G	G	A	C	A	A	C	C	A	C	A	A	A	A	T	C	G	A
Minor (2)	-	-	-	-	-	-	-	-	-	-	G	-	-	-	T	-	-	-	-	-	-

447

448 Figure legends

449 Fig. 1. Haplotype NETWORKs based on partial mtCOI sequences from *A. vasorum* recovered from
450 London foxes. (A) mtCOI sequences from Dataset 1 (single worm per fox) compared with
451 published sequences derived from parasites hosted by dogs, foxes and a coyote (accession
452 numbers GQ982734-GQ982876). The diameter of the circle is proportional to the number of
453 individuals presenting each haplotype. The colour of each node indicates geographic origin and
454 worm host. (B) mtCOI sequences from Dataset 2 (multiple worms per fox), including between 7
455 and 11 worms recovered from each of six London foxes. The diameter of the circle is
456 proportional to the number of individuals presenting each haplotype. The colour of each node
457 indicates host identity. Nodes circled in red were not previously detected in Dataset 1.

458

459

460 Supplementary Fig. 1. The location of foxes sampled in this study at the time of culling used in
461 datasets 1 (single worm per fox, black squares; the number indicates the sample size per
462 postcode) and 2 (multiple worms per fox, red circles) from the Greater London area.

463

464 Supplementary Fig. 2. An example of a Maximum Likelihood phylogenetic tree representing Dataset
465 1, illustrating relationships between the single worm dataset from London (highlighted) and
466 published sequences (accession numbers GQ982734-GQ982876). GenBank sequence suffixes
467 include the initial of the country of parasite origin and the initial of the host it was isolated from.
468 Specifically, c = Canada, d = Denmark, f = France, g = Germany, I = Ireland, n = Netherlands,
469 p= Portugal, and u = UK; while F = fox, C = coyote, D = dog. NJ and MP phylogenies presented
470 similar topologies, with a lack of structure within each branch.

471