First notification in Italy of cardiopulmonary filariosis (heartworm disease) in a wolf (*Canis lupus*)

Ilaria Pascucci, Rosario Fico, Anna Rita D'Angelo, Sabrina Serini & Cesare Cammà

Summary

The authors report on the first notification of filariosis (heartworm disease) caused by *Dirofilaria immitis* in a wolf (*Canis lupus*) in Italy. On account of this exceptional finding, the parasite was typed not only using traditional methods, such as stereomicroscopic examination, but also using highly innovative diagnostic methods, such as scanning electron microscope and molecular identification with the application of various recently developed methods (polymerase chain reaction and sequencing). Certain aspects regarding the epidemiology of the disease are discussed in the light of this first case in Italy that occurred in an area in which cardiopulmonary filariasis had not previously been reported in wild or domestic carnivores.

Keywords

Canis lupus, *Dirofilaria immitis*, Filariosis, Helminth, Italy, Parasite.

Introduction

Filariosis caused by *Dirofilaria immitis* (order *Spirurida,* family *Onchocercidae*), is a parasitic disease caused by helminths that affects carnivores. It is transmitted by vectors (*Culicidae*) and is widespread in temperate areas (2, 9, 23).

The parasite has been reported on all continents (1, 9). In Europe, the highest prevalence has been reported in the Mediterranean Basin, namely: in north Italy, central France and Corsica, Greece, Portugal, Spain (particularly the islands) and Turkey.

The nematodes that carry the disease belong to the family *Onchocercidae*, species *D. immitis.*

Adult *D. immitis* live mainly in the right ventricle and pulmonary artery and its branches, although there have also been reports of aberrant locations (9).

Occasional infestations of sterile females have also been described in humans, characterised by subcutaneous and pulmonary nodules (2).

Clinical signs of filariosis, including coughing, intolerance of physical exertion, weight loss, heart failure, liver failure, coagulopathy, vomiting and ascites, are well known and described in cats and dogs, but as yet there is no description of the clinical symptomatology in wild animals. In some cases, the presence of high numbers of parasites in the right heart can cause the sudden death of the host (1, 23).

The principal factors that affect the spread and epidemiology of filariosis are climatic conditions which affect the biology of the vectors and the density and availability of mosquitoes, their degree of zoophilia and vector competence and the presence and density of end-hosts that are able to act as a reservoir. The Po plain offers ideal ecological conditions for the persistence of filariosis (9). The prevalence within the area ranges between 22% and 68%, but can reach 80% if no preventive chemical measures are taken (19).

In the light of the spread of the disease northwards and along the rivers leading to the

Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Via Campo Boario, 64100 Teramo, Italy
i.pascucci@izs.it

Tyrrhenian sea (filariosis is present in some

areas of Tuscany), probably due to climate change, it is interesting to note its general absence (at least in the clinical form) in the centre and south of Italy, where only sporadic occurrence has been recorded.

Although dogs have long been recognised as the main reservoir, the role of canids and other wild carnivores in the epidemiology of filariosis is yet to be established. It appears to be of secondary importance, but there is little available data in the literature. Marconcini *et al.* (15) presented a study conducted in Tuscany where 50 of a total of 523 European red foxes (*Vulpes vulpes*) examined presented adult *D. immitis* specimens in the heart chambers, whereas low levels of microfilaraemia were encountered in only a few animals.

In contrast, filariosis is present in wild canids throughout the area of spread and sporadic cases have been observed in other wild mammals.

Various reports of *D. immitis* have been made in wild carnivores in continental Europe: adult *D. immitis* were reported in the 1990s in the European red fox in the Ebro Valley (12) and in Tuscany (15). Previously (in 1980), some foxes culled in the Province of Grosseto were found positive for microfilariae (13).

Only recently (2001) was *D. immitis* encountered for the first time in Europe in a wolf (a single female specimen of *D. immitis* was gathered from 47 wolves examined in north-eastern Spain between 1993 and 1999) (20) and, during the same period, *D. immitis* was reported in a wolf (*Canis lupus*) and in two jackals (*Canis aureus*) in Bulgaria (11). In contrast, there have been no reports of *D. immitis* in wolves in Italy.

In 2004, *D. immitis* was reported in the Eurasian otter (*Lutra lutra*), that were reintroduced into areas of south-western Europe (22).

Most reports of *D. immitis* in wild animals are observed in post-mortem examinations of free-ranging animals found dead or those that have died in captivity in zoos.

In recent years, the development of molecular diagnostic techniques, initially developed with the specific aim to distinguish parasites involved in occasional infestations of humans, has led to increased knowledge of the epidemiology of filariasis caused by *D. immitis* and *D. repens*.

Materials and methods

At the end of November 2003, the carcass of a wolf (*Canis lupus*) from the Regional Park of Matese (Province of Caserta) was sent to the *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale'* in Teramo to investigate the cause of death by anatomopathological examination. The wolf presented clear canid bite injuries in various areas of the body that were probably the cause of death. Numerous adult nematodes were present in the right heart and pulmonary artery and branches (Fig. 1). Furthermore, the right heart was enlarged and extensive visceral congestion was observed. Whole parasites and fragments were collected and preserved in 70% ethanol.
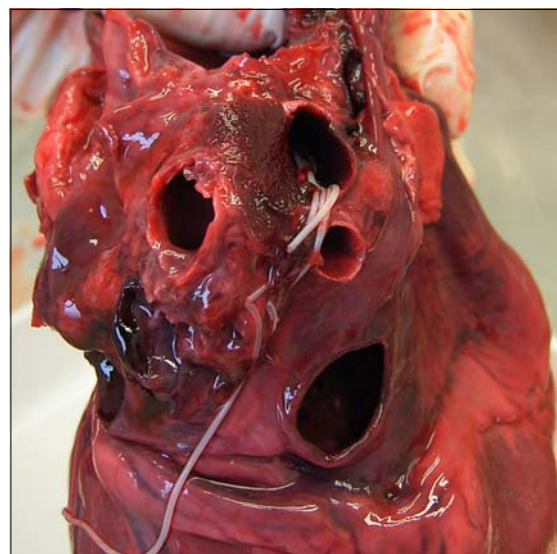

Figure 1
Adult *Dirofilaria immitis* in the heart and pulmonary artery of a wolf

Given the location, morphology and size of the helminths, filariosis caused by *D. immitis* was suspected. Given this exceptional finding, being the first case of filariosis in a wolf in Italy and one of the first described in Europe in a free-ranging animal, molecular identification was also performed.

This wolf came from an area in which the clinical form of filariosis had never previously been described.

Determination of age

During the anatomopathological examination, the first lower premolar tooth was extracted and examined histologically to determine the age of the animal.

Stereomicroscopic examination

A male specimen preserved in 70% ethanol was examined at increasing magnifications by stereomicroscope to reveal anatomical characteristics, such as the spiral form of the tail and the presence of caudal papillae.

Scanning electron microscope examination

The caudal end of the same specimen was then examined using a scanning electron microscope (SEM) (Zeiss DSM 940-A) after dehydration in ethanol, through steps at increasing concentrations, that then underwent critical point drying before being sputter-coated with gold. Examination at magnifications from ×6 to ×210 enabled an assessment of the number and shape of caudal papillae.

Polymerase chain reaction

DNA was extracted from an approximately 1 cm fragment of the parasite preserved in 70% ethanol by extraction in phenol-chloroform (18).

The parasite was identified using various polymerase chain reaction (PCR) methods with the simultaneous amplification of DNA extracted from a *D. repens* specimen, as described below.

- The first, developed by Favia *et al.* in 1996 (7) with the aim of distinguishing *D. repens* from *D. immitis* in human biopsy samples, was modified as follows: the PCR was conducted using 2 mM of MgCl$_2$, 1 IU of AmpliTaq Gold (Applied Biosystems) and 2 μl of DNA extracted from 40 amplification cycles. Agarose gel electrophoresis enabled identification of the parasites from two characteristic bands of 348 bp and 747 bp for *D. immitis*, and one band of 325 bp, in

addition to a series of equal sized bands at multiples of 175 bp for *D. repens*.

- The second, described by Murata *et al.* in 2003 (17) was developed and used to identify helminth specimens taken from the heart and pulmonary artery of a snow leopard (*Uncia uncia*) as *D. immitis.* The amplification reaction was modified using a primer concentration of 0.2 μM and 1.75 IU of AmpliTaq Gold (Applied Biosystems). The PCR protocol was also modified as follows: 10 min of initial denaturation at 94°C followed by 40 cycles with 30 sec at 94°C, 30 sec at 50°C and 30 sec at 72°C, and a final extension of 7 min at 72°C. This method amplifies a fragment of 656 bp in the region coding for cytochrome oxidase (COI) of the mitochondrial DNA, the sequence of which is known for numerous nematodes. The PCR product was purified using the QIAquick gel extraction kit and QIAquick PCR purification kit (Qiagen, Germany). The reaction sequence of the amplified purified product was followed using the Big Dye Terminator kit 3.1 (Applied Biosystems) and purified with Montage™ SEQ$_{96}$ Sequence Reaction Cleanup kit (Millipore). The electrophoresis was conducted on the ABI PRISM 3100 avant sequencer (Applied Biosystems). Sequences were analysed by EditSeq and Megalign Software (DNA Star Inc.).

- Finally, a third PCR was performed, amplifying a segment of 246 bp, specific for *D. repens* (14). Here too, certain modifications were made, as follows: 1.5 IU of AmpliTaq Gold (Applied Biosystems) and 2 μl of DNA were added to 50 μl of reaction volume. The PCR was conducted using the following protocol: 10 min of initial denaturation at 94°C, followed by 40 cycles with 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C and a final extension of 5 min at 72°C.

Results

Determination of age

A histological examination of the first lower premolar revealed the age of the wolf to be approximately five years.

Stereomicroscopic examination

Stereomicroscopic and SEM examination revealed certain morphological features that were characteristic of *D. immitis*, such as the coiled tail of the male (Fig. 2) and the caudal papillae (Fig. 3) (21).

Figure 2
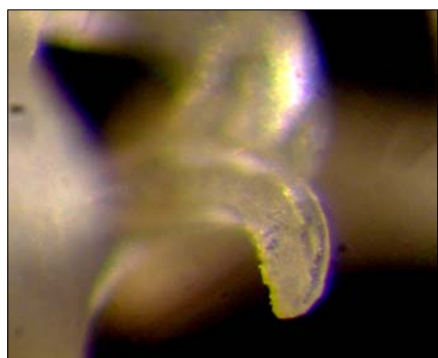Coiled tail of a male *Dirofilaria immitis*

Figure 3
Detail of caudal end of a male *Dirofilaria immitis*

Scanning electron microscope examination

SEM examination enabled a more precise evaluation of the cranial and caudal morphology, in particular, the number and shape of papillae on the tail of the male, from 4 to 6 pairs with a roughly ovoid shape (21) (Figs 4 and 5).
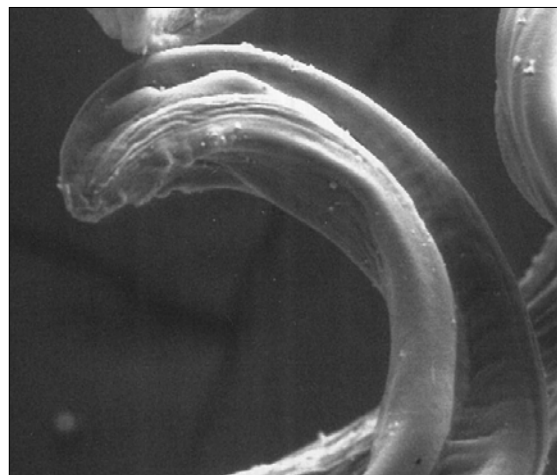

Figure 4
Caudal end of a male *Dirofilaria immitis*
(200 × 20 kV)

Figure 5
Caudal end of a male *Dirofilaria immitis*
(210 × 10 kV)

Polymerase chain reaction

With the first method, the presence of two PCR products of 348 bp and 747 bp led to the identification of the parasite as *D. immitis* (Fig. 6). In the case of *D. repens*, the PCR produced a band of 325 bp and a series of equal sized bands of multiples of 175 bp (Fig. 7).

The method developed by Murata *et al.* (17) identified a characteristic band corresponding to a fragment of 656 bp (Fig. 8). The PCR product sequence provided 100% homology with the complete sequence of the *D. immitis* mitochondrial DNA stored in the database
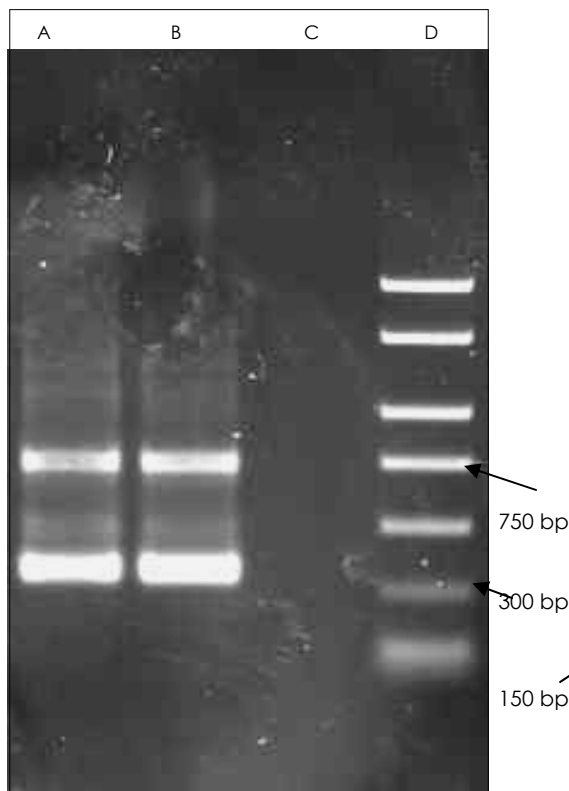
Figure 6
Gel electrophoresis of polymerase chain
reaction that discriminates *Dirofilaria immitis*
from *D. repens* (7)
Lanes A and B: DNA of wolf parasite
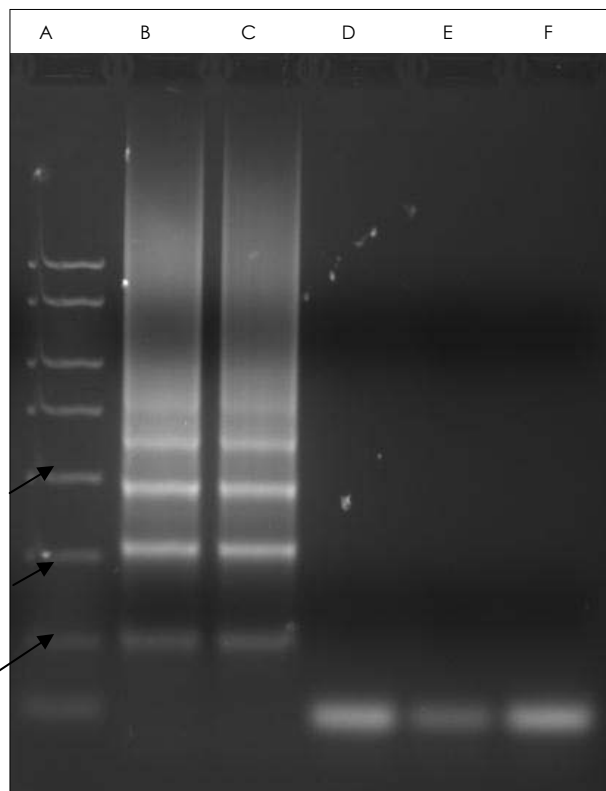Lane C: DNA of Dirofilaria repens
Lane D: PCR marker (Sigma)

Figure 7
Gel electrophoresis of polymerase chain reaction
that discriminates *Dirofilaria immitis* from *D. repens* (7)
Lane A: PCR marker (Sigma)
Lanes B and C: DNA of *Dirofilaria repens*
Lanes D and E: DNA of wolf parasite
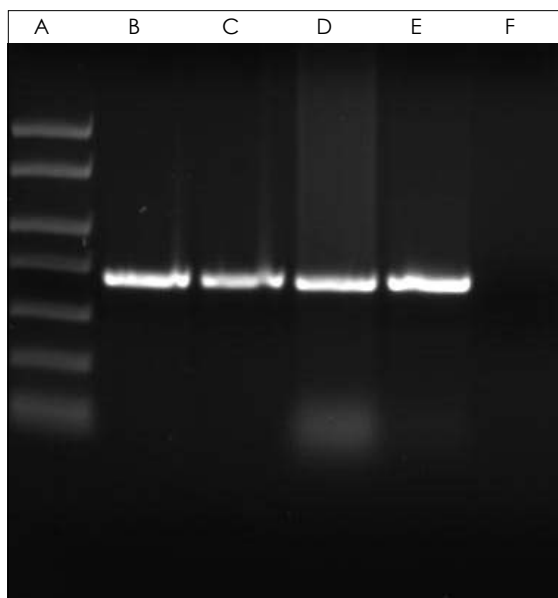Lane F: negative control

Figure 8
Gel electrophoresis of polymerase chain
reaction that amplifies 656 bp of cytochrome
oxidase (17)
Lane A: PCR marker (Sigma)
Lanes B and C: DNA of wolf parasite
Lanes D and E: DNA of *Dirofilaria repens*
Lane F: negative control

(AJ537512) and that published by Casiraghi *et al.* in 2001 (4) (AJ271613). The DNA extracted from the *D. repens* specimen amplified with the same PCR method and then sequenced was 99% homologous with the reference sequence (AJ271614). Both sequences were submitted to the GenBank database with the following codes: DQ358815 for *D. immitis* and DQ358814 for *D. repens*.

Finally, the method of Lee *et al.* (14), specific for *D. repens*, was negative for the DNA extracted from the parasites of the wolf, but was positive for the *D. repens* specimen (Fig. 9).

Conclusion

This case of filariosis in a wolf in Italy confirms the presence of *D. immitis* in wild Italian carnivores (already described in foxes), should be considered as exceptional.

The role of this species in the epidemiology of this disease should be investigated. Although
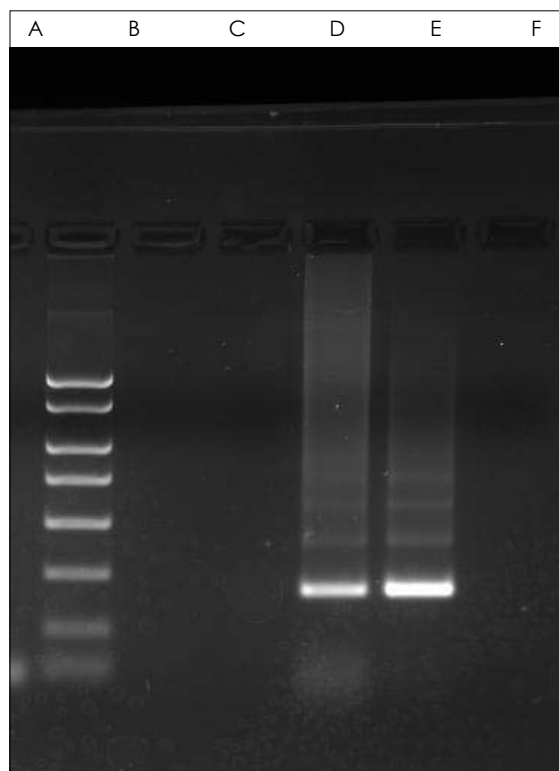
Figure 9
Gel electrophoresis of specific polymerase
chain reaction for *Dirofilaria repens* (14)
Lane A: PCR marker (Sigma)
Lanes B and C: DNA of wolf parasite
Lanes D and E: DNA of *Dirofilaria repens*
Lane F: negative control

it is unlikely that the wolf population can act as a reservoir, given the relatively low population of this species, it can be postulated that in this host, the ancient forebear of the domestic dog, the disease may behave biologically in the same way as it does in dogs, thus reaching and maintaining high long-term levels of microfilaraemia. It should also be noted that in this case, in contrast with most cases described in the literature, where a post-mortem finding of *D. immitis* occurs completely by chance and is not related to the cause of the death of the host, it is possible that the presence of filariosis was a concomitant cause in the death of this wolf (the disease would have produced subclinical cardiorespiratory failure in the host, reducing its physical exertion tolerance making it easy prey for aggressors).

The report of filariosis in central southern Italy is also exceptional, as the disease is considered as sporadic in this area, even in domestic carnivores. However, there have been reports of microfilariae outside the area where the disease is endemic. Reports have been made of *D. immitis* and *D. repens* microfilariae in 2 asymptomatic dogs in a study of 351 dogs in the area surrounding Vesuvius (6), while in a recent study (2003), Cancrini *et al.* (3) described the presence of *D. immitis* and *D. repens*, revealed by PCR, in *Aedes albopictus* specimens collected at Focene (coastal Lazio, Province of Rome).

Studies of the prevalence of filariosis in Europe using models based on geographic information system (GIS) technology have also demonstrated that conditions in southern Europe are favourable for the spread of filariosis, with an estimated maximum of 10 generations of *D. immitis* per year in the population of competent vectors (10). However, in common with other studies based on the analysis of the literature (24), these models postulate a higher level of the disease in south-eastern Europe than has actually been encountered.

This discrepancy is probably linked to the high prevalence in southern Europe of other filarioses caused by *Dipetalonema reconditum* and *D. repens*; according to Genchi *et al.* (8), the high prevalence of *D. repens* in dog populations may inhibit the simultaneous development of *D. immitis*. Despite this situation, it is possible that cases of filariosis may occur sporadically in areas where *D. repens* does not reach high levels and where *D. immitis* is circulating. A final element of significance is that wolves have an enormous home range of approximately 200 km^2 (5, 16), and can therefore cross environments with different ecological and epidemiological conditions in a very short time.

The presence of filariosis in an Italian wolf is not only exceptional but may be a threat for this species in endemic areas. Further knowledge of the epidemiology of this disease in populations of wild European carnivores is therefore necessary.

The use of molecular methods, such as PCR and sequencing which enable a distinction to

be made between different species of similar morphology and distribution, proves to be of great value in identifying parasites.

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