Mappe Parassitologiche

Series Editor
Giuseppe Cringoli

Registered office
Veterinary Parasitology and Parasitic Diseases
Department of Pathology and Animal Health
Faculty of Veterinary Medicine
University of Naples Federico II
Via della Veterinaria, 1
80137 Naples
Italy

Tel +39 081 2536283
e-mail: cringoli@unina.it
website: www.parassitologia.unina.it

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Dirofilaria

Mappe Parassitologiche 8

Dirofilaria

Dirofilaria immitis and *D. repens* in dog and cat and human infections

Editors
Claudio Genchi, Laura Rinaldi, Giuseppe Cringoli

Dirofilaria

Free Communications presented at the First European *Dirofilaria* Days
(Zagreb, Croatia, February 22-25, 2007)

The last years have seen an increasing use of cartographic representation of the distribution of parasitic diseases, particularly vector-borne (arthropod- and snail-borne) diseases.

Geographical Information Systems (GIS) are finding increasing use in the study of geographical epidemiology and their recent application to medical and veterinary parasitology is rapidly advancing.

MAPPE PARASSITOLOGICHE is a volume series aimed primarily to publish Disease Maps and parasitological field researches dealing with applications of GIS.

This Volume, the number 8th of the series, after a chapter dedicated to the use of GIS in health applications with particular regard to filariosis, contains the most important topics of canine, feline and human *Dirofilaria* infections and free communications presented at the First European *Dirofilaria* Days, held in Zagreb (Croatia), 22nd – 25th February 2007.

The contents deal the different aspects concerning filarial worms, specifically biology, pathogenesis, diseases, treatment of *Dirofilaria immitis* and *D. repens* parasitizing dogs and cats; their vectors; the role of *Wolbachia* for immunopathogenesis and diagnosis; human dirofilariosis; and epidemiological updates of dirofilariosis in Italy, Spain, Hungary, Czech Republic, Bulgaria, Romania, Serbia, and Turkey.

We thank the leading European experts and colleagues from the board of the American Heartworm Society for their contributions, and Pfizer Animal Health for having supported the costs of the Volume.

We hope that the information presented in this Volume serves as inspiration for scientists and practitioners to join in the further development of activity and studies on filarial worms in veterinary and human field.

Giuseppe Cringoli
Series Editor

Although significant contributions to the understanding of canine and feline heartworm disease have come from many North American scientists, *Dirofilaria immitis* infection in the dog was first reported in southern Europe, in the Po River Valley of Italy. In 1626, Francesco Birago published a treatise on hunting (Trattato cinegetico, ouero della caccia. V. Sfondrato, Milano, pp. 77,1626) in which he refers to the presence of the worm in the right heart of a dog and of another worm (probably *Dioctophyma renale*) in the kidney of the same dog. Since then, the parasite has been recognized in many countries of the world and European researchers mainly in Italy and France have been active in the study of *Dirofilaria* spp. infections.

Climatic changes, together with an increase in the movement of cats and dogs across Europe, have caused an increase in the geographical range of *Dirofilaria* worms and in the risk of infection for both animals and humans. At the same time, greater attention to animal welfare and improved social and economic conditions in many countries have contributed to an increase in pet owners' awareness of potentially life- threatening conditions, such as canine and feline heartworm disease, or of less serious but equally distressing infections like subcutaneous dirofilariosis, both in animals and humans.

This manual, which has been published on the occasion of the First European *Dirofilaria* Days, held in Zagabria (Croatia) in February 22nd-25th 2007, is intended as a contribution to and an update of current knowledge of *Dirofilaria* infection from leading European experts and colleagues from the board of the American Heartworm Society. The most important aspects of *Dirofilaria* infections are reviewed, from biology to prevention and treatment. Several chapters offer insights into new topics such as the application of the Geographic Information Systems (GIS) to *Dirofilaria* distribution in Europe and the role of *Wolbachia* endosymbionts in host immune response. The clinical aspects of the disease are described with the aim of giving practitioners suitable guidelines for diagnosis, management, prophylaxis and treatment of the disease. The last part includes a selection of short contributions presented during the meeting in Zagreb.

Finally, we would like to thank Pfizer Animal Health for having supported the costs of this publication without any constraints to the opinions expressed by the contributors.

Claudio Genchi

President International Scientific Comitee

Albert Marinculić

President Organizing Comitee

Mary B. Ard
Department of Pathology, College of Veterinary Medicine, University of Georgia, Athens, Georgia, USA

Relja Beck
Veterinary Faculty, University of Zagreb, Zagreb, Croatia

Gabriella Cancrini
Dipartimento di Scienze di Sanità Pubblica, Sezione di Parassitologia, Università "La Sapienza", Roma, Italy

J.A. Corbera
Facultad de Veterinaria, Animal Medicine and Surgery, Universidad de Las Palmas de Gran Canaria, Canary Islands, Spain

Giuseppe Cringoli
Dipartimento di Patologia e Sanità Animale, Facoltà di Medicina Veterinaria, Via della Veterinaria 1, 80137, Napoli, Italy

Annarita Di Cerbo
Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy

Dan Domingo
Animal Health Group, Pfizer Inc., New York, New York, USA

Michael T. Dzimianski
Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA

Éva Fok
Department of Parasitology and Zoology, Faculty of Veterinary Science, Szent István University H-1400 Budapest, P.O.Box 2, Hungary

Simona Gabrielli
Dipartimento di Scienze di Sanità Pubblica, Sezione di Parassitologia, Università "La Sapienza", Roma, Italy

Claudio Genchi
Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy

Marco Genchi
Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy

Giulio Grandi
College of Veterinary Medicine, University of Parma, Parma, Italy

Jorge Guerrero
Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

Richard Hack
Animal Health Group, Pfizer Inc., Lee's Summit, Missouri, USA

M.C. Juste
Facultad de Veterinaria, Animal Medicine and Surgery, Universidad de Las Palmas de Gran Canaria, Canary Islands, Spain

Laura H. Kramer
College of Veterinary Medicine, University of Parma, Parma, Italy

Maria Teresa Manfredi
Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy

Abdelmoneim E. Mansour
TRS Labs, Inc., Athens, Georgia, USA

John W. McCall
Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA

Scott D. McCall
TRS Labs, Inc., Athens, Georgia, USA

J.A. Montoya
Facultad de Veterinaria, Animal Medicine and Surgery, Universidad de Las Palmas de Gran Canaria, Canary Islands, Spain

M. Morales
Facultad de Veterinaria, Animal Medicine and Surgery, Universidad de Las Palmas de Gran Canaria, Canary Islands, Spain

Rodrigo Morchón
Laboratorio de Parasitología, Facultad de Farmacia, Universidad de Salamanca, Salamanca, Spain

Vincenzo Musella
Dipartimento di Patologia e Sanità Animale, Facoltà di Medicina Veterinaria, Via della Veterinaria 1, 80137, Napoli, Italy

Silvio Pampiglione
Dipartimento di Sanità Pubblica Veterinaria e Patologia Animale, Università di Bologna, Bologna, Italy

Laura Rinaldi
Dipartimento di Patologia e Sanità Animale, Facoltà di Medicina Veterinaria, Via della Veterinaria 1, 80137, Napoli, Italy

F. Rivasi
Dipartimento di Scienze Morfologiche e Medico Legali, Università di Modena e Reggio Emilia, Italy

Fernando Simón
Laboratorio de Parasitología, Facultad de Farmacia, Universidad de Salamanca, Salamanca, Spain

W.L. Steffens
Department of Pathology, College of Veterinary Medicine, University of Georgia, Athens, Georgia, USA

Nonglak Supakorndej
TRS Labs, Inc., Athens, Georgia, USA

Prasit Supakorndej
Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, Georgia, USA

Luigi Venco
Clinica Veterinaria Città di Pavia, Viale Cremona 179, 27100 Pavia, Italy

Tatjana Živičnjak
Veterinary Faculty, University of Zagreb, Zagreb, Croatia

1

Geographical Information Systems in health applications: experience on filariosis

Laura Rinaldi, Vincenzo Musella, Claudio Genchi, Giuseppe Cringoli

Introduction

The concept that geographic location can influence health is a very old one in medicine. As far back as the time of Hippocrates (460-377 BC), it was observed that certain diseases tend to occur in some places and not in others. Disease mapping and environmental risk assessment using geographical information systems (GIS) and remote sensing (RS) technologies are now established as basic tools in the analysis of both human and veterinary health (for a review see Cringoli et al., 2005b). However, epidemiologists have needed time to become convinced that the prospects offered by GIS and RS could be useful in their own discipline. The last 15-20 years have seen an ever increasing reliance on cartographic representation of infectious diseases, particularly parasitic infections and their vectors (Bergquist, 2006). In fact, during the past two decades the publication of original research articles and reviews in veterinary and human health with an emphasis on GIS and/or RS has followed an exponential trend (Hendrickx et al., 2004). In addition, recent GIS/RS symposia organized at national and international conferences and several thematic issues on this topic published in the peer-reviewed international literature (e.g. special theme issues in *Advances in Parasitology* in 2000 and 2006; *Parassitologia* in 2005) demonstrate the wide array of applications and benefits of these tools (Cringoli et al., 2005b; Rinaldi et al., 2006). Furthermore, the publication of thematic books pertaining GIS and RS, as well as international peer-reviewed journals, including the current launch of *Geospatial Health* (www.geospatial-health.unina.it) attest to the increased interest in these new technologies. The establishment and maintenance of websites as a platform for sharing data and exchanging opinions, experiences and expertise on GIS and RS, with an emphasis on animal and public health, is also worth mentioning (for example http://www.gnosisgis.org). In this paper, we first summarize general aspects of GIS and RS and emphasize the most important applications of these tools in veterinary parasitology, with particular regard to filarial worms. Then, disease mapping, ecological analyses and predicting parasite occurrence/seasonality as useful tool for surveillance are summarized in the next section.

Brief history of GIS and RS

Depending upon the application area, a number of discordant definitions of GIS have appeared in the literature (Tim, 1995). One of the most widely used definitions of GIS is that proposed by Burrough (1986): "*a powerful set of tools for collecting, retrieving at will, transforming, and displaying spatial data from the real world*". Overall, a GIS is a platform consisting of hardware, software, data and people and encompasses a fundamental and universally applicable set of value-added tools for capturing, transforming, managing, analyzing, and presenting information that are geographically referenced (geo-referenced). Digital GIS data may be presented in map form using so called data layers repre-

senting the information collected. Two approaches can be used, namely:

(i) a vector data model, and

(ii) a raster data model.

The former model stores a table containing coordinates of points together with instructions on which points are alone and which points belong to a common set. In a vector data model, all lines are represented by chains of vectors, and all areas by polygons (Fig. 1). Attributes are coded in separate tables using alphanumeric characters as a label for a specific class or category of properties. The raster data model uses a net of adjacent polygons (termed "cells") to provide a virtual cover of a given part of a territory. The cells are often called pixels, and attribute-values of the objects that the cells represent are assigned to corresponding pixels (Daniel et al., 2004). Raster data are typically utilized to represent continuous phenomena, e.g. land cover maps, phenoclimatic maps, and digital elevation models (Fig. 2).

Fig. 1. Vector map of Europe and attribute (spreadsheet), used in this example to extract Italy.

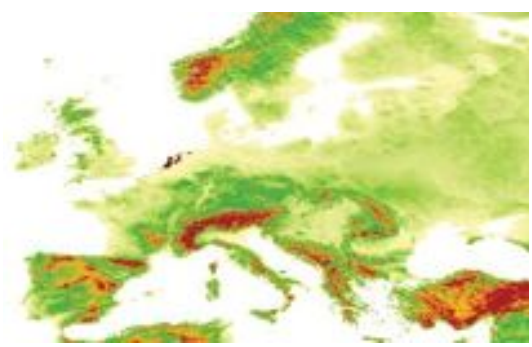

Fig. 2. Raster map of Europe, digital elevation model.

The use of GIS in epidemiology can aid in answering some important questions such as "what is?" and "where is?". In addition, because also the time domain - i.e. "when" - is important in most environmental and epidemiological processes, it has also been suggested that GIS should be replaced by STIS, which is an abbreviation for space-time information systems (Kistemann et al., 2002; Hendrickx et al., 2004). There is currently a movement towards regarding GIS as a science (geographical information science) rather than a simple technology (Goodchild, 2000; Kistemann et al., 2002).

One of the first major uses for GIS was in 1964 when the Canadian Geographical Information System (CGIS) was launched in an effort to assess the productivity of Canadian farmland. Subsequently, GIS has become widely and effectively used in natural-resource management. Prominent examples are timber management, mine permitting, water quality assessment, wildlife and habitat management, land use planning, zoning, and transportation design; predictive modelling of earthquakes, forest fires, and flooding; utilities management, routing analysis; marketing and demographic analyses, etc. (Cox and Gifford, 1997). The capacity of these systems justifies their wider application including veterinary and human medi-

cal sciences. Several types of analyses routinely used in GIS can be very useful for spatial epidemiology. These include:

(i) neighbourhood analysis, i.e. all features which meet certain criteria and are adjacent to a particular feature are found and listed;
(ii) buffer generation, i.e. generation of buffer zones around or along certain features;
(iii) overlay analysis, i.e. merging of two or more layers or maps to identify areas of intersection;
(iv) network analysis, i.e. modelling of networks and calculation of parameters such as the shortest distance between two locations;
(v) surface area and distance calculations, and
(vi) three-dimensional surface modelling (Ward and Carpenter, 2000).

A very useful function of GIS is the kriging, i.e. a linear interpolation method that predicts the values of a variable, at non-sampled locations, based on observations at known locations, using a model of the covariance of a random function (Berke, 2004). Kriging is widely used in meteorology in order to interpolate values of climate data from observing stations, and have also been used in epidemiology to model the distribution of various parasites/diseases, e.g. *Ixodes scapularis* that transmits Lyme disease (Nicholson and Mather, 1996), malaria (Kleinschmidt et al., 2000), alveolar echinococcosis (Conraths et al., 2003; Pleydell et al., 2004), tsetse flies that transmit human African trypanosomosis (Sciarretta et al., 2005), *Calicophoron daubneyi,* the causative agent of paramphistomosis in ruminants (Biggeri et al., 2004), *Oncomelania hupensins*, the intermediate host snail of *Schistosoma japonicum* (Zhang et al., 2005), co-infection with *S. mansoni* and hookworm among schoolchildren in Côte d'Ivoire (Raso et al., 2006), human filariosis (Brooker and Micheal, 2000) and canine filariosis (Genchi et al., 2005; Vezzani and Carbajo, 2006).

The term "remote sensing" (RS) was used - for the first time - in the US during the 1960s to designate the technique allowing the study of objects without any direct contact, through image capture. In 1970, in an article titled "*New eyes for epidemiologists: aerial photography and other remote sensing techniques*" Cline recognised that RS could have applications in detecting and monitoring disease outbreaks (Cline, 1970, 2006). In the following years, scientists of the National Aeronautics and Space Administration (NASA) in the US, used colour infrared aerial photography to identify the habitats of *Aedes sollecitans* (Hay, 2000).

While aerial photographs were the first source of RS data, the subsequent development of satellite measuring instruments significantly improved both the spatial and temporal coverage of the earth surface, generating a continuous and almost complete cover.

Satellites may be divided into two groups based on the orbit they follow, namely:

(i) geostationary satellites (e.g. GOES, Meteosat, GMS), and
(ii) near-polar-orbiting (or sun-synchronous) satellites (e.g. NOAA, Landsat, MODIS, SPOT, IKONOS, Quickbird).

The former group, which consists of satellites in orbits parallel with the rotation of the earth, is dedicated to meteorological applications, while the latter follows an elliptical orbit between 681 and 915 km, with a different ground track after each rotation, lasting about 100 minutes. The revisit period varies from 1 to 41 days but can be lowered. Most of the satellites are sun-synchronous and offer different spatial resolutions (Herbreteau et al., 2005). However, very few have found any application in epidemiology, the most important being the Landsat and the NOAA series (Durr and Gatrell, 2004). Satellites have several sensors recording radiation in different wavelengths that allow combination of spectral signals (Cringoli et al., 2005a; de La Rocque et al., 2005). RS provides a unique source of data that can be exploited to characterize climate and land surface variables at different spatial resolutions. It permits the calculation of vegetation indices, land surface temperatures, atmospheric and soil moisture, rainfall indices, etc. Among the vegetation indices obtained from RS, the most widely employed one is the normalized difference vegetation index (NDVI). It is defined as the difference between the visible (*red*) and near-infrared (*nir*) bands of satellite information over their sum: NDVI = (*nir* – *red*)/(*nir* + *red*). NDVI is a specific measure of chlorophyll abundance and light absorption, but its use has been extended to quantify herbaceous vegetation biomass, vegetation primary productivity, vegetation coverage and phenology.

The RS data are increasingly used for investigations in the field of environmental health sciences for mapping and prediction, surveillance and monitoring, particularly for vector-borne diseases (Beck et al., 2000). Since the disease vectors have specific requirements regarding climate, vegetation, soil and other edaphic factors, and are sensitive to changes in these factors, RS can be used to determine their present and future and predict distribution. Land cover classification refers to the natural vegetative cover, function of the topography, soil or local climate, and can differentiate between different covers up to the species communities. Land use classification refers to the description of human uses of the land, or immediate actions modifying the land cover, such as agriculture (e.g. used for crop production, lying fallow, irrigated, etc.), human settlements (e.g. urban, rural, isolated infrastructures, etc.), protected areas (e.g. national parks, forest reserves, etc.) (for recent reviews see Daniel et al., 2004 and Herbreteau et al., 2005). In Europe, the corine (Coordination of Information on the Environment) land cover (CLC, European Commission, 2000) is widely used, which is a map of the European environmental landscape based on interpretation of satellite images. CLC provides comparable digital maps of land cover for each country for much of Europe. It is useful for environmental analysis and comparisons over large scales (spatial resolution = 1 km). When studies are focused on small areas, aerial photographs can be used because of their capability to afford very high spatial resolution images (<1 m). The analysis of historical sequences of aerial photographs is an important method for determining

the medium-term dynamics of land cover on a landscape scale (Acosta et al., 2005). Most developed and developing countries have governmental programmes for aerial surveys but campaigns are not reproduced on a regular time basis, and ground coverage is often limited to areas of discreet interest.

However, aerial photography must be considered as a supplementary source of information (Herbreteau et al., 2005). Classifications of landscape from digital aerial photographs have been scarcely used in veterinary parasitology (Rinaldi et al., 2006), with the majority of applications focusing on mosquito surveillance (see for example, Kline and Wood, 1988; Brown and Sethi, 2002).

Disease mapping

One of the most useful functions of GIS in epidemiology continues to be its utility in basic mapping.

Usually, when data are collected either routinely or through purposely-designed surveys, they are presented in tabular forms, which can be exploited for analytical usage. However, the reading and interpretation of such data is often a laborious and time-consuming task and does not permit easy decision-making (Paolino et al., 2005). On the other hand, representation of these data in the form of a map facilitates interpretation, synthesis and recognition of frequency and clusters of phenomena. Today, GIS, RS and Global Positioning Systems (GPS) are well-known tools of the trade and few scientists working in the fields mentioned can manage without them. As we enter the era of Global Earth Observation Systems (GEOS), the old adage that a picture is worth more than a 1000 words rings truer than ever (Bergquist, 2006).

The oldest examples date back more than 200 years and consist of a world map of diseases put forward by Finke in 1792 (Barrett, 2000), and a map of yellow fever occurrences in the harbour of New York issued in 1798 (Stevenson, 1965). One of the most prominent examples is the mapping of cholera victims in relation to the location of water supplies in London's Soho district carried out by John Snow (1854). The street addresses of cholera victims were recorded and close proximity to putative pollution sources (i.e. water supply pumps) was identified as the key risk factor. With respect to parasites, in two papers published in 1903, Smith and Stiles independently presented maps of Texas, which displayed the prevalence of hookworm infection and demonstrated that infection was typically restricted to the eastern part of the state where the soils contain the most sand (Brooker and Michael, 2000).

Development of methods for mapping diseases using GIS has progressed considerably in recent years.

Disease maps can be drawn to a demographic base or to a geographic base. In the first case, they are related to the population and the epidemiological information they show are presented in relation to population size. Geographically based maps are constructed according to the shape of a country or a region or any administrative unit. They may be qualitative e.g.

point maps, distribution maps, point distribution maps (PDMs), indicating location without specifying the amount of disease; or quantitative e.g. distribution maps with proportioned peaks, proportional circle maps, choroplethic maps, choroplethic maps with proportioned peaks, PDMs with proportioned peaks, PDMs with proportioned circles, isoplethic maps, displaying the number of cases of disease, the population at risk, infection prevalence or intensity or incidence (Thrusfield, 1995; Cringoli et al., 2005c; Rinaldi et al., 2006). With the appropriate data at hand, producing a map using GIS can be undertaken literally in a matter of minutes; but therein lies one of the problems with GIS - one needs the spatially explicit data - and collecting these may take months or even years (Durr, 2004). GIS is, however, not only a digital map representation but is indeed an informational and analysing tool which permits the processing of space-related data. Certainly, maps themselves keep on playing a prominent role and are, of course, the most frequently used output of GIS (Kistemann et al., 2002).

Disease maps drawn by GIS can be the results of both meta-analysis and dedicated surveys.

Regarding maps derived from meta-analysis, examples on human filariosis are present in literature. For example, the global geographical distribution of human filariosis prevalences, estimated for 96 endemic countries in the 1993 World Bank Global Burden of Disease study (Michael et al., 1996), showed different patterns for the two lymphatic filariosis, namely Brancroftian filariosis (caused by *Wuchereria brancofti*) and Brugian filariosis (caused by *Brugia malayi*) (Brooker and Michael, 2000). More recently, GIS and a database on the distribution of lymphatic filariosis have been used to develop the first maps at district-level in India (Sabesan et al., 2000) and Egypt (Hassan, 2004). In addition, GIS and RS, combined with a landscape epidemiological approach, established the geographical patterns of river blindness (caused by *Onchocerca volvulus*) in southern Venezuela (Botto et al., 2005) and Ethiopia (Gebre-Michael et al., 2005), and the relationship between environmental features and infection prevalence was also assessed.

GIS is also the tool used by the African Programme for Onchocerciasis Control (APOC) in order to delineate zones of various levels of endemicity, and this is considered a fundamental step in the planning process for onchocerciasis control (Noma et al., 2002; Seketeli et al., 2002).

With respect to canine and feline filariosis, on the basis of a systematic review of the literature, in a recent paper, Trotz-Williams and Trees (2003) provided the first evidence-based GIS maps of the distribution of the major vector-borne infections, including *Dirofilaria immitis* and *Dirofilaria repens* in dogs and cats in Europe by a thorough investigation of published work on their distribution, prevalence and incidence, which should be useful both to practitioners and researchers in endemic areas and to those in non-endemic areas.

Regarding maps derived from dedicated surveys, GIS has become an important tool for designing a study and territorial sampling. The fundamental steps which can be used to produce

quality descriptive survey-based maps within GIS are the following:

(i) selection of the study area;
(ii) selection of the study population and calculation of the sample size, using as parameters the study population, the expected prevalence, the confidence level, and the standard error;
(iii) selection of the sample in the study area (e.g. random sampling, systematic sampling, proportional allocation, use of grids, transect sampling, etc.);
(iv) laboratory and/or field survey;
(v) geo-referencing of the study units (e.g. individuals, group of animals, counties, municipalities, regions, or any other administrative unit); and finally
(vi) drawing maps by GIS (Cringoli et al., 2005c).

The sampling procedures in the study area play obviously a key role in disease mapping. As indicated at point 3 above, several sampling methods can be used. For example, in order to study the distribution of filarial worms in dogs from a certain geographical area, proportional allocation can be used, i.e. the number of dogs to be tested in each administrative unit (e.g. neighbourhood, municipality, etc.) is proportional to the dog population in that administrative unit, or, if the dog population is unknown, is proportional to the administrative unit surface area.

This latter approach has been used by us in order to study canine filariosis in the Mt. Vesuvius area of southern Italy (Cringoli et al., 2001) and in the city of Naples (Rinaldi et al., 2004). Regarding the former study, conducted in an area comprising 51 municipalities (Fig. 3), microfilariae were detected in 63 of the 351 dogs surveyed (prevalence = 17.9%); in particular, 56 dogs (15.9%) showed only microfilariae of *Acanthocheilonema* (*Dipetalonema*) *reconditum*, three dogs (0.8%) only microfilariae of *D. repens*, two dogs (0.6%) microfilariae of both *A. reconditum* and *D. repens* and two dogs (0.6%) microfilariae of both *D. immitis* and *D. repens* (Cringoli et al., 2001). In the study conducted in the city of Naples (divided into 21 neighbourhoods, Fig. 4), microfilariae were detected in 8 of the 358 dogs surveyed (prevalence = 2.2%); in particular, 5 dogs (1.4%) showed only microfilariae of *A. reconditum*, three dogs (0.8%) only microfilariae of *D. repens*, and no *D. immitis* positive dogs were found (Rinaldi et al., 2004).

Figures 5-9 are distribution maps with proportioned peaks, which use the municipality (for the Mt. Vesuvius area) or the neighbourhood (for the city of Naples) as the geographic unit of reference and display the following information:

(i) filarial species studied (*D. immitis, D. repens* and *A. reconditum*);
(ii) total study area divided into municipalities/neighbourhoods;
(iii) municipalities/neighbourhoods with positive dogs;
(iv) municipalities/neighbourhoods without positive dogs; and
(v) municipal/neighbourhood prevalence (%) determined as follows:
(number of positive dogs in the municipality/neighbourhood)/ (number of dogs examined in the total study area) x 100.

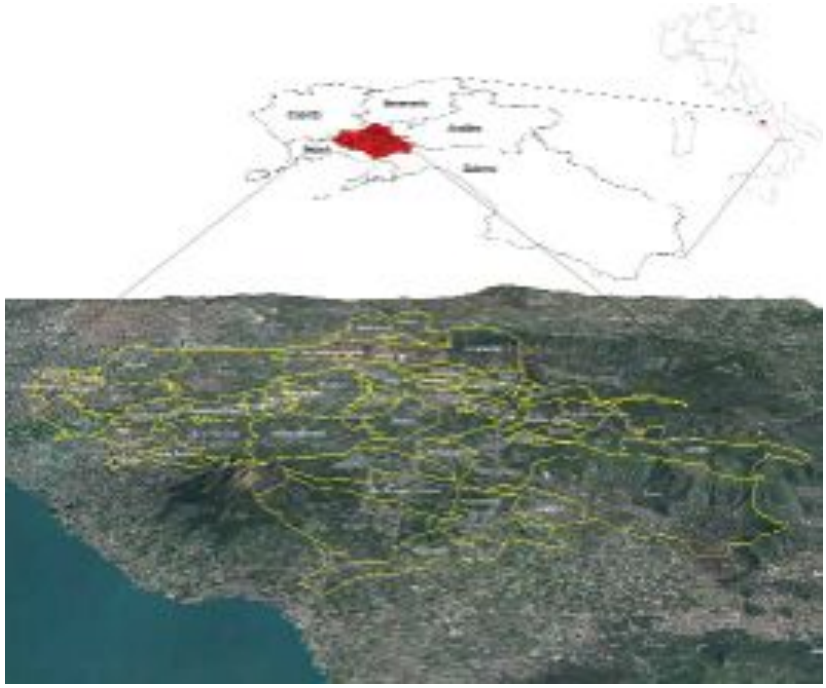

Fig. 3. The Mt. Vesuvius area of southern Italy divided into 51 municipalities (administrative boundaries on digital aerial photographs).

Fig. 4. The city of Naples divided into 21 neighbourhoods (administrative boundaries on digital aerial photographs).

In both the studies, the general trends of the maps showed that *D. immitis* and *D. repens* were present only in a few municipalities/neighbourhoods, whereas *A. reconditum* was widely and homogeneously spread throughout the entire study areas, thus indicating that this filarial worm is the predominant filarial species in dogs in the Campania region of southern Italy.

The information derived from descriptive maps derived from dedicated surveys provides an operational tool for planning, monitoring and managing control programmes, and for deriving inferences about the relationship between the environment and diseases. In fact, without good descriptive maps of disease agent/vector species' distribution, based on ground observation, we do not have the essential starting point to generate predictive maps based on statistical pattern-matching (Randolph, 2000). The derivation of detailed epidemiological maps, at the relevant spatial resolution, is being increasingly recognized as vital to the effective design and implementation of successful for the control of parasites and their vectors (Sabesan et al., 2000).

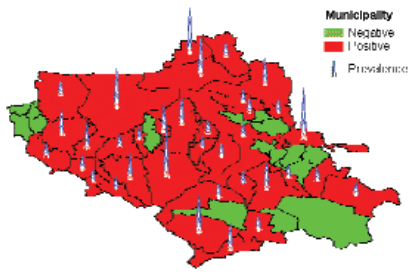

Fig. 5. Distribution map with proportioned peaks. *Acanthocheilonema* (*Dipetalonema*) *reconditum* in dogs from the Mt. Vesuvius area of southern Italy (from Cringoli et al., 2001).

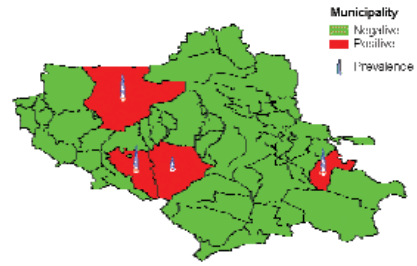

Fig. 6. Distribution map with proportioned peaks. *Dirofilaria repens* in dogs from the Mt. Vesuvius area of southern Italy (from Cringoli et al., 2001).

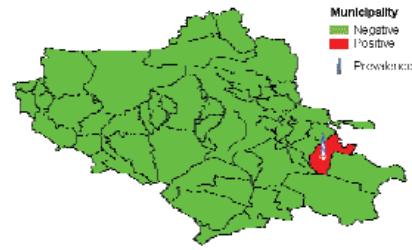

Fig. 7. Distribution map with proportioned peaks. *Dirofilaria immitis* in dogs from the Mt. Vesuvius area of southern Italy (from Cringoli et al., 2001).

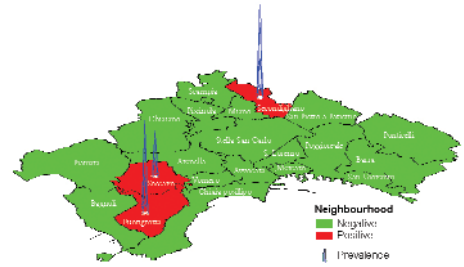

Fig. 8. Distribution map with proportioned peaks. *Acanthocheilonema* (*Dipetalonema*) *reconditum* in dogs from the city of Naples (from Rinaldi et al., 2004).

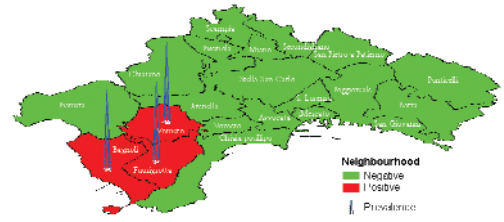

Fig. 9. Distribution map with proportioned peaks. *Dirofilaria repens* in dogs from the city of Naples (from Rinaldi et al., 2004).

Ecological analysis

Ecological analysis is targeted on the description of relations existing between the geographic distribution of diseases and environmental risk factors and their analysis by means of statistical procedures (Kistemann et al., 2002). A wide number of papers have been published on the analysis of the relationship between disease indicators (e.g. positivity, incidence and prevalence) and the explanatory environmental and climatic variables (Herbreteau et al., 2005). In order to make ecological analysis, the following fundamental steps can be utilized:

(i) GIS construction for the study area utilizing data layers on environmental and climatic features (Fig. 10);
(ii) geo-referencing the geographic units of interest;
(iii) creation of buffer zones of a given diameter centered on these geo-referenced points;
(iv) extrapolation of values for each environmental feature within each buffer zone;
(v) databases with environmental and parasitological data;
(vi) statistical analyses (univariate, multivariate, etc.) and individualize of environmental risk factors and/or development of forecast models (Cringoli et al., 2005c; Rinaldi et al., 2005, 2006).

A part from the administrative boundaries, the data layers on environmental and climatic features most commonly used for ecological analysis in veterinary epidemiology are: NDVI, land cover and land use, elevation, slope, aspect, lithofacies map, presence of lakes, rivers and other water bodies, temperatures, rainfall and humidity. The extrapolation of environmental variables can also be directly extracted from the study units instead of the buffer zones.

Ecological analysis has been previously performed by us in order to study the climatic and environmental factors that influence the distribution of the rumen fluke *C. daubneyi* (Cringoli et al., 2004) and of the protozoan *Neospora caninum* (Rinaldi et al., 2005) in southern Italy.

Environmental factors that may govern filarial worm distribution may be expected to affect the life history parameters of both the mosquito vector and the filarial species. Traditionally, attention in this area has focussed primarily on temperature, humidity and elevation (Attenborough et al., 1997; Brooker and Michael, 2000). For example, GIS functions have been recently used by Sowilem et al. (2006) to identify environmental indicators of high-risk village for human filariosis in Egypt, as indicated by mosquito density, human infection rate, vector species composition, mean life expectancy, as well as environmental variables (geology, hydrology, soil types) and meteorological factors (temperature, rainfall and humidity); in this study, the most important landscape

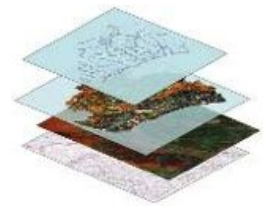

Fig. 10. Data layers used for ecological analysis.

elements associated with filarial prevalence were water and different vegetation types. In other recent study conducted in India, a range of geo-environmental variables were selected, and customized on GIS platform to develop a geo-environmental risk model for determining the areas of potential transmission of lymphatic filariosis (Sabesan et al., 2006).

Studies on ecological analysis regarding canine filariosis are very scant in literature, probably due to the difficulties in obtaining geo-referenced data.

We made an attempt to determine the environmental and climatic features that influence the distribution of *D. repens* in the Lazio region of central Italy (Scaramozzino et al., 2004). A GIS for the study area was constructed utilizing the following data layers: elevation, slope, aspect, NDVI, land cover and minimum, maximum and mean temperature average covering a 30-years period. Data on each of the above variables were then calculated for 3 km diameter buffer zones centered on the geo-referenced point of dog sampling (Fig. 11). However, statistical analysis did not show any significant association between the positivity to *D. repens* and the environmental data. This was probably due to the non-uniform spatial distribution of the sample (the data used were obtained from a meta-analysis, without a sampling design), and to the low number of positive dogs (1.9%). These latter results point out once again that the use of GIS does by no means overcome the two major concerns of any empirical research: data availability and data quality (Kistemann et al., 2002). This issue should always be kept in mind by all GIS/RS users.

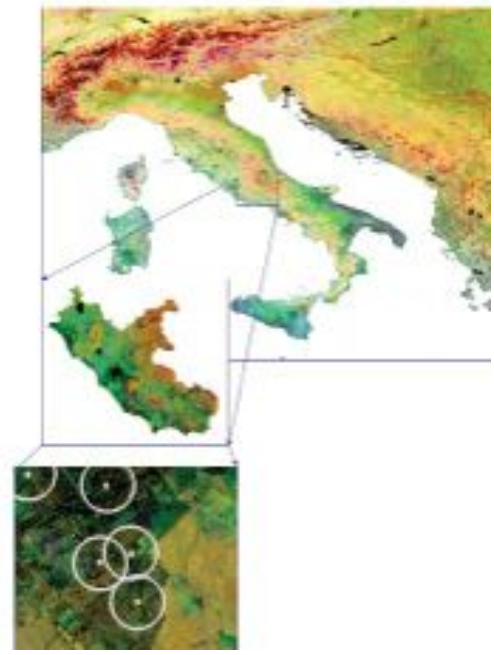

Fig. 11. NDVI map of the Lazio region obtained from Landsat-5 TM (spatial resolution = 30 x 30 m) and 3 km diameter buffer zones centered on the dog sampling points.

Predicting parasite occurrence/seasonality: an useful tool for surveillance

GIS and RS can be also used to predict disease seasonality based on the climatic and/or environmental characteristics of a certain area and the information about the climatic and/or environmental requirement(s) of a certain species. Climate-based forecast systems, employing the concept of growing degree days (GDD), have been developed for different diseases of parasitological importance, such as fasciolosis, schistosomosis, onchocerciasis and malaria (Malone, 2005; Gebre-Michael et al., 2005; Yang et al., 2006).

GIS have been also used in order to predict the occurrence and seasonality of *D. immitis* in Europe by us (Genchi et al., 2005) and in Argentina (Vezzani

and Carbajo, 2006). The heartworm (HW) disease models are based on the fact that climate dictates the seasonal occurrence of *D. immitis* transmission. The rate of maturation to infective third-stage larvae (L3) in the mosquitoes depends mainly on the environmental temperature, and there is a threshold of about 14°C below which development will not proceed (Fortin and Slocombe, 1981). The total environment heat required for development may be expressed in terms of degrees days in excess of this threshold (Heartworm Development Units – HDUs) (Fortin and Slocombe, 1981; Slocombe et al., 1989). The seasonal HW transmission model formulated by Slocombe et al. (1989) and re-evaluated by Lok and Knight (1998) in Canada and US, respectively, assumes a requirement of 130 HDUs for larvae to reach infectivity and a maximum life expectancy of 30 days for a vector mosquito. The model performed by us (Genchi et al., 2005) was applied using temperature records from 1846 European Meteorological stations (Fig. 12), spanning a 14-year period, in order to assess the theoretic transmission timing of HW in Europe. In addition, a GIS based on thermal regime was constructed in order to produce predictive maps which assess the duration of the HW transmission risk period, and the efficient timing of HW chemoprophylaxis and scheduling of diagnostic testing. The following maps were obtained from the GIS: 1) monthly maps showing the stations that reached the 130 HDUs at least once in the studied years (for example, see July in Fig. 13); 2) monthly maps showing the average predicted number of generations (for example, see July in Fig. 14); 3) a map showing the yearly average predicted number of HW generations (Fig. 15); 4) a map showing the start and end of the HW transmission period in some European localities (Fig. 16).

The results of the model showed that HW transmission is markedly seasonal in Europe, with peaks in summer, from June to September. The study provided HW risk assessment maps for Europe and suggests that if the actual trend of temperature increase continues, filarial infection should spread into previously infection-free areas (Genchi et al., 2005).

The recent models performed by Vezzani and Carbajo (2006) in order to assess spatial and seasonal *D. immitis* transmission risk throughout Argentina, showed that also in the Southern Hemisphere, HW transmission is markedly seasonal, with peaks in January and February (summer).

The *D. immitis* risk maps performed in Europe and Argentina could be used by practitioners and health authorities to efficiently direct surveillance and control efforts. From a general point of view, epidemiological surveillance employing the use of GIS is characterized by an integrated set of planned epidemiological activities whose aim is to identify and prevent new cases of disease. Such a system is justified provided the natural history of the disease and its determinants are known and effective preventive strategies are available (Rinaldi et al., 2006).

Conclusions

The trends over the past two decades and their effects in the fields of hard-

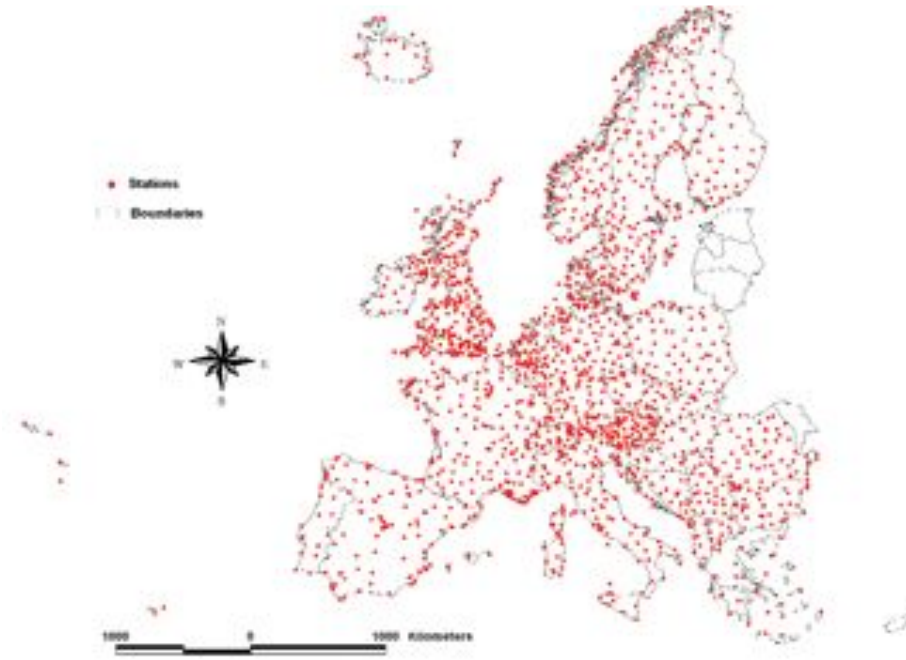

Fig. 12. European meteorological stations (n. 1846) used for predict HW transmission in Europe (from Genchi et al., 2005).

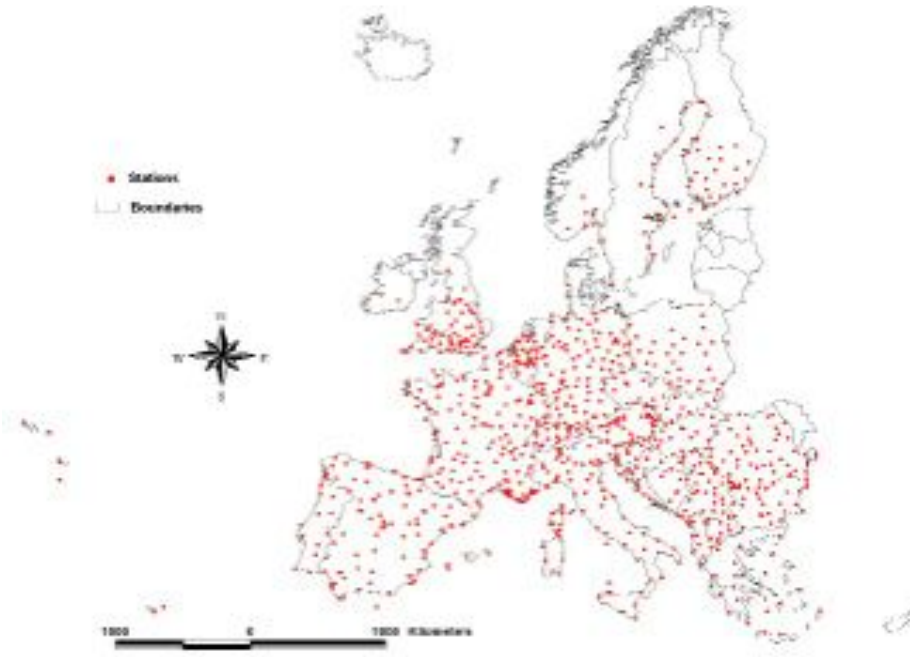

Fig. 13. HW predictive model based on growing degree days. Stations reaching the 130 HDUs at least once in the studied years in July (from Genchi et al., 2005).

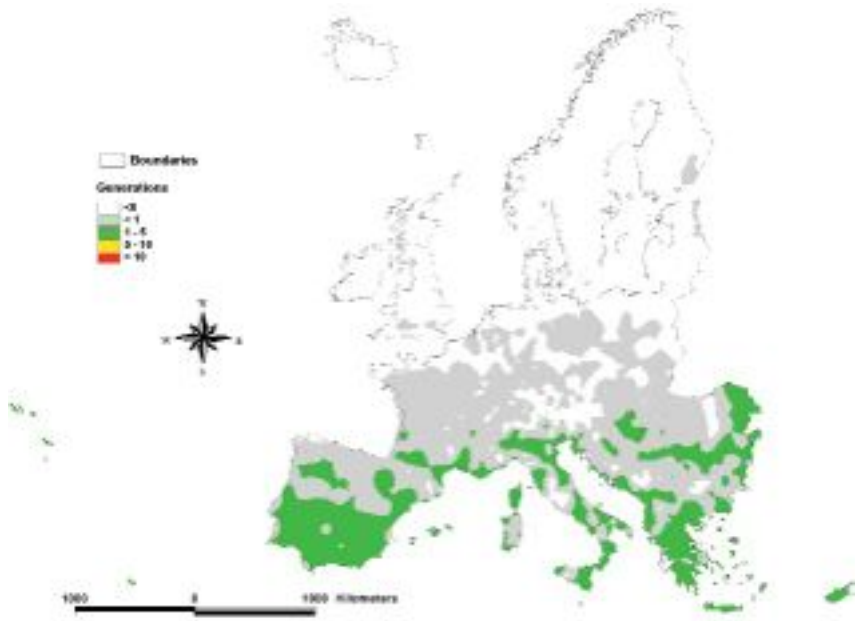

Fig. 14. HW predictive model based on growing degree days. Average predicted number of *D. immitis* generations in July (from Genchi et al., 2005).

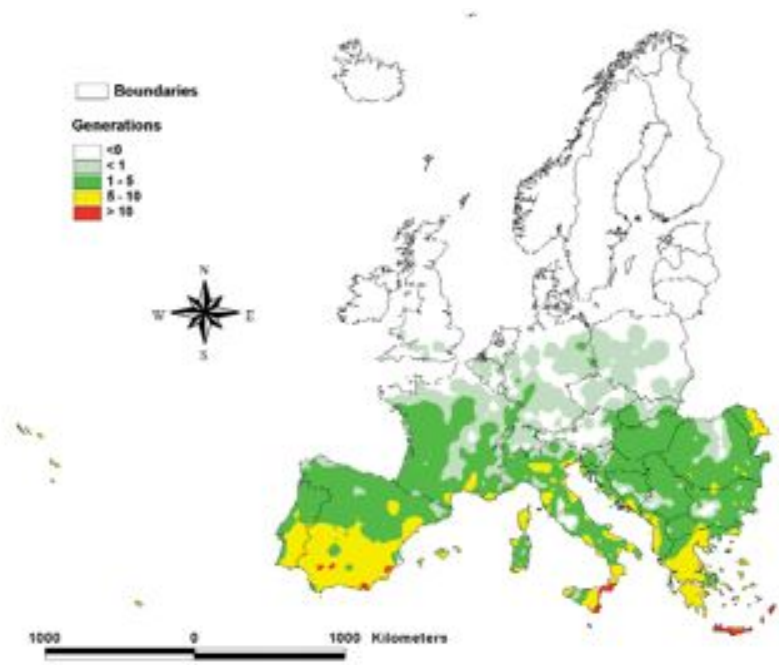

Fig. 15. HW predictive model based on growing degree days. Yearly average predicted number of *D. immitis* generation in Europe (from Genchi et al., 2005).

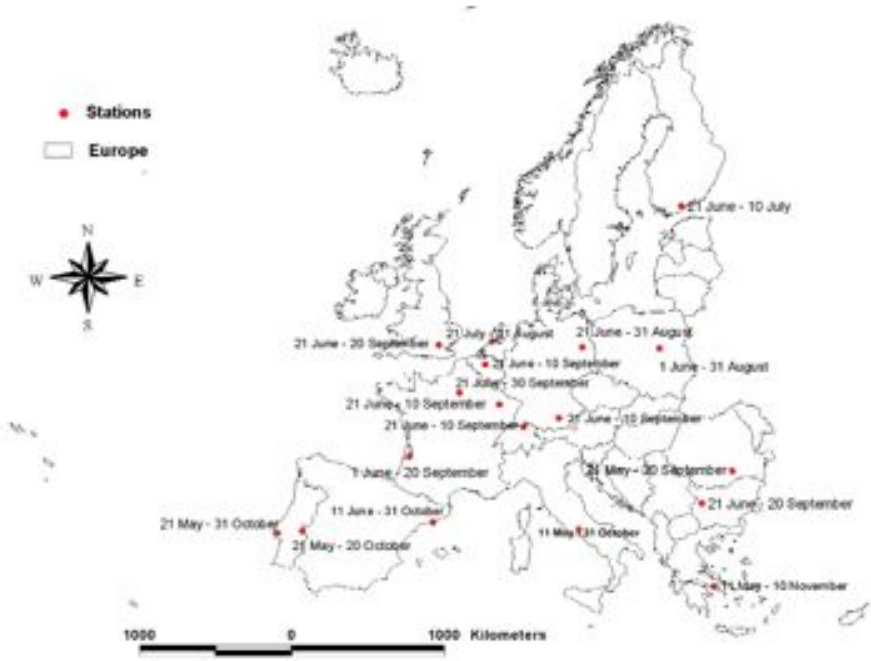

Fig. 16. HW predictive model based on growing degree days. Starting and ending HW transmission period in some European localities (from Genchi et al., 2005).

ware, software and network technology, in particular in the internet domain, have created the prerequisites for a broad acceptance of GIS and RS within the health sciences, including veterinary science.

Recent studies showed that GIS – in addition to their broad utility reviewed here – holds promise in capturing data on medicine usage (Ryan et al., 2005), as well as in organizing spatial information involving the distinction of anatomic relationships (Ganai et al., 2006). However, epidemiology remains the main application field of GIS in veterinary and public health and, since epidemiology is inextricably bound to "place", it seems reasonable to expect that GIS will further advance as science (Jacquez, 2000). Perhaps one of the most powerful benefits of GIS is its ability to integrate different databases into a single environment; GIS may be though of as a *database of databases* (Cox and Gifford, 1997). In conclusion, the present review showed that, with the appropriate data at hand, GIS can be very useful to study the epidemiological patterns of filarial infections in veterinary and public health.

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2

Biology of filarial worms parasitizing dogs and cats

Maria Teresa Manfredi, Annarita Di Cerbo, Marco Genchi

The nematodes of genus *Dirofilaria* belong to family Onchocercidae and subfamily Dirofilariinae of the order Spirurida (Anderson, 2000). They are slender nematodes and the larval stages are generally found in the definitive host distant from their site of origin. Embryos (microfilariae) develop in the uteri of the female worm and are surrounded by the egg membrane. Females are ovoviparous and lay microfilariae in the blood stream, where they become available to haematophagous vectors.

Microfilariae are able of responding to physiological changes in the host and may flood into the peripheral circulation at a certain time of the day or night and disappear from the peripheral circulation at other time. Microfilariae have structures that allow their passage through the fine blood capillaries and through the narrow mouthparts and the gut wall of the arthropod vectors.

The genus *Dirofilaria* is divided in two subgenera. The subgenus *Dirofilaria* includes *Dirofilaria immitis* and the subgenus *Nochtiella* includes *Dirofilaria repens*.

The nematodes of *Dirofilaria* subgenus are large filarial worms without cuticular ridges except in the ventral surface of the caudal extremity of the male; the caudal papillae of the males show only slight asymmetry in number and distribution.

The nematodes of the subgenus *Nochtiella* are relatively small filarial worms with cuticular longitudinal ridges; the caudal papillae of the males show a distinct asymmetry in number and distribution (Canestri Trotti et al., 1997).

Description of *Dirofilaria immitis* (Leidy, 1856) Railliet and Henry 1911

Dogs are the main hosts, however it has been reported in wolves, dingoes, coyotes, foxes, various felines (including the domestic cat), pinnipeds, mustelids (e.g. ferrets), bears, pandas, beavers, coatis, rabbits, deer, horses, non-human primates and humans.

Most of these infections are sporadic and the microfilaremia could be absent.

The distribution is worldwide and has been found in tropical, subtropical and temperate regions (Lok, 1988).

Morphology of adults

D. immitis is a long, whitish thin filiform nematode. Mouth opening is terminal without lips and leads into the oesophagus differentiated into muscular and glandular regions without distinction. The oral aperture is surrounded by 6 small median papillae and 2 lateral papillae (amphids). The adult females measure 250 to 310 mm in length and 1 to 1.3 mm in width and the males are 120-200 mm long and 0.7-0.9 mm wide.

Females have an obtuse caudal end; the anus is subterminal and the vulvar opening is located just posterior to the junction of the oesophagus and intestine. The male caudal end is spirally coiled and has 2 lateral narrow alae. The cloaca opens to 0.13 mm from the tip of the tail. The ventral side bears pre-anal, per-anal and post-anal papillae. The spicules are unequal and with different structure, enlarged at proximal end and acute at distal end. The left spicule is 300-375 µm and the right 175-299 µm; the gubernaculum is absent.

The cuticle is smooth and ridges and striations are only present on the ventral surface of the last coil of the male tail (Uni and Takada, 1986; Lichtenfels et al., 1987).

The adults of *D. immitis* feed on plasma and they can live several months or years in their hosts.

This nematode is ovoviviparous, and females release unsheathed microfilarie into the bloodstream. They are 290-330 µm long and 5-7 µm wide; the cephalic extremity is tapered and the tail is straight. The tip of the tail is pointed. The microfilarie can survive from 2 to 18 months in the bloodstream.

Biology

The microfilariae develop to L3 (infective stage) in the intermediate host, a mosquito of the genera *Culex*, *Aedes*, *Psorophora*, *Mansonia* or *Anopheles*. However, the most important intermediate hosts are those species without the bucco-paryngeal armature, which is able to damage the microfilarial cuticle preventing its development to the third larval stage.

The microfilariae ingested by mosquitoes throughout the blood meal migrate to the Malpighian tubules where they moult into second larval stage and the third larval stage. Infective L3 migrate from the tubules to the lumen of the labial sheath in the vector mouthparts. Development in the mosquito is temperature dependent, requiring approximately two weeks at temperature ≥ 26°C.

Infection is transmitted to a new host when an infected mosquito takes a further blood meal in a competent host.

The larvae are deposited by the mosquito throughout a drop of infected hemolymph in the proximity of bite wound. L3 enter the bite wound and penetrate the connective tissues, migrate from the subcutaneous or subserosal tissues to the muscles and undergo the 3rd and 4th moults. The fourth stage larva is 8.29-13.2 mm long (Lichtenfels et al., 1985). They undertake extensive migration through the subcutis, which continues for some 60-90 days until the final to L5 (immature adult). Then, the juvenile worms migrate from muscular tissues and thoracic/abdominal cavities to pulmonary arteries and right heart within a few days of their final moult, presumably carried by the venous circulation (Kume and Itagaki, 1955; Webber and Hawking, 1955; Lichtenfels et al., 1985; Anderson, 2000). The immature adults penetrate the jugular or other veins leading them directly to hearth and grow to a maximal length of 20 cm. Final maturation and mating occur in the pulmonary arteries. After copulation, the females produce unsheathed microfilariae approximately 6 and half months after infection (Table 1). Microfilariae are then released into the circulation.

Description of *Dirofilaria repens*

D. repens Railliet and Henry, 1911 is a parasite of the subcutaneous connective tissues mainly of dogs. There are reports from domestic cats, genet cats (*Genetta tigrina*), lions and foxes. Cases have been reported in humans.

This species occurs in southern and Eastern Europe, Africa and Asia.

Table 1. Development of *Dirofilaria immitis* in the mosquito and in dogs.

Days in the mosquito	Days in the dog	Stage	Length cm	Hosts	Localization
		Mf	0.030	dog	blood
1		Mf	0.030	mosquito	middle gut
5		*sausage*	0.015	mosquito	Malpighian tubule cells
10		L2	0.05	mosquito	Malpighian tubule lumen
15		L3	1	mosquito	proboscis
	1-15	L3	1.5	dog	subcutaneous tissue
	3-80	L4	3-7	dog	subcutaneous/muscle tissues
	70-150	L4-L5	4-13	dog	muscle/subcutaneous tissues
	100-160	L5	4-20	dog	thoracic/abdominal cavities/ pulmonary arteries/right heart
	150-270	gravid female/ mf	25-30	dog	pulmonary arteries/right heart

Mf: Microfilariae

Note: In cats the life cycle is about 30 days longer

Morphology of adults

Adults of *D. repens* are smaller than *D. immitis*. The cuticle is white with distinct longitudinal and tender transverse striations. Adult females are 100 to 170 mm long and 4.6 to 6.5 mm wide and males are 50-70 mm long and 3.7-04.5 mm wide. The male caudal end curves slightly toward the ventral side. The tail is obtuse, with two lateral alae and oblong pedunculate papillae. The spicules are unequal; the larger spicule is 0.43 mm long and has an acute tip; the smaller spicule is strongly chitinized, measures 0.175 mm in length and has an obtuse end. In the females, the vulva is situated 1.84 to 1.92 mm from cephalic end and it is encircled by slightly projecting labia. The tail has an obtuse tip and curve slightly toward ventral side.

D. repens is ovoviviparous and produce unsheathed microfilariae living in bloodstream. Microfilarie have the anterior end obtuse, the posterior end tin and pointed ending curved in form of an umbrella handle. The microfilarie are 300-360 µm long and 6-8 µm wide and should be differentiated by other microfilariae which can be found in the bloodstream such as *D. immitis*, *Acanthocheilonema* (syn. *Dipetalonema*) *reconditum* and *Dipetalonema dracunculoides.*

Biology

D. repens life cycle requires a mosquito of genera *Aedes*, *Anopheles*, *Culex, Armigeres* and *Mansonoides* as intermediate host. The duration of development depends on the species of intermediate host (min 10 days max 21 days at a temperature of 24 to 27°C).

Under experimental conditions larvae developed also in horseflies and sand-

flies (Coluzzi, 1964).

Microfilariae of *D. repens* ingested by a blood meal reach the stomach of mosquitoes and migrate to the body cavity within 1.5 days and settle in the Malpighian tubules where have 2 moults. After attaining the infective stage larvae migrate to the head and mouthparts of the mosquito.

In the definitive host the infective larvae were found in sections of the subcutaneous connective tissue up to 48 hours after inoculation. The larvae do not have long migrations in the subcutaneous connective and reach the maturity here (Table 2). The macrofilariae are found between the connective layers in most part of the body. They were often found on the trunk and few were found in the legs and head (Webber and Hawking, 1955). Usually, microfilarie do not show periodicity in the peripheral blood, but microfilaremia has a tendency to increase seasonally (from August to September in Italy) (Cancrini et al., 1975). The prepatent period is 27-34 weeks. Adults can live several years.

Table 2. Development of *Dirofilaria repens* in the mosquito host and in dogs.

Days in the mosquito	Days in the dog	Stage	Length cm	Hosts	Localization
		Mf	0.035	dog	blood
1		Mf	0.035	mosquito	middle gut
2-7		sausage	0.015	mosquito	Malpighian tubule cells
6-9		L2	0.05	mosquito	Malpighian tubule lumen
10-20		L3	1.00	mosquito	proboscis
	1-15	L3	1.00	dog	subcutaneous connective tissue
	3-70	L4	n.d.	dog	n.d.
	70-150	L5	n.d.	dog	n.d.
	180-240	gravid female/ mf	10-17	dog	subcutaneous tissue/ perimuscular connective fasciae

Mf: Microfilariae

Note: The duration of the life cycle is unknown

Dirofilaria immitis
Caudal end of male, ventral view

Dirofilaria immitis
Cephalic end of female, ventral view

Dirofilaria immitis
Cephalic end of female, ventral view

Dirofilaria immitis
Anterior end of female, ventral view

Dirofilaria immitis
Posterior end of female, lateral view

Dirofilaria immitis
adult female (over) and male (under)

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3

Vectors of *Dirofilaria* nematodes: biology, behaviour and host/parasite relationships

Gabriella Cancrini, Simona Gabrielli

In the last century many important aspects of the diseases due to *Dirofilaria immitis* and *D. repens* have been studied and elucidated, including pathogenesis and parasite transmission. However, there are still many unanswered questions concerning the species that can act as vector of these parasites in the world.

Experimental studies have shown that dirofilarial nematodes can develop, more or less completely, in numerous arthropods but, on the basis of current knowledge, only the insects, namely the mosquitoes (order Diptera, suborder Nematocera, family Culicidae), act as vector.

The Culicidae have a cosmopolitan distribution, with over 3500 species throughout the world. The high degree of adaptability of these insects has assured their presence in every type of environmental habitat. In fact, mosquitoes are found in low-lying plains, hilly areas, in mountainous zones and marine habitats, where each species has adapted its egg laying activity and larval development to the types of water surfaces there available. Some species lay their eggs only in fresh rain water, others in stagnant pools or in any vessel that contains water; others lay in quiet pools at the edges of streams, and some even in salt water.

In tropical areas, mosquitoes are active all year round, whereas in temperate climates adults are active during the late spring and summer, and have different seasonal rhythms of activity. Environmental and climatic changes, in particular the predicted rise of the mean temperature (+0.2°C in 10 years) as a consequence of the "global warming" (Romi, 2001; Genchi et al., 2005) are now strongly influencing the activity patterns of mosquitoes in temperate areas.

The male mosquito feeds mainly on fruit juices, whereas the female is also hematophagous. This blood-sucking habit allows to the female participate to the filariosis transmission.

Finding a host and host preference

To find resting sites or environment suitable for mating or ovoposition, or to find a source of blood host, mosquitoes move owing to phototropism and in response to chemical stimuli. They are attracted to carbon dioxide and odour produced by the host, which is then localized by the warmth and moisture radiated from its body. Following these stimuli, some mosquito species travel only a few hundred meters from their larval habitats, whereas others (several species of *Aedes*) can go as far as 50 km (Hocking, 1953). They may cover long distances carried mostly by winds (passive movement) or by flying actively in successive stages.

The host-seeking behaviour of the mosquito follows different patterns, depending on the species. Some are active only during the nocturnal hours (*Cx. pipiens* and most of the *Anopheles*) whereas others predominantly in the early morning (like *An. gambiae, An. balabacensis, An. maculipennis*), or on the daytime (*Ae. albopictus*). Species such as *Ae. aegypti, Ae. caspius, Ae. vexans* and *Cx. modestus* show two peaks of feeding activity, one at sundown and the other at dawn (Matting, 1969; Di Sacco et al., 1992; Pollono et al., 1998). These

biological rhythms are not a fixed rule but the most common behaviour of the species, often the result of co-evolution with the parasite transmitted, and therefore usually correlate to the possible major success to fed on parasitized animals. During this species-specific circadian window, other factors may modulate the expression of activity. For example, the age of the female can influence activity levels. Shortly after the emergence, there may be a brief period during which the insect is oblivious to host stimuli, and holder individuals may respond much differently than when they were younger (Nayar and Sauerman, 1973). In general, the older female is more epidemiologically important by virtue of its increased opportunity to acquire and transmit pathogens. Nutritional state also affects the intensity of activity peaks; as the period of blood deprivation lengthens, activity patterns become more intense (Klowden, 1996). When all factors contribute to the increase in activity levels, they make the female more likely to encounter host stimuli, and it may then find itself in the vicinity of a host.

Based on their preference to feed outdoors or inside dwellings, mosquito species can be divided into exophagic and endophagic, respectively. As far resting habits, species can be further classified as endophilic (remaining after feeding within human habitations for most of the gonotrophic cycle) or exophilic (spending most of their time outside). It is clear that the different behaviour patterns can influence several epidemiological factors: the availability of suitable hosts; the temperature at which the filarial larvae develop within the mosquito; the exposure to potential predators (therefore the life span of the mosquito).

As far the host preference, several species are strictly zoophilic or "specialists" (limit feeding to specific types of hosts), several are less restrictive and more largely zoophilic or "generalists" (have the ability to identify and feed on a wide variety of host types) and even anthropophilic. The last species are important for the dirofilariosis transmission from animals to humans. Host use patterns may vary seasonally between geographic regions and, as the relative abundance of different hosts, changes in an area.

Mosquito feeding preferences may depend on several factors, including the behaviour and attractiveness of the host. Actually the mosquito never seeks a host as such, but simply responds to host kairomones by orienting in their direction. These kairomones are released as discontinuous filaments or packed of stimuli, broken up by the wind as they move downwind, like the dispersion pattern of a smoke plume from a chimney. The host seeking steps may be bypassed in the laboratory experiments, but are of critical importance in nature. In fact, those individuals that feed when placed directly on a host are not necessarily those that will seek it from a distance.

Dog and cat baited traps, operated to investigate on the species that can act as vectors, showed that the dog attracts a large number of *Ae. caspius, Ae. scapularis, Ae. taeniorhynchus, Cx. pipiens, Cx. quinquefasciatus, Cx. declarator, Cx. nigripalpus, An. maculipennis* and *Cs. annulata* and, under the same field conditions, larger than does the cat (Di Sacco et al., 1992,

1994; Genchi et al., 1992; Iori et al., 1990; Pollono et al., 1994; Labarthe et al., 1998) (Fig. 1). It is likely that the dog does not represent the preferred host of all individual mosquitoes of these species (for example, *Cx. pipiens* and *Cs. annulata* were originally ornithophilic and have adapted to biting man and other mammals; *Ae. caspius*, on the other hand, is highly anthropophilic), but is however an important food source. Moreover it has been shown that *Culex* species are not particularly attracted to the cat, but other studied species are even less so (Pollono et al., 1998; Labarthe et al., 1998). The feeding activity of these mosquito species begins at sundown and lasts throughout the night. Dogs have a habit of sleeping during the nocturnal hours and their resting places offer an ideal environment for the insect (warmth, carbon dioxide). Cats, on the other hand, in natural conditions, are nocturnal hunters and move about quite a lot. It is possible that the mosquito, who needs sufficient contact time with the host in order to feed, is disturbed by the cat movement. This, along with the cat smaller body mass, may explain the insect feeding preference for dogs and the higher prevalence of infection observed in dogs compared to cats, who likely play only a marginal role as reservoir for the disease. In fact, cat infections induced by simulated natural exposure to *Ae. aegypti* experimentally infected with *D. repens* or *D. immitis* confirmed that mosquitoes do not feed willingly on the cat (Cancrini et al., 1979; Cancrini and Iori, 1981; Mansour et al., 1995).

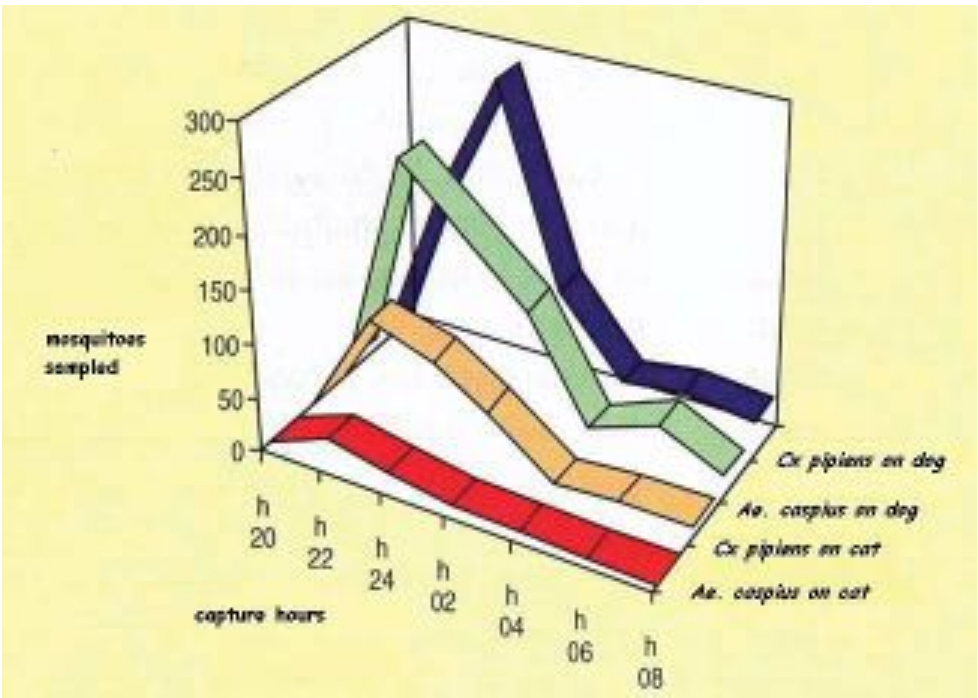

Fig. 1. Activity patterns and feeding preferences of *Cx. pipiens* and *Ae. caspius* as regards the dog and the cat, taken from bait-capture studies in Pavia province (Italy).

Development of *Dirofilaria* parasites in the invertebrate host

When the female mosquito fed on *Dirofilaria* infected animals, it takes up with the blood embryos (microfilariae) that can develop into infecting L3 only in the "competent" invertebrate host.

Moreover, the number of larvae that can successfully complete their development depends on the vector efficiency of the mosquito individual.

During the blood meal the microfilariae (270-365 µm in length and 6-8 µm wide, depending on the species) pass through the pharynx and reach the mid-gut where they remain for approximately 24 hours. They then migrate to the Malpighian tubules and invade the cells of the distal end (Fig. 2), where they undergo transformation to the so-called "sausage stage"(first stage larva or L1, about 70 µm in length), mute to the L2 stage and finally, into L3. The L3 (1,100 µm in length) leaves the Malpighian tubules by perforating their distal end, migrate through the haemocele to reach the *labium*. During the mosquito probing and blood feeding on the vertebrate host infective L3 emerges from the folded *labium* and rests on the skin of the host immersed in a drop of haemolymph to avoid dehydration. It then enters the host when the insect has pierced the skin. It is impossible to distinguish by morphology between the developing larvae of *D. immitis* and *D. repens* (Nelson, 1959; Nelson et al., 1962).

The time required for larval development in the mosquito is temperature-dependent: it takes 8-10 days at 28-30°C, 11-12 days at 24°C, and 16-20 days at 22°C. Larval development ceases at temperatures below 18°C, but it can resume if the mosquitoes are placed at higher temperatures (Cancrini et al., 1988) (Fig. 3).

The initial invasion of the Malpighian cells by L1 and the penetration of the tubule walls by the exiting L3 are both critical moments for the mosquito sur-

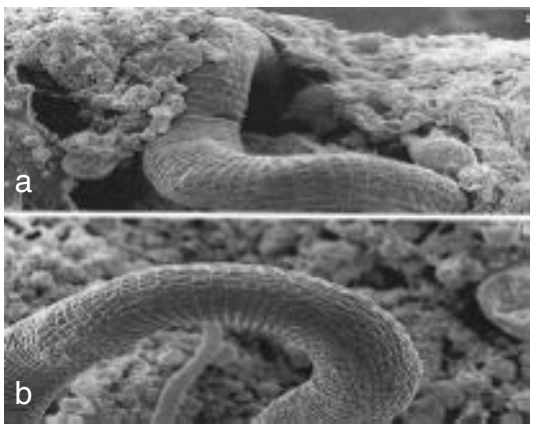

Fig. 2. Scansion electron microscopy at 2 days after experimental infection: a microfilaria of *D. repens* is penetrating in a primary cells of the Malpighian tubules with the cefalic end (a), whereas its caudal end is still free in the lumen of the tubules (b).

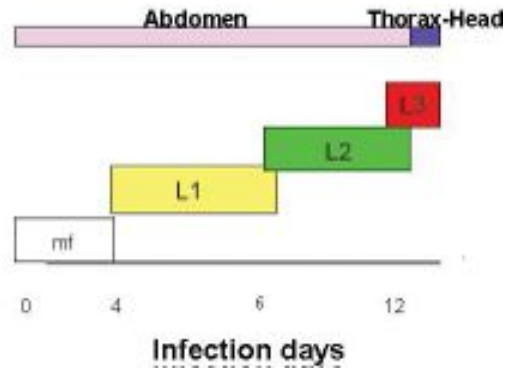

Fig. 3. Experimental infection of *Cx. pipiens* with *D. repens*: development times and location of the larvae observed.

vival. When the parasite load is heavy, tubule function is compromised and the insect dies. The maximum number of larvae compatible with the insect survival depends on the species of mosquito and the species of *Dirofilaria* and it is lower for *D. immitis* than for *D. repens* (Le Coroller, 1957; Coluzzi, 1964; Christensen, 1978; Russell and Geary, 1996).

Host-parasite relationship and vector efficiency

The percentage of microfilariae taken up with a blood meal that complete their development to infective L3 can vary from 0 to 100%. In general, the efficient vector is the female that moderates the parasite invasion and allows the development of the maximum number of infective larvae compatible with its survival. Therefore, different species or different individuals within a species can be more or less efficient vectors of the parasite, and it depends on various factors.

Parasitic burden can affect the mosquito survival, so mosquitoes have different defence mechanisms they use to block larval development and consequently to control infective larval load. The presence of the cibarial armature, for example, is an efficient mechanical tool for damaging microfilariae as they pass through the pharynx (Fig. 4). The rythmic opening-closing action of the cibarial pump, armed with sharp teeth, can provoke serious lesions to the cuticle of the microfilariae, leading to embryonic death and elimination (Coluzzi and Trabucchi, 1968) (Fig. 5). The coagulation time of the blood

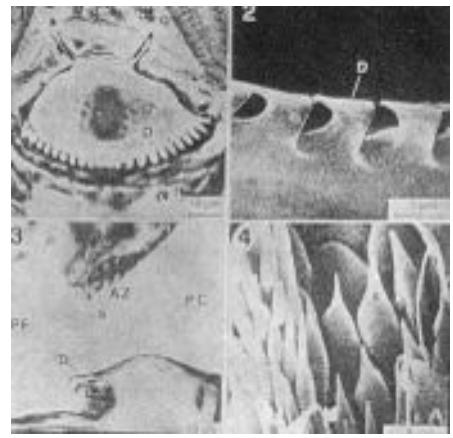

Fig. 4. Cibarial armature of *Cx. pipiens*, showing smooth teeth in a brush-like structure and pointed thorn-like projections. 1) Cross section of the pharingeal valve with teeth clearly evident (D). 2) Cibarial armature observed from the cibarial pump. 3) Longitudinal section through the area zigrinata (AZ) with thorn-like projections on top and cibarial armature, situated between the cibarial (PC) and pharingeal pumps (PF). 4) Thorn-like projections (S) in the area zigrinata (from Coluzzi et al., 1981).

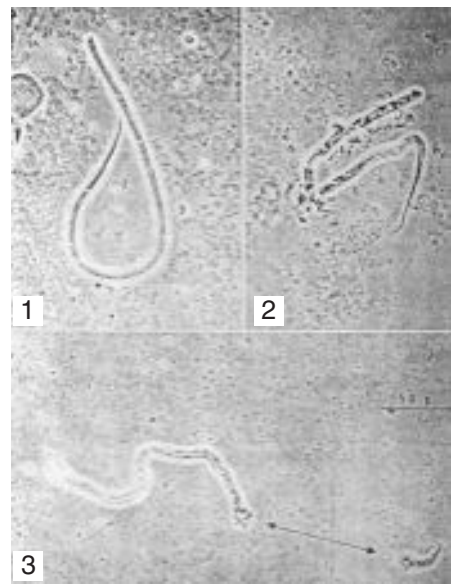

Fig. 5. *D. repens* microfilariae after ingestion by a mosquito without (1), and with (2,3) cibarial armature. In the first case, the larva is intact, whereas in the second, lesions to the cuticle are clearly evident (2), even causing a complete break in the larva's body (3) (from Coluzzi and Trabucchi, 1968).

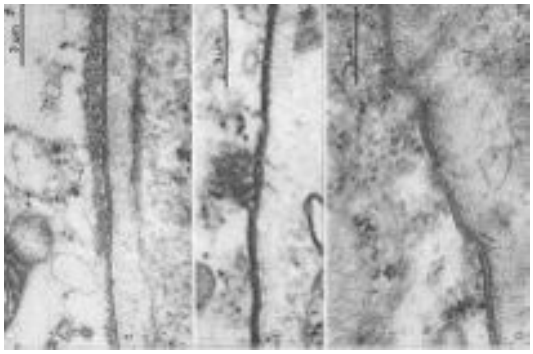

Fig. 6. Cross-section of *D. immitis* microfilaria within a primary cell of the Malpighian tubules of *Ae. aegypti*. Transmission electron microscopy at 6 days after experimental infection: the host cell (on the left of each image) has produced electron-dense material that has begun to deposit on the cuticle wall of the embryo (mf, on the right of each image), causing lysis.

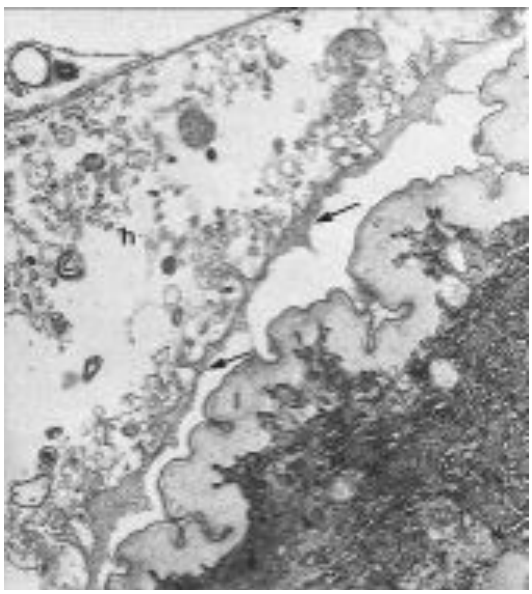

Fig. 7. Transmission electron microscopy of *D. repens* L3 (cross-section) 10 days after experimental infection of *Ae. aegypti*, situated in the haemocele. A cytoplasmic projection from a haemocyte (h), adhering to the L3 (l), has produced and deposited an electron-dense substance that has not, however, caused any damage to the cuticle.

taken up during the meal is another factor that influences vector competence. In fact, if the blood coagulates quickly, the microfilariae remain trapped in the clot, and can not reach the tubules (Frizzi and Pedrotti, 1957; Grieve et al., 1983). Therefore, mosquitoes that produce anticoagulant substances (such as *An. quadrimaculatus*) are more receptive to infection because they allow the microfilariae to more easily reach the Malpighian tubules and go on their development.

Other defence mechanisms have been observed in different mosquito species. For example, *Ae. aegypti* can secrete substances that induce epicuticle lysis (Fig. 6) and, like *Ae. scapularis* and *Ae. taeniorhynchus,* is able to encapsulate in a melanotic reaction microfilariae present in Malpighian tubules, causing microfilaria death (Kartman, 1953; Beernsten et al., 1994; Forton et al., 1985; Bradley and Nayar, 1985; Vegni-Talluri et al., 1993; Vegni-Talluri and Cancrini, 1994). On the contrary, haemocytes, are not able to cause any damage to the L3 that, having left the tubules (Fig. 7), migrate towards the head of the insect (Vegni-Talluri and Cancrini, 1991). Another defence mechanism, even if somewhat less efficient, is based upon the mosquito ability to control development times for the larvae, that may become incompatible with the insect life span (Cancrini and Iori, 1981; Cancrini et al., 1995).

The receptiveness/refractoriness to infection and the efficiency of the mosquito as vector for both *D. immitis* (Zielke, 1973; McGreevy et al., 1974) and *D. repens* (Coluzzi and Cancrini, 1974) is genetically determined and it is controlled by a sex-linked recessive

allele. The genes that control the trait are different for *D. repens* and *D. immitis*. Variations in vector competency have been identified in both natural and laboratory mosquito populations.

The importance of a mosquito in the epidemiology of dirofilarial infections not only depends upon the receptiveness to infection and on the efficiency in transmitting infective larvae, but also on the size of the vector population, its feeding pattern, life span and seasonality. In fact, in order to transmit the infection, the vector must take at least two blood meals, and in this respect autogenous species, which can use protein reserves accumulated during the larval stages to produce the first batch of eggs, are less important as vectors than those species that require several blood meals for egg laying, thus increasing the chances for larval uptake and transmission. The life span of the insect must be long enough to guarantee larval development to the infective stage and is thus influenced by the exophagic and exophilic behaviour of certain species. The seasonal activity is also important and those species that reproduce actively all year round or several times during the summer are more efficient. Ambient temperature, humidity and predation are further factors that influence the mosquito survival.

Arthropod vectors

Most of the data on the arthropod species that can act as vector derives from laboratory experiments started in the last century (Grassi and Noè, 1900). Experimental infections have shown that dirofilarial nematodes can develop in numerous insects (several mosquito species and biting flies) and that varying levels and modes of resistance to infection exist in other biting flies, simulides, phlebotomes, culicoids, fleas, and in ticks (Coluzzi, 1964).

Then, it has been demonstrated that some mosquito species are more competent than others to transmit the parasite, and that within a single species some individuals or strains are more efficient vectors than others (Coluzzi and Trabucchi, 1968; McGreevy et al., 1974; Bemrick and Moorehouse, 1968). Capture studies carried out in endemic areas to identify natural vectors have suggested a possible role of some species of Culicidae on the basis of their abundance. However, crucial progress in knowledge on the species actually involved in the parasite transmission has been made through the fieldwork based on animal-baited traps. This method allows to restrict the interest only to species that are effectively attracted to the host, and to evaluate the biological factors that affect the actual dirofilaria infection risk. These capture studies on host animals (dog, cat, and/or man) have been carried out in the United States of America, Italy and Brasil, and have identified as possible vectors *Cx. erraticus, Cx. modestus, Cx. nigripalpis, Cx. pipiens, Cx. quinquefasciatus, Ae. canadensis, Ae. caspius, Ae. excrucians, Ae. scapularis, Ae. sierrensis, Ae. sollicitans, Ae. stimulans, Ae. taeniorhynchus, Ae. trivittatus, Ae. vexans* and *An. maculipennis* s.l. (Sauerman, 1985; Favia et al., 1996). Further species, like *Ae. cantans, Ae. cinereus, Ae. geniculatus, An. claviger, Cq. richiardii, Cx. declarator, Cx. pipiens-restuans, Cx. sultanensis,*

Cx. territans and *Cs. annulata* feed less willingly on dogs and cats, and therefore could be of minor interest in the parasite transmission (Di Sacco et al., 1992; Genchi et al., 1992).

Several studies have reported the mosquito species that, by microscopy, have been found in nature infected with dirofilarial larvae but, unfortunately, it is not possible to morphologically distinguish developing larvae belonging to the 27 recognized species of *Dirofilaria*. First unambiguous results have been achieved through the analysis of the collected insects by molecular techniques recently adjusted (Favia et al., 1996), which allows to reliably distinguish *D. immitis* and *D. repens* larval stages developing in the invertebrate host (Cancrini and Kramer, 2001). On this basis, entomological investigations to date carried out in Italy and performed using PCR-based technologies have shown that *Ae. albopictus, Cx. pipiens* and *An. maculipennis* s.l. proved natural vectors for both *D. immitis* and *D. repens,* whereas *Cq. richiardii* is almost certainty vector for *D. immitis.* Moreover, abdomens of *Cx. modestus, Cx. torrentium, Ae. punctor, Ae. cinereus, Ae. detritus,* and *Ae. geniculatus* have been found positive to *D. immitis.* Those species could act as vectors, but their role needs further confirmations, being the abdomen a location common to either just ingested or infectious larvae (Rossi et al., 2002; Cancrini et al., 2003a,b, 2004, 2006). A more extensive application of the molecular techniques will allow a clearer and more comprehensive understanding of the epidemiology of subcutaneous dirofilariosis and of feline and canine heartworm infection, in particular the seasonal transmission patterns in the different geographical areas and the monitoring of infection rates among different vectors.

These first results have been obtained by studying several dirofilariosis areas in northern and central Italy. They are of concern because the main natural vector (to date *Ae. albopictus)* is a species proven very efficient in the parasite transmission, "generalist" in the host choice and highly anthropophilic.

The presence now stable of this mosquito in Italy suggests that the infection risk for animals and humans is increased, at least because of the simultaneous presence of vectors showing diurnal and nocturnal activity patterns.

The same concern could be addressed to Croatia. In fact, although to the best of our knowledge no data are available for dirofilariosis natural vectors in the Country, the existence of a culicidofauna almost overlapping to that present in Italy (Ramsdale et al., 2001; Ramsdale and Show, 2006; Merdic and Boca, 2004) suggests the possible importance of the same species.

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4

Pathogenesis of *Dirofilaria* spp. infections

Giulio Grandi, Tatjana Živičnjak, Relja Beck

Introduction

Dirofilaria spp., helminths belonging to Dirofilariinae superfamily, are able to cause a wide range of diseases. The best known and studied is heartworm disease, common in dogs and cats in areas where *Dirofilaria immitis* is widespread. Other species of *Dirofilaria*, like *D. repens*, are less important from a pathogenic point of view, but their differentiation from *D. immitis* is a fundamental diagnostic task. The zoonosic risks related to each species is also extremely important.

Pathogenesis of heartworm disease (*D. immitis*)

D. immitis infection is characterised by several different clinical pictures, caused both by adults and first stage larvae (microfilariae). However, the pathophysiology of heartworm disease is mainly due to the presence of adult worms in the pulmonary arteries. The primary lesions occur in the pulmonary arteries and lung parenchyma and are mostly attributable to the intravascular adult parasites. They cause pulmonary hypertension that, if not treated, progresses inevitably to congestive heart failure. Other syndromes are related to the disturbance of blood flow due to the location of heartworms in the right atrium at the level of tricuspidal valve.

This event causes massive haemolysis and related haemoglobinuria, responsible for the vena caval syndrome (liver failure disease) (Ishihara et al., 1978; Kitagawa et al., 1986). Microfilariae play a relatively minor pathogenic role but may cause clinically significant pneumonitis and glomerulonephritis.

Some individuals develop a hypersensitivity to microfilariae. Occasionally, aberrant migration results in parasites becoming trapped in ectopic locations like the anterior chamber of the eye (Weiner et al., 1980) or systemic arteries (Liu et al., 1966; Slonka et al., 1977). Data regarding immunopathogenesis and in particular the role of cytokines, mediators as well as cellular components of the immunity in the development of heartworm-related lesions have been recently revised (Grandi et al., 2005). Although the spectrum of pathologies related to heartworm infection is broad, the most important clinical manifestation in dogs is congestive heart failure (cor pulmonale).

Congestive heart failure (CHF, syn.: cor pulmonale)

Heartworms are primary agents of vascular disease, rather than the cause of simple obstruction and/or blood flow disturbances. Intimal proliferation occurs in arteries occupied by living worms and embolic worm fragments trigger thrombosis, both of which may completely obstruct segments of the pulmonary arteries (Adcock, 1961). These effects, both leading to pulmonary hypertension, are strongly correlated to worm burden, in turn related to the degree of their distribution through lung parenchyma (Knight, 1980).

The juvenile adult heartworms are only about 2.5 cm long when they reach the systemic venous circulation. They passively embolize the pulmonary arteries and are disbursed in propor-

tion to the lobar blood flow. Generally, the larger right caudal lobar artery accumulates more worms than the left (Atwell and Rezakhani, 1986). Contact between the parasite and the intima of the pulmonary arteries is an important, if not essential, initial step in the development of the endovascular lesions.

The earliest lesions are limited to the small peripheral branches where the worms first come to rest. As the parasite grows, lesions occur in more proximal segments. Intimal thickening and narrowing of the vessel lumen in small peripheral branches of the pulmonary arteries are the major cause of obstructed blood flow and pulmonary hypertension. The intimal proliferation is caused by migration of medial smooth muscle cells through the internal elastic laminae (Munnel et al., 1980; Patterson and Luginbuhl, 1963; Schaub et al., 1981).

The pathogenesis of the arteritis caused by heartworms remains a matter of speculation. Disruption of endothelial cell junction and denuding of the intimal surface are characteristics of the first lesions that occur only after a few days of presence of worms. Physical trauma, metabolic and immune-mediated cytotoxicity induced by the parasite (Keith et al., 1983a), as well as other less likely mechanisms (Schaub and Rawlings, 1980) have been considered. Several facts seem to be more consistent with mechanical trauma as the initiating event rather than cytotoxicity or induction of host immune responses. The evidence suggests that injury to the endothelium occurs immediately upon arrival of the parasite, too soon for the components of an immune response to fall in place without prior sensitisation.

Furthermore, the disappearance of endothelial cells occurs without evidence of degeneration and is followed by an aggressive build-up of cells and structural elements. This suggests that cells have been dislodged rather than destroyed *in situ*. Macrophages, granulocytes, and platelets are attracted to the site of endothelial damage and adhere to the exposed subendothelium. Shortly after their arrival, vascular smooth muscle cells migrate into the intima and a very active process of myointimal proliferation produces rapid growth of the lesions. The integrity of the endothelium covering the intraluminal villous-rugose growths is restored and thrombosis is not a feature at this stage of the disease (Munnel et al., 1980; Schaub et al., 1981). The prominence of platelets in the acute lesions and their documented ability to stimulate growth of the vascular smooth muscle, through the release of platelet-derived growth factors (PDGFs) (Ross, 1986), are hypothesized to be a likely mechanism for triggering and sustaining the growth of these lesions (Schaub and Rawlings, 1980; Schaub et al., 1981). The circumferential lesions of the peripheral distributing arteries transcend into more discontinuous rugose ridges in the larger elastic branches (Keith et al., 1983b).

In cross section, these ridges have a villous appearance and are considered pathognomonic for heartworm infection. Although the lesions thicken the wall of these large elastic vessels and produce a rough texture on the intimal surface, they do not obstruct blood flow by narrowing the lumen. On the contrary, the large distributing arteries actually dilate as pulmonary hypertension becomes increasingly severe.

Pulmonary blood flow is impeded primarily by the reduction in cross-sectional area of the arterial vascular bed, caused by obliterative endarteritis of small peripheral branches. Recently, in heartworm infected dogs it has been demonstrated that there are markedly increased plasma level of endothelin-1, a mediator that induces acute vasoconstriction and chronic vascular remodelling. It is probable that both these events contribute in turn to the development of pulmonary hypertension (Uchide and Saida, 2005). Thrombosis and thromboembolism may further compromise the pulmonary circulation.

As worms accumulate, lesions also develop in the large distributing arteries, which dilate and become stiffer as pulmonary blood pressure rises. The decreased distension of the large vessels significantly increases cardiac work by coupling the right ventricle directly to the high vascular resistance in the obstructed peripheral vasculature.

Right ventricular hypertrophy is a compensatory response to the increased pressure load. As heartworm infection impedes flow in an increasing number of branches, the pulmonary vascular reserve diminishes. For a time, normal pulmonary blood pressure is preserved at rest and rises only modestly during exercise as patent arteries reach full distension. Eventually, the pulmonary arterial tree is restricted to the point that it assumes the characteristics of a system of rigid tubes and pulmonary vascular resistance becomes fixed. This occurs about the time pulmonary blood pressure becomes elevated at rest (Knight, 1977). At this stage, pressure rises in direct proportion to further increase in flow. Consequently, the more severe the disease and active the patient, the more cardiac work must be performed. In advanced cases of heartworm disease, low-output congestive heart failure develops as a result of the right ventricle's inability to generate and sustain the high perfusion pressures required to move blood through the lung. At a molecular level, in the myocardium of heartworm infected dogs it has been observed a decrease of extracellular collagen matrix; this event can contribute to the dilatation of the ventricle, thereby markedly affecting the systolic and diastolic functions of the heart (Wang et al., 2005). Frequently, dogs at this stage experience syncope when attempting to suddenly increase cardiac output. Right-sided congestive heart failure (R-CHF) with ascites, hepatomegaly, and cachexia is a late sequela and may be precipitated by an acute episode of pulmonary thromboembolism.

D. immitis infection in the cat

The general pulmonary pathology in the cat is similar to the one observed in dog. Muscular hypertrophy, villous endarteritis, and cellular infiltrates of the adventitia are typically more severe in the caudal pulmonary arteries (Dillon, 1998). A characteristic feature of the reaction in felines in response to heartworm infection is the development of severe muscular hypertrophy in the smaller arteries (McCall et al., 1994). The host's response to the parasite is intense and the role of inflammation is very important, being platelet factors release less important in the cat rather

than in the dog (Furlanello et al., 1998). The cause of the acute and often fatal crisis in the cat is lung injury resulting in respiratory distress. Frequently this event is associated with the death of as little as one adult heartworm. The lung can become acutely edematous and respiratory failure, instead of heart failure, becomes the life threatening event. Being pulmonary hypertension an occasional event, right sided heart failure and severe cor pulmonale is uncommon in feline heartworm infection (Dillon, 1998; Genchi et al., 1995).

D. repens infection

Much less is known about the pathogenesis of *D. repens* infection, which usually is characterized by the occurrence of painless subcutaneous nodules in which adult parasites reside (Bredal et al., 1998). The localization of adult worms can vary, and recently it has been reported also the presence of a female of *D. repens* in the conjunctiva of a dog (Hermosilla et al., 2006). This asymptomatic pattern of infection, which seems to be the most common, transforms the discovery of *D. repens* infection in an accidental finding. This occurs mainly in dogs (and cats) that are showing other symptoms or diseases, not related to the presence of these nematodes in the subcutaneous tissues. Indeed, the majority of infected animals do not present any clinical sign, notwithstanding the occurrence of persistent microfilaremia, therefore making it difficult to assess the pathogenicity of *D. repens* (Soulsby, 1982).

Some authors classified the rare clinical manifestations observed in association with the presence of *D. repens* into two clinical syndromes (Scarzi, 1995). The first one is characterized by a nodular multifocal dermatitis, mainly localized in facial region (Scott and Vaughn, 1987), the second instead is characterized by the presence of several pruriginous papulae; this one is considered similar to sarcoptic mange (Halliwell and Gorman, 1989), although the clinical experience of one of us (Tatjana Živičnjak) suggests that pruritus rarely occurs in *D. repens* microfilaremic dogs. The most frequent reports, however, are of dermatological signs (generalized dermatitis, localized alopecia, scratching and rubbing) associated with the presence of adults and microfilariae in the skin (Lee Gross et al., 1992; Kamalu, 1986; Mandelli and Mantovani, 1966; Scarzi, 1995; Živičnjak et al., 2006).

It has been suggested that the pathology may be significant in cases of massive infection (Kamalu, 1991; Mandelli and Mantovani, 1966; Mantovani, 1965; Restani et al., 1962). In the few cases found to be massively infected with adult worms and with simultaneously high microfilaremia, gross and histopathological changes have been reported in many organs, like spleen, liver, kidneys, lungs, heart and brain (Kamalu, 1991; Mandelli and Mantovani, 1966, Restani et al., 1962).

The nature of these lesions suggests combined mechanical and immunopathological effects elicited by both micro- and macro-filariae (Mantovani, 1965), also if it is not possible to determine what underlying mechanisms may be involved, also due to the lack of experimental infection with reproduction of these clinical signs. Nothing is

currently known about the potential ability of microfilariae to cause hypersensitivity and inflammatory reactions at the cutaneous level, although this is likely one of the major mechanisms involved. Furthermore, it has been suggested that *D. repens*-related dermatitis is a conditioned pathology, that requires the presence of other infectious agents or stress (Bonvicini, 1910; Beaufils and Martin-Granel, 1987; Cazelles and Montagner, 1995). Single case reports of acute liver failure in the cat (Schwan et al., 2000) and cutaneous hyperpigmentation in a dog have been attributed to *D. repens* infection (Kamalu, 1991).

Conclusions

The pathogenesis of *D. immitis* is well known, while that of *D. repens* is still far from being understood, also if in the last years this parasite has been suspected to be not as harmless as usually considered. However, both parasites are important agents of zoonosis, in particular *D. repens*, and veterinarians play an essential role in protecting humans from infection. For this reason, it is essential that clinical signs are recognized, correct diagnosis is made and efficient treatment and prophylaxis are guaranteed not only to safeguard the health and well-being of our pets, but also that of our clients, neighbours and ourselves.

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5 Immunopathogenesis of filarial infections in dogs and cats: a role for *Wolbachia* endosymbiont?

Laura H. Kramer

Introduction

Dirofilaria immitis is the causative agent of canine and feline heartworm disease. Adult worms live in the pulmonary arteries, females produce first-stage larvae ("microfilariae") which are taken up by mosquitoes who then in turn transmit the infection to other animals. In dogs, the infection is chronic and leads to congestive heart failure. *D. immitis*, like most filarial worms studied so far, harbour bacteria called *Wolbachia* which are thought to play an essential role in the biology and reproductive functions of their filarial hosts. *Wolbachia pipientis*, the only species thus far identified in the genus, are gram-negative bacteria belonging to the order Rickettsiales (just like *Ehrlichia* spp and *Anaplasma* spp; Bandi et al., 2001). In adult *D. immitis*, *Wolbachia* is predominantly found throughout cells in the hypodermis, which is directly under the worm's cuticle. In females, *Wolbachia* is also present in the ovaries, oocytes and developing embryonic stages within the uteri. This suggests that the bacterium is vertically transmitted through the cytoplasm of the egg.

What does *Wolbachia* do in its filarial host?

There are several reasons why it is thought that the presence of *Wolbachia* is essential for a filarial worm's survival:

(i) in those species of filarial worms that have been identified as harbouring *Wolbachia*, all of the individuals are infected: i.e. 100% prevalence;

(ii) the evolution of the bacteria match that of the filarial worms, and phylogenic studies have shown that the two organisms have been "walking hand-in-hand" for millions of years;

(iii) the bacteria are transmitted from female to off-spring and in this way *Wolbachia* increases its own fitness by increasing the fitness of the host that is involved in its transmission;

(iv) removal of *Wolbachia* (antibiotics/radiation) leads to sterility of female worms and eventual death of adults.

It is still unclear however exactly what *Wolbachia* does to make it so important for its filarial host. It has been suggested that, while the filarial worm likely supplies the bacteria with amino acids necessary for growth and replication, *Wolbachia* on the other hand may produce several important molecules that are essential for heartworms, like glutathione and haeme (Foster et al., 2005). It is indeed a "one hand washing the other" situation that may, however, be the key to novel strategies for the control/treatment of filarial infection, including canine and feline heartworm disease.

The role of *Wolbachia* in the inflammatory and immune response in *D. immitis*-infected animals

As a gram-negative bacteria, *Wolbachia* has the potential to play an important role in the pathogenesis and immune response to filarial infection. The possible consequences of the massive release of *Wolbachia* in the filarial-

infected host has been evaluated.

Wolbachia are released both by living worms and following worm death through natural attrition, microfilarial turnover and pharmacological intervention (Taylor et al., 2001). In human and murine models of infection, the release of bacteria has been shown to be associated with the up-regulation of pro-inflammatory cytokines, neutrophil recruitment and an increase in specific immunoglobulins. Ongoing studies in dogs with heartworm disease may shed light on what happens when infected animals come into contact with the bacteria. For example, we recently tested the hypothesis that *D. immitis*-infected dogs come into contact with *Wolbachia* either through microfilarial turnover or natural death of adult worms (Kramer et al., 2005). In our study, positive staining for *Wolbachia* was observed in various tissues from dogs who had died from natural heartworm disease. Bacteria were observed in the lungs and particularly in organs like the kidney and liver, where microfilariae normally circulate (Fig. 1). Furthermore, when we looked at specific antibody responses to *Wolbachia*, we observed a stronger response in dogs with circulating microfilariae compared to dogs with occult infection, supporting the hypothesis that microfilarial turnover is an important source of *Wolbachia* in dogs with heartworm disease. Furthermore, *Wolbachia* from *D. immitis* has been shown to provoke chemiokinesis and pro-inflammatory cytokine production in canine neutrophils (Bazzocchi et al., 2003, 2005). Cats with heartworm disease also produce antibodies to *Wolbachia*. Interestingly, it has recently been reported that the development of a strong antibody response against *D. immitis* occurs after one-two months of infection and this may have important implications for early diagnosis (Morchon et al., 2004).

Areas of future research should include the possible diagnostic use of specific immune responses to

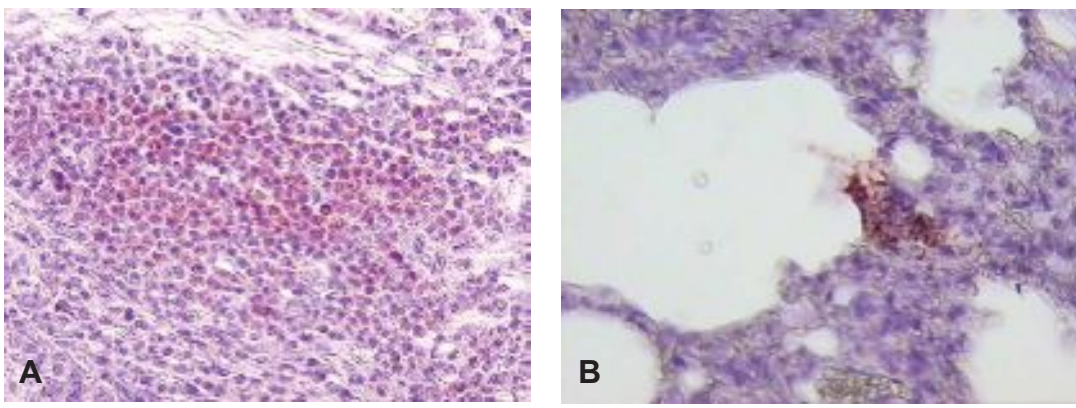

Fig. 1. Lung tissue from a *D. immitis* infected dog. A: The Wolbachia Surface Protein (WSP) is present within many neutrophils surrounding a pulmonary artery (ABC-HRP, X40). B: Free bacteria are also present in tissue (ABC-HRP, X100).

Wolabchia, it's potential immunomodulatory activity (prevention) and the effects of antibiotic treatment in infected animals.

What are the effects of antibiotic treatment on filarial worms?

Wolbachia can be eliminated from filarial worms through antibiotic therapy of the infected host. Numerous studies have shown that various treatment protocols/dosages (tetracycline and synthetic derivatives appear to be the most effective), are able to drastically reduce if not completely remove the endosymbiont from the worm host. Such depletion of *Wolbachia* is then followed by clear anti-filarial effects, including:

(i) inhibition of larval development: it has been shown that antibiotic treatment of filarial-infected hosts can inhibit moulting, an essential process in the maturation of worms from larvae to adult;

(ii) female worm sterility: antibiotic treatment leads first to a reduction and then to the complete and sustained absence of microfilariae. Researchers at the University of Milan, Italy have reported that *D. immitis* adults taken from naturally-infected dogs that had been treated with 20 mg kg^{-1} day^{-1} of doxycycline for 30 days showed morphlogical alterations of uterine content with a dramatic decrease in the number of pretzels and stretched microfilariae, indicating that bacteriostatic antibiotic treatment was able to block embryogenesis (Genchi et al., 1998; Bandi et al., 1999);

(iii) adulticide effects: this a particularly intriguing aspect of antibiotic treatment of the filarial worm-infected host and one that merits strict attention.

Recently, clinical trials in human filariasis have reported extremely promising results: a recent placebo-controlled trial in humans infected with *Wuchereria bancrofti* has demonstrated a clear macrofilaricidal effect of doxycycline (Taylor et al., 2005). When administered for 8 weeks at 200 mg/day, the treatment resulted in a complete amicrofilaremia in 28/32 patients assessed and a lack of scrotal worm nests (where adult worms reside) at 14 months post-treatment, as determined by ultrasonography in 21/27 patients. In the other patients, the number of worm nests declined. This was significantly different from placebo patients, where lack of worm nests was only observed in 3/27. This is the first report of adulticide activity in a human filarial worm with antibiotics. Could antibiotic treatment have the same effect on *D. immitis*? Research is underway to answer this very important question.

What are the effects of antibiotic treatment on the filarial worm-infected host?

It is very likely that antibiotic treatment will have some beneficial effects on subjects with filariasis. First of all, those effects described above on the worm will themselves lead to improved

clinical presentation: for example, a reduction in circulating microfilariae. However, if we consider *Wolbachia* as a potential cause of inflammation in the course of filarial disease, depletion of the bacteria may be beneficial, independently of its effect on the worm. There is little data concerning the effects of antibiotic treatment in dogs with natural heartworm disease. We know however that such treatment drastically reduces *Wolbachia* loads in *D. immitis*. Preliminary trials in naturally infected dogs have shown that doxycycline treatment before adulticide therapy with melarsomine may help reduce pro-inflammatory reactions due to the death of adult worms (as seen by lower antibody levels against *Wolbachia* and lower levels of interleukin 8, an inflammatory cytokine involved in nuetrophil recruitment). These results have encouraged us to continue evaluation of the clinical benefits of antibiotic treatment in naturally infected dogs.

Given the recent and very promising developments in the use of tetracyclines for micro-macrofilaricidal therapy in human filariosis, it is hoped that similar attention will be given to canine and feline heartworm disease that could greatly benefit from alternative therapeutic strategies.

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6

A possible role for *Wolbachia* in the diagnosis of *Dirofilaria* infections

Fernando Simón, Laura H. Kramer, Rodrigo Morchón, Claudio Genchi

Introduction

Dirofilaria immitis is the aetiological agent of cardiopulmonary dirofilariosis in dogs and cats, and pulmonary dirofilariosis in man, while *Dirofilaria* (*Nochtiella*) *repens* causes subcutaneous dirofilariosis in animal reservoirs and subcutaneous/ocular dirofilariosis in man. Like other filarial species, *D. immitis* and *D. repens,* harbour intracellular bacteria of the genus *Wolbachia* (Rickettsiales), whose participation in the development of the inflammatory pathology of dirofilariosis has been extensively investigated. Nevertheless, the role of *Wolbachia* as a possible diagnostic tool has been poorly studied until now.

Here we present our experience related to *Wolbachia* in *Dirofilaria* spp. canine, feline and human infections, as well as in a murine model of immunization, and its possible importance for the diagnosis of *Dirofilaria* infection in humans.

Wolbachia interacts with *Dirofilaria*-infected hosts

There is direct and indirect evidence that *Wolbachia* comes into contact with hosts infected with *D. immitis* and *D. repens*. Direct evidence has been obtained in dogs and humans by immunohistochemistry techniques, while indirect evidence is based on the presence of specific antibodies and inflammatory mediators observed in dogs, cats and humans, as well as in mice immunized with *Dirofilaria* spp. or *Wolbachia*-derived antigens.

Mice: The first evidence of interaction between *Wolbachia* and the host immune system was obtained in mice immunized with crude *D. immitis* adult somatic and L3 antigens. These mice develop specific antibodies against the *Wolbachia* surface protein (WSP) between 25 and 90 days post-inoculation (Marcos-Atxutegi et al., 2003). In a different experiment, mice immunized with WSP and/or a heat shock protein (GroEl) from *Wolbachia* showed a strong specific-antibody response, demonstrating the contact of *Wolbachia* molecules with murine immune cells, and an intense expression of different cytokines and innate inflammatory mediators a few days after inoculation (data not published).

Dogs: Both microfilaremic and amicrofilaremic naturally-infected dogs developed a high production of IgG antibodies against WSP. Microfilaremic dogs showed significantly higher IgG antidody levels than amicrofilaremic dogs (Kramer et al., 2005a). Eighteen out of 128 dogs living in an endemic area were positive to a validated, commercial kit for circulating antigens. Among these, 15 were also positive for IgG anti-WSP. Moreover, other 43 dogs that were negative for circulating antigens showed elevated levels of IgG anti-WSP antibodies (data not published).

Evidence of the presence of *Wolbachia* itself or its molecules in *Dirofilaria*-infected hosts was obtained by an inmunohistochemistry using a polyclonal antiserum against the *Wolbachia* surface protein (WSP). Positive staining for WSP (Fig. 1a and 1b) was obtained in kidney tubules, glomerules, lung tissue (free bacteria) and alveolar macrophages and hepatic monocytes of naturally infected dogs

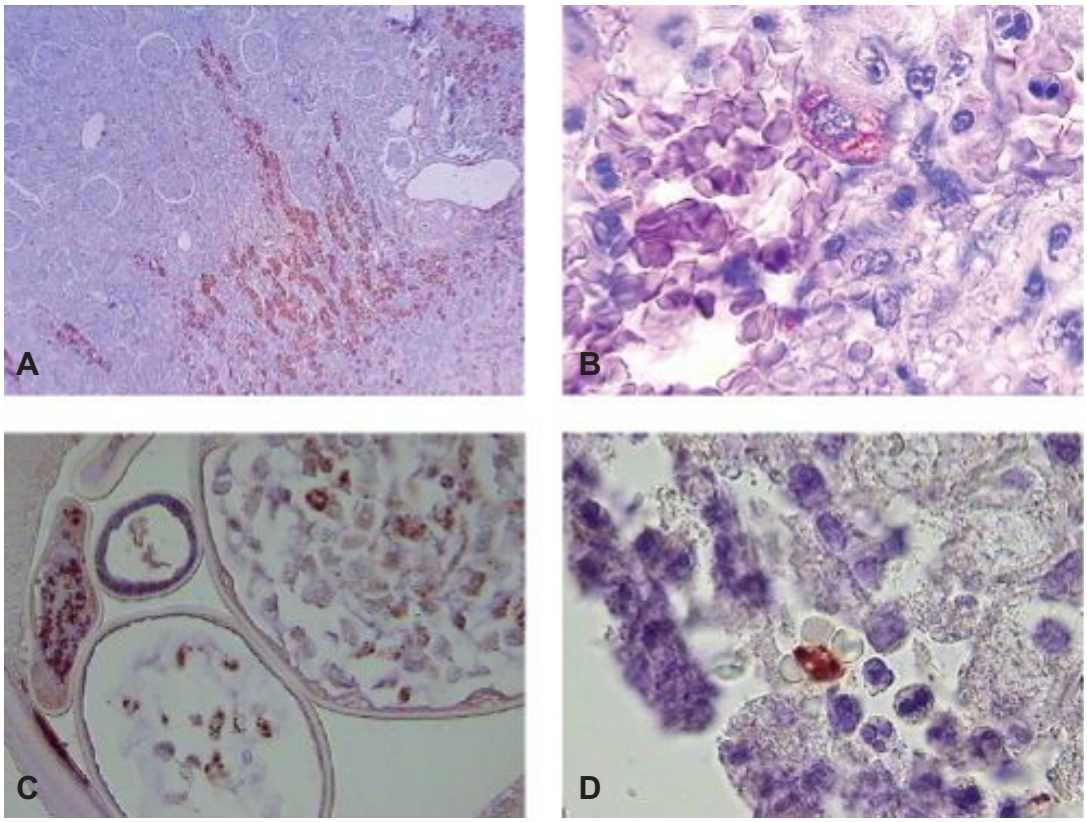

Fig.1. Immunohistochemistry (ABC-HRP method) for the localization of Wolbachia surface protein (WSP) in dogs and humans naturally infected with *Dirofilaria immitis*. A. Kidney tubule. B. Alveolar macrophage (dogs). C. Hypodermic chord. D. Morulae surrounded by immune cells (worms in subcutaneous nodules from humans).

(Kramer et al., 2005a,b).

Cats: Specific anti-WSP IgG antibodies were detected in experimentally infected cats and in cats naturally exposed to a high risk of heartworm infection. Nine out of 49 cats living in an endemic area of the Canary Islands (Spain) were positive to a validated, commercial antibody kit and to an experimental ELISA for the detection of antibodies against synthetic peptides derived from molecules of 22 and 30 kDa of the adult worms. Among these cats, 5 were positive to IgG-ELISA against WSP. Other 8 cats were positive to anti-WSP IgG but negative to the ELISA test for anti-WSP antibodies (Morchón et al., 2004).

Humans: Specific IgG antibodies against WSP were identified in patients diagnosed as having clinical dirofilariosis (Fig. 2). The level of antibody response was significantly higher in patients with pulmonary or subcutaneous dirofilariosis than in clinically healthy individuals living in endemic areas of canine dirofilariosis; among these only a part developed anti-WSP

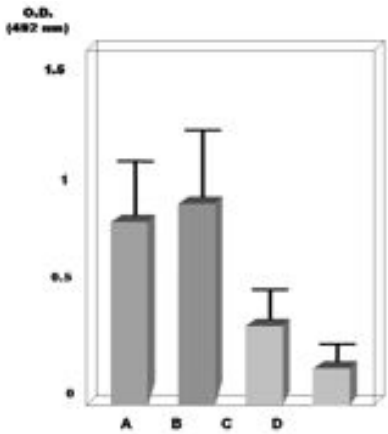

Fig. 2. Specific IgG anti-WSP antibody response in humans. A. Individuals diagnosed as having pulmonary dirofilariosis. B. Humans diagnosed as having subcutaneous dirofilariosis. C. Healthy donors residing in an endemic area. D. Healthy donors living in a non endemic area.

antibodies (Simón et al., 2003 and data not published).

In humans diagnosed as having subcutaneous dirofilariosis, positive staining for WSP was present in inflammatory cells (Fig. 1c and 1d), in very close proximity to WSP+ oocytes and in one case WSP+ morulae were seen free within inflammatory tissue. In those nodules where worms were undergoing degeneration, there were many cells within the inflammatory granuloma that stained intensely positive for WSP (data not published).

An alternative to demonstrate that a worm removed from an infected person is a *Dirofilaria*, is the polymerase chain reaction (PCR). Amplification of *Wolbachia* DNA with specific primers can be achieved and may be applied directly on worms extracted from nodules, or samples obtained by different histological techniques from nodules in which the worms have been destroyed by the granulomatous reaction.

Wolbachia stimulates a Th1-type response

Several studies have been conducted to identify the type of immune response stimulated by *Wolbachia* in different hosts of *Dirofilaria* spp. Mice immunized with WSP alone or in combination with GroEl developed a strong response of IgG2a antibodies (related to a Th1-type response in this species) immediately after inoculation. The highest levels were observed on day 20 of the experiment (13 days after the last inoculation) and similar levels were observed 10 days latter (data not published). In dogs with natural *D. immitis* infections, both microfilaremic and amicrofilaremic individuals showed significantly higher IgG2 antibody response against WSP than negative controls. The response is significantly higher in microfilaremic than in amicrofilaremic dogs (Kramer et al., 2005b). Humans diagnosed as having pulmonary dirofilariosis also develop significantly higher IgG1 antibody levels (Th1-type response in humans) than healthy individuals living in endemic areas. No antibodies related to Th2-type response were detected in any human hosts (Marcos-Atxutegi et al., 2004).

Specific anti-*Wolbachia* antibody levels are modified by drug treatment

Dogs with naturally acquired heartworm disease and treated with melarsomine showed a strong increase of specific IgG anti-WSP antibodies a few days after treatment (Fig. 3a). The maximum antibody levels were detect-

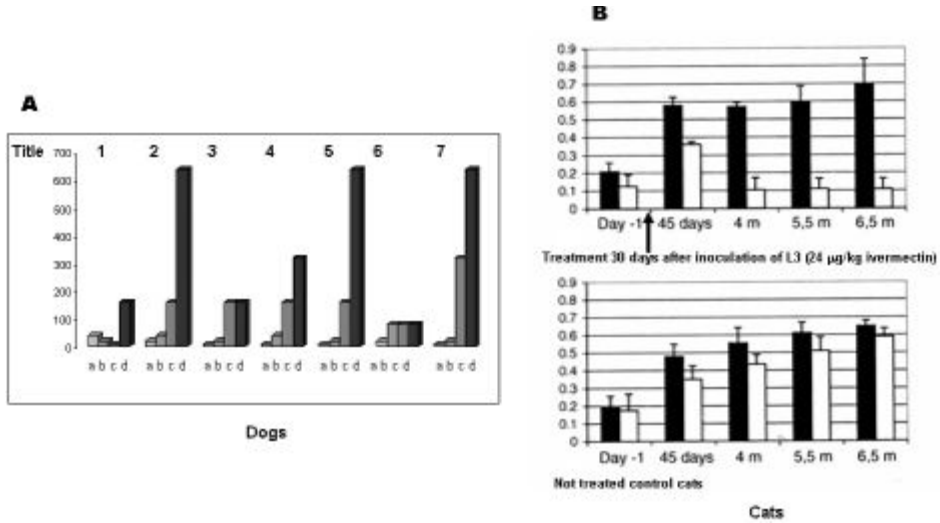

Fig. 3. Changes in specific IgG anti-WSP antibody levels in *Dirofilaria immitis* naturally infected dogs treated with melarsomine (A): a. Previous to treatment; b. One day after the treatment; c. 1 week (1-6) or 2 weeks (7) after the treatment; d. 2 weeks (1-6) or 6 weeks (7) after the treatment. Experimentally infected cats treated with ivermectin (B).

ed at the end of the study (21 or 63 days post-treatment depending the protocol employed) (data not published).

Change in the levels of anti-*Wolbachia* and anti-*Dirofilaria immitis* antibodies following filaricide treatment was observed in a group of experimentally infected-, ivermectin treated-cats (Morchón et al., 2004). A dramatic increase of specific IgG antibodies against both WSP and *Dirofilaria immitis* peptides (Dipp) was found 15 days after ivermectin treatment. At 3.5 months post-treatment the anti-Dipp antibodies decreased to values seen before infection, while specific anti-WSP antibodies continued to increase till the end of the study 5.5 months post-treatment. In the control group (infected-, untreated cats) the antibody response against both WSP and Dipp increased continuously throughout the study (Fig. 3b).

Human pulmonary or subcutaneous dirofilarioses do not require treatment. Nevertheless, the antibody response to both *Dirofilaria* and *Wolbachia* antigens was measured during the follow-up of a subcunjuntival case due to *D. repens*, until 6 months after the removal of an intact, live female worm (Ruiz-Moreno et al., 1998). An ELISA test for antibodies against *D. repens* adult worms was positive until 3 months after the removal of the worm, while the ELISA for anti-WSP antibodies was positive before removal, but was negative in the subsequent three blood samples (1, 3 and 6 months after removal of the worm).

Conclusions

The accurate diagnosis of canine and feline *D. immitis* infections is the first step towards the correct prevention of the spread of the disease in the animal reservoirs and human infections. The development of the parasite and the

signs it causes are different in dogs and cats. Thus, the methods validated for the diagnosis in these two hosts are also different. Considering the excellent performance of existing commercial kits for the detection of antigens and antibodies for canine and feline dirofilariosis, respectively, the moderate/low correspondence of *Wolbachia* antibody tests with these kits, and the existence of other diagnostic techniques like detection of microfilariae and echocardiography, the application of serologic diagnostic tests based on the detection of anti-*Wolbachia* antibodies in animal dirofilariosis is not useful.

Nevertheless, the detection of anti-*Wolbachia* antibodies could be of interest in:

(i) epidemiological studies for evaluation of the real pressure of infection in an endemic area. In this case, risk levels are represented not only by active infection (detected by commercial kits), but also by prepatent infections, mainly in feline dirofilariosis. In these cases, a test for the detection of specific antibodies against *Wolbachia* could be an invaluable diagnostic tool, as shown by the results discussed above;

(ii) evaluation of the efficacy of macro/microfilaricidal treatment. The death of filarial worms is the main factor for the release of *Wolbachia*, increasing the stimulus of the host's immune system. The detection of a rise of anti-*Wolbachia* antibody levels, together with a fall of the anti-*Dirofilaria* antibodies can be interpreted as sign of drug efficacy. Results of serological follow-up in the human subconjuntival case previously presented confirms that massive release of *Wolbachia* occurs when worms are physically destroyed, because in this case the removal of whole worm causes an immediate and clear fall of specific anti-*Wolbachia* antibodies.

In the case of human pulmonary and subcutaneous/ocular dirofilariosis, the suspicion of neoplasia caused by the presence of a nodule makes differential diagnosis of prime importance. This implies that once the dirofilarial origin of a nodule is demonstrated, the specific identification is a secondary matter (Simón et al., 2005). When worms are present inside the nodules, the specific identification is possible by histology, but if they have been destroyed by the inflammatory reaction or the physician takes a conservative attitude, evidence for the presence of *Wolbachia* can contribute to the identification of the filarial origin of the nodule. This can be obtained by antibody tests, PCR or immunohistochemical techniques. In the first case invasive techniques are not necessary, while in the other two, tissue samples must be obtained.

In conclusion, in spite of the existence of validated methods for the diagnosis of dirofilariosis in animal reservoirs based on the use or detection of *Dirofilaria* molecules, the identification of *Wolbachia* by both direct and indirect methods, seems an excellent complementary data and constitute an effective tool for epidemiological studies and research related to

inflammatory pathology. In humans the detection of anti-*Wolbachia* antibodies can be a fundamental tool in the differential diagnosis for the identification of the genus *Dirofilaria* in cases in which the worms have been previously destroyed by the granulomatous reaction of the host.

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7

Human dirofilariasis due to *Dirofilaria (Nochtiella) repens*: an update of world literature from 1995 to 2000*

S. Pampiglione, F. Rivasi

* Paper from Parassitologia 42: 231-254, 2000

Introduction

Following on from their 1995 review (Pampiglione et al.) of the world literature on human dirofilariasis due to *Dirofilaria (Nochtiella) repens* the authors have attempted to gather all the new cases published in the scientific literature from 1995 to 2000 for an update on the subject. *Dirofilaria (Nochtiella) repens* (Nematoda: Filarioidea, Onchocercidae) is a habitual parasite of the subcutaneous tissue of dogs and other carnivores. It is transmitted by Culicidae and can also accidentally affect man. Of the species of filariae found in the temperate regions of the world it is the one most commonly observed. It is present only in the Old World, notably in Italy, where 181 human cases were reported up to 1995 distributed practically over the whole of the national territory. Endemic zones were also identified in France, Greece, Turkey, Sri Lanka, Ukraine, the Russian Federation, Uzbekistan and other countries for a total of 410 cases spread over 30 different nations.

Materials and methods

The cases published in the world literature during the last 5 years have been classified by nation and the essential data for each one (year of publication, author's name, patient's sex, age, place of residence, location of the parasite) set out in tabular form. In some cases in which the authors did not provide the full anamnestic and morphological details necessary for a correct documentation we have limited ourselves to recording what findings were reported and have accepted them on trust.

A few of other cases that occurred before 1995 but which escaped mention in our previous enquiry have now been included so as to offer as complete a general picture as possible of the overall number and geographical distribution of subjects affected.

In this context two recent reviews of cases occurring in the ex-Soviet Union (Avdiukhina et al., 1996, 1997) have been extremely useful and have enabled us to include in our statistics many cases published in Russian journals that are unlikely to be available in European or American libraries. Similarly, as regards Sri Lanka, the review recently published by Dissanaike et al. (1997), together with their prompt personal communications of subsequent updates, have been of great value. The data missing for some of the cases reported in our 1995 review have now been included (age, sex, locality and location, together with bibliographical reference, if applicable).

They appear in the numerical listing with the number 0, since they were already counted in the previous review.

Results

Geographical distribution

There do not appear to be any significant variations in the geographical distribution of the parasite in the dog and other carnivores from that reported in the previous review, exceptions being 5 animals in Tenerife (Canary Isles) (Stenzenberger and Gothe, 1999), 3 in Hungary (Fock et al., 1998; Szell et al., 1999), several dogs (number not stated) in the South African Republic

(Bredal et al., 1998; Schwan, personal communication), all considered autochtonous infections by the respective observers.

Imported cases from other endemic countries are reported in Germany (Zahler et al., 1997; Glaser and Gothe, 1998) and also in the south of Switzerland (Bucklar et al., 1998), even though in the latter instance it may be suspected that the parasite's life cycle completed itself *in loco* given the mildness of the climate in recent years. In fact, Petruschke et al. (1998) report the presence of *D. repens* in the low-lying Canton Ticino in 2 dogs that apparently had never been outside the area. The geographical distribution of canine dirofilariasis in France has been illustrated by Beuguet and Bourdasseau (1996) and by Chauve (1997). The latter claims that the parasitosis is present in 19 of the 62 districts investigated and that it can affect as many as 22% of the animals examined. Generally, in France *D. repens* appears to be more common than *D. immitis*, while neither species has been reported there in cats. Cazelles and Montagner (1995) report 2 cases of dogs simultaneously affected by leishmaniasis and dirofilariasis due to *D. repens* in Aveyron and Gard (southern France).

In Spain, the presence of canine dirofilariasis is reconfirmed in Alicante, Cadiz and Salamanca, albeit with a prevalence of less than 2%, and, in the Ebro delta only, of 9.4% (Anguera Galiana, 1995; Lucientes and Castello, 1996). Aranda et al. (1998) found no evidence of the parasitosis in 188 dogs examined in the Barcelona area. In the Alicante and Elche areas (southeastern provinces of Spain) Cancrini et al. (2000a) claim that dogs are more affected by *D. repens* than by *D. immitis*, in contrast to a previous study by Rojo Vasquez et al. (1990), basing their results on 114 canine blood samples tested with Knott technique, PCR and ELISA for specific IgG detection.
In Bulgaria, Kanev et al. (1996) report 2 dogs testing positive out of 192 examined.

In Greece, prevalence is between 6.7 and 22% (Vakalis and Himonas, 1997).

The geographical distribution of both *D. immitis* and *D. repens* in the European Union in dogs is illustrated in a map by Muro et al. (1999).
The possibility that the biological cycle of *D. repens* completes itself on Israeli territory, and that therefore the human cases diagnosed there are autochtonous, is confirmed by the discovery of a dog affected by the parasite but which had never left Israel (Harrus et al., 1998).

In northern Italy, a study involving 2628 dogs (Rossi et al., 1996; Pollono et al., 1998) confirms the high incidence of *D. repens* in Piedmont with a prevalence of up to 20.5%. In the province of Varese (Lombardy), Petruschke et al. (1998) report a prevalence of 6.9% out of 216 dogs examined. In Sicily, it is confirmed that autochtonous cases due to *D. immitis* are rare, maybe even non-existent, whereas *D. repens* is widespread (Pennisi and Furfaro, 1997; Giannetto et al., 1999), particularly in the province of Trapani, where Giannetto et al. (1997) diagnosed a prevalence of 22.8% for this latter species. In the communes of Vesuvius (province of Naples), microfilariae of *D. repens* were found in 3% of 351 dogs examined (Rinaldi et al., 2000). In Tuscany

(central Italy), where canine dirofilariasis due to *D. repens* affects 25-39% of dogs, Marconcini et al. (1996) found microfilaraemia in 1% of 523 foxes examined, while autopsy revealed adult nematodes in 3 out of 28 foxes examined. However, the authors in question do not consider the fox to be a reservoir of parasitosis.

In South Africa, the presence of *D. repens* has been found for the first time in a cat suffering from hepatic insufficiency (Schwan et al., 2000). Tarello (2000a,b) claims to have found microfilariae of *D. repens* in the blood of 10 cats associated with pruritic dermatosis and haemobartonellosis in Alessandria (northern Italy) and in another cat affected by ulceration on both flanks.

In Romania, Olteanu, in a congressional communication (1996) reports *D. repens* not only in the dog but also in the fox, the wolf and the cat, as well as in man, but without specifying the number of cases. By way of confirmation of the parasite on Romanian territory, said author quotes other researchers (Motas, 1903; Popovici, 1916) who have, in the past, discovered it in dogs.

In Sri Lanka the high prevalence of *D. repens* in dog, formerly signalled (Dissanaike, 1961, and others) is confirmed to reach 60-70% in some provinces by Dissanaike et al. (1997).

Roncalli (1998), in a paper presented at the XX Congress of the Italian Parasitology Society, described the history of canine dirofilariasis from the 17th century to the present with some observations on the geographic distribution for the past times.

Cases of human dirofilariasis reported since 1995 number 372 in all, involving 25 countries. In Europe: Belgium, Bulgaria, France, Greece, Hungary, Italy, Romania, Serbian Republic, Slovakia, Slovenia, Spain, Ukraine; in Africa: Kenya and Tunisia; in Asia: Georgia, India, Iran, Israel, Kazakhstan, Malaysia, Russia, Sri Lanka, Turkey, Turkmenistan and Uzbekistan. The countries most affected have been Italy (117 cases), Sri Lanka (101), Russia (Siberia included, 61), Ukraina (23) and France (23). In the few cases found in Austria and Germany the infection was picked up in other countries. In Belgium, where the parasite has never previously been observed, a curious episode of dirofilariasis is reported (van den Ende et al., 1995) involving three members of the same family; it may have been caused by a mosquito transported in luggage from an endemic zone or from a nearby international airport. Parasitological diagnosis (itching, cutaneous *striae* and a nodule containing a female of *D. repens*) was possible in only one of the three cases, but the presence of the same type of cutaneous *striae* persisting for months in the other two family members would suggest a cryptic infection of the same nature.

In a large part of Africa and in other developing regions of the world, as Orihel and Eberhard (1998) point out, it must be borne in mind that it is not always possible to establish with any degree of certainty whether infections due to *D. repens* in man are rare or frequent because their diagnosis is determined by the level of development and the efficiency of the health services and hence by the diagnostic resources available. The only countries where the geographical distribution of human dirofi-

lariasis has been traced are France, Greece, Italy, the Russian Federation and Sri Lanka. In Italy, cases of the infection have been reported in 9 regions, prevalently in Piedmont. Takings the cases recorded in the previous enquiry into account, the parasitosis is found to be present in 19 regions of Italy out of 20. In Greece (Pampiglione et al., 1996d), when the recent cases are added to those reported previously, human dirofilariasis is also seen to affect virtually the whole of the country. In France, Raccurt (1999, 2000) claims that there are at least 14 departments affected by *D. repens* with the highest incidence in the Mediterranean coastal regions, although sporadic occurrences on the Atlantic seaboard and above the 46° latitude North have been reported (Guillot et al., 1998; Weill et al., 1999). In Sri Lanka (Dissanaike, 1996), the zones most affected are those of the western provinces. In the ex-Soviet Union, today the Russian Federation, although there is no distribution map properly covering this vast area, it appears from the sketchy literature and, in particular, from the reviews of Avdiukhina et al. (1996 and 1997), that the regions where the parasitosis is most endemic are the Caucasus (in the south of the Russian Republic), which account for 62% of the cases cited, and Ukraine, accounting for 21%. Kazakhstan, Uzbekistan and Georgia each account for 4% and Turkmenistan for 3%, while other small states account for the remaining 2%. The northern limit to the spread of *D. repens* seems to be the 62nd parallel in the Russian Federation.

Distribution by age and sex

The age-range of the subjects affected goes from 4 months to 100 years, most of them being in their 40s. In Sri Lanka, Dissanaike et al. (1997) report a considerable number of cases in children less than 10 years old, a phenomenon not found in any other country. There is a greater prevalence in females than in males (186 of the 322 cases in which the patient's sex is specified, representing 57.8%), but it is not statistically significant.

Carriers

Extensive research into the Culicidae captured with canine bait in a zone endemic for *D. repens* in Piedmont (north-western Italy) (Pollono et al., 1998) has shown that this natural reservoir is very attractive to *Culex molestus* and *Aedes caspius*, but less so to *C. pipiens*, *Ae. vexans* and *Anopheles maculipennis.* The Culicidae are at their most active, at least in Piedmont, during the month of July. Talbalaghi (1999) has shown an increase in the density of *Ae. caspius* in the same area over the last 3 years and an increase in the spread of larval foci. *Ae. caspius* is the species most aggressive towards man; it is capable of travelling 20 km without wind assistance and bites all day long. Using both canine and human bait in endemic zones of the Veneto and Friuli-Venezia Giulia (north-eastern Italy), Pietrobelli et al. (2000) demonstrated a strong attraction for *C. pipiens* and *Ae. caspius*, much reduced for *Ae. vexans*. In Nigeria, Anyanwu et al. (2000) between 6 different species of Culicidae tested (*Culex pipiens fatigans, C. p. pipiens, Anopheles gambiae,*

Mansonia africana, *Aedes vittatus*, *Ae. aegypti*) have found that this last is the most suitable vector for a local strain of *D. repens*. In order to select strains of *Ae. aegypti* refractory and otherwise to *D. repens*, Favia et al. (1998) used molecular biology techniques with apparently reliable results. Pampiglione and Gupta (1998) reported the presence of immunocytes (plasmatocytes) of Culicidae, probably *Aedes* sp., in a mammary nodule enclosing an immature specimen of *D. repens*. The authors think it likely that, during the passage of the infecting larvae from the mosquito to man, the carrier's immunocytes are often borne along by the larvae themselves and so penetrate the subcutaneous tissue, only to be regularly destroyed by the defensive reactions of the host.

Clinical history and symptomatology

In the dog, the parasitosis is generally asymptomatic even when microfilariaemia is fairly developed. There may, however, be cutaneous manifestations such as erythematous patches, papulae, localised alopecia, eczema accompanied by pruritus and, rarely, nodule and/or ulceration (Bredal et al., 1998; Tarello, 1999).

In man, the carrier's bite has sometimes been described as painful, followed by slight local acute phlogosis (erythema, swelling, pruritus) lasting for 5-8 days. Generally speaking, however, no particular sensation attributable to the insect bite is recalled by the patient other than that of being bitten by mosquitoes. The subsequent formation of the nodule in which the nematode remains trapped by the defensive reaction of the host is an essential feature, typical of the parasitosis and of which it may prove to be the sole clinical manifestation. It is noticed after a period of approximately 2-12 months from the penetration of the parasite, although much longer periods cannot be ruled out since it is often impossible to recall the moment when infection took place. At times the nodule appears without any external phlogistic manifestation, at others it is accompanied by local erythema and pruritus, localised or more widespread urticarial manifestations lasting a few days but recurring for as much as a year and a half (Thérizol-Ferly et al., 1996). The nodule may also suppurate and assume the appearance of an abscess. There have been very occasional reports of erysipeloid phlogistic reactions and satellite lymphoadenopathies as well as of rises in temperature of modest entity or with hyperpyrexia due to infectious complications that are quickly brought under control with antibiotics. In eye cases, complications such as detached retina, crystalline opacity, glaucoma, uveitis, episcleritis and a limited loss of vision have exceptionally been reported (Mizkievic and Leontieva, 1961; Avdiukhina et al., 1996). In one case affecting the orbit, Braun et al. (1996) report monolateral exophthalmos lasting for a year. Patients have reported subcutaneous migrations of the parasite sometimes over considerable distances, such as from the lower limbs to the neck or head, or from one side of the body to the other, during periods of many months (Delage et al., 1995; Azarova et al., 1995; Artamonova and Nagornyi, 1996). In one patient, migrations in the tissues of the head had caused trigeminal neuralgia, which dis-

appeared when the nematode, suddenly appearing under the bulbar conjunctiva, was promptly removed (Avdiukhina et al., 1997).

The speed of these migrations, when visible, has been estimated at 30 cm in two days. Jelinek et al. (1996) report 2 cases of patients whose insistently complaints of "a worm under the skin" had led the doctors to diagnose delusory parasitosis and have them admitted to psychiatric ward, until the appearance of a nodule and its subsequent histological analysis cleared up the matter. A sensation of a nematode travelling under the skin had been reported by 10% of the Russian patients included in the review. Artamonova and Nagornyi (1996) report a case with cutaneous manifestations as in a true *larva migrans*. The application of hot steam compresses or ultrasound therapy appears to stimulate the movements of the nematode under the skin. There are rare cases of 2 nodules being present in the same patient, either appearing contemporaneously or months apart (Degardin and Simonart, 1996; Jelinek et al., 1996; Orihel et al., 1997). The nodules had appeared 5 years earlier (Degardin and Simonart, 1996) in one case, 2 years earlier in others. The maximum duration of the parasitosis is reported as being 12 years (Avdiukhina et al., 1996). The spontaneous emergence of the nematode from the suppurating nodule is reported on at least 10 occasions. In one case, localised near the junction of the lips, squeezing the nodule, which had been mistaken for a boil, caused the emergence of the nematode together with pus, after which the lesion healed in the space of a few days. In others involving the subconjunctiva or eyelids, vigorous rubbing of the affected eye by the patient caused the spontaneous emergence of the nematode (Avdiukhina et al., 1966, 1967). Peripheral hypereosinophilia is reported only rarely, with values generally little more than 10-15% of the leukocyte formula (Thérizol-Ferly et al., 1996; Pampiglione et al., 1996). However, Petrocheilou et al. (1998) report a Greek case, diagnosed merely on account of the presence of microfilariae in the bloodstream defined by the authors as *Dirofilaria*-like, in which the eosinophil count amounted to 26% of the leukocyte formula. A case of subconjunctival dirofilariasis in an HIV-positive patient is reported in France (Basset et al., 1996) without there being any apparent correlation between the development of the parasitosis and the presence of HIV.

Locations

In the dog, reports of localised nodular formations enclosing the nematode are rare, since the parasite is almost always more or less elongated and free in the web of subcutaneous tissue.
Localisation in the sternal region, the neck and the flanks has nevertheless been reported by Bredal et al. (1998) and Tarello (1999). A dog with *D. repens* in the heart has been observed by Isola et al. (2000), thus demonstrating that the species can penetrate the bloodstream.

In man, on the other hand, the parasite nodule is always present, if one excludes localisation in the subconjunctiva where the nematode can be considered to be migratory and not yet trapped by the host's reaction. In the

vast majority of cases, the nodule is located in the subcutaneous tissue, the deep dermis or the submucosa. There are rare cases of location in muscle tissue (Pampiglione et al., 1996c; Gros et al., 1996), in the lymph nodes (Alain,1997; Shekhar et al., 1996; Auer, pers. comm., 1998; Cancrini et al., 1999) and in the deep viscera. More frequent are reports of localisation in the upper half of the body (74%), in particular the ocular region (eyelid, subconjunctiva, orbit) (35.3%) and also the upper limbs (11%). In the cases reported by Avdiukhina et al. (1997) localisation in the ocular region accounts for 45% of the total observed in the Russian Federation. Localisation in the vitreous body in 3 cases and in the crystalline lens in one case is reported respectively in the Slovakian Republic (Vasilkova et al., 1992) and Uzbekistan (Mizkievic and Leontieva, 1961). A case of preretinal localisation is also reported in Kazakhstan (Glinciuk et al., 1992), although the morphological description may cast doubt on the actual species. In Italy, two cases are reported of localisation in the orbital cavity, operated on by means of anterior orbitotomy (Strianese et al., 1998). Another case of localisation in the orbital cavity was operated on transnasally (Braun et al., 1996; Groell et al., 1999), the authors producing a remarkable videotape of the removal of the nematode. A subconjunctival localisation is reported by Bianchi Rossi et al. (1991) where the parasite had previously passed through the retrobulbar area causing exophthalmos. In Sri Lanka, Dissanaike and coworkers (1997) report relatively frequent cases affecting the male genitals (scrotum, epididymis, spermatic chord, penis), particularly in children below 5 years of age; this is in contrast with what occurs in other geographical regions, where the few cases reported are restricted to adults. One case affecting the subcutaneous tissue of the penis is reported from Corsica (Pampiglione et al., 1999b), another from Israel (Stayermann et al., 1999) and two more from Sri Lanka (Dissanaike et al., 1997). Pulmonary cases are reported from Greece (Pampiglione et al., 2000d) and from Italy (Pampiglione et al., 1996e, 2000b).

Jelinek et al. (1996) and Fueter and Gebbers (1997) report 2 cases and one case, respectively, of pulmonary infection, one from Corsica, one from Italy and the third in a globetrotter possibly affected in America – all treated in Germany. These authors attribute the infection to *D. immitis*; however, it is possible that, by analogy with many other cases reported from Corsica and Italy, these are due to *D. repens*, although, given the advanced state of decomposition of the nematodes, precise identification was not possible. Rare cases affecting the omentum and the mesentery have been casually observed during laparotomy in Italy and the Russian Federation (Avdiukhina et al., 1997; Dorofeiev et al., 1997; Mastinu et al., 1998; Pampiglione et al., 2000b).

There are single instances of localisation in the parotid gland (Hoop, 1997; H. Braun, pers. comm.), in the submucosa of the mouth, of the base of the tongue, of the isthmus of the fauces and within a granuloma at the root of a tooth (Avdiukhina et al., 1997).

Table 1. World distribution of human infections by *Dirofilaria repens*. The dates refer to publications. Abbreviations: n.s.=not specified; p.c.=personal communication; 0=cases already counted in the previous review (1995).

No.	Date	Author(s)	Sex	Age (yrs)	Locality	Location
				EUROPE		
Belgium						
1	1995	van den Ende et al.	F	33	n.s.	upper eyelid*
* Two other suspected cases occorred in the same family. The locality where infections took place remained unknown.						
Bulgaria						
1 to 4	1996	Kaven et al.	n.s.	n.s.	n.s.	n.s.
France						
1	1995	Nozais and Huerre	M	39	Languedoc	scrotum
2	1995	Delage et al.	M	43	Ardèche	eyelid
3	1996	Hautefort (cit. by Basset et al., 1996)	n.s.	n.s.	Arles	thigh
4	1995	Basset et al.	M	37	Narbonne	subconjunctival
5	1996	Masseron et al.	M	61	Gironde	subconjunctival
6	1996	Gros et al.	M	19	Corsica	arm
7 to 10	1996	Rabodonirina et al.	n.s.	n.s.	Lyon?	n.s.
11	1996	Thérizol-Ferly et al.	F	35	Sologne? Côte méditerranéenne	subconjunctival
12	1996	Jelinek et al. (surgery in Germany)	F	46	Corsica	lung (*D. immitis*?)
13	1997	Weill et al.	F	70	Côte méditerranéenne	subconjunctival
14	1997	Alain (cit. by Raccurt, 2000)	F	22	Loire and Cher	groin lymphnode
15	1998	Guillot et al.	F	12	Gironde	forehead
16	1998	Desruelles et al.	F	47	Var	subcutaneous n.s.
17	1999	Weill et al.	M	66	Côte d'Azur or Corsica	cheek
18	1999	Weill et al.	F	39	Charante Maritime	orbital region
19	1999	Pampiglione et al.	M	64	Pinarello (Corsica)	foot
20	1999	Pampiglione et al.	M	23	Porto Vecchio (Corsica)	penis
21	1999	Rouhette et al.	M	65	Nice	subconjunctival
0	1999	Morassin et al.*	F	26	Tarn or Herault	subclavicular region
* This case was already recorded by Kramer 1993 (Raccurt, 2000).						
22	1999	Calvet et al.	M	47	Var	arm
23	1999	Dei-Cas (cit. by Raccurt, 2000)	F	10	Nice or Corsica	abdominal wall
Greece						
0	1996	Pampiglione et al.	M	45	Athens	abdominal wall
1	1996	Jelinek et al. (surgery in Germany)	n.s.	adult	n.s.	hand, thigh
2	1997	Arvanitis et al.	M	68	n.s.	subconjunctival
0	1998	Petrocheilov et al.	M	70	Ionian islands	worm not recovered*
* Only microfilariae (210-230 µm long) were detected in peripheral blood with diagnosis of *Dirofilaria*-like.						

continued

No.	Date	Author(s)	Sex	Age (yrs)	Locality	Location
3	1998	Vakalis and Himonas	n.s.	n.s.	n.s.	nose region
4	1998	Vakalis and Himonas	n.s.	n.s.	n.s.	thoracic wall
0	1998	Vakalis and Himonas	M	3	n.s.	scrotum
0	1998	Vakalis and Himonas	M	48	Athens or Epirus	hand
5	1998	Vakalis et al.	F	42	Messinia (Peloponnesos)	breast
6	1999	Pampiglione et al.	M	31	Aigios Peloponnesus	lung
Hungary						
1	2000	Parlagi et al.	F	57	n.s.	eyelid
2	2000	Szénasi, p.c.	M	56	Szeged province	subconjunctival
3	2000	Elek et al.	F	52	Budapest province	upper eyelid
4	2000	Elek et al.	M	48	Hodmezovasarhely	eye-brow
5	2000	Elek et al.	F	64	Tisza river bank	pelvis region
6	2000	Elek et al.	F	76	Csobaj (Miscolc)	arm
Italy						
0	1986	Spina et al.	M	60	Biancavilla (Catania)	orbital region
0	1986	Spina et al.	F	73	Ramacca (Catania)	lower eyelid
0	1987	Toniolo et al.	M	23	Cadore (Trento)	jaw
1	1990	Stemberger H (cit. by Auer et al. 1997; surgery in Austria)	M	adult	Italy (locality n.s.)	periocular region
2	1991	Bianchi Rossi et al.	F	79	Laiatico (Pisa)	subconjunctival
0	1993	Bay et al.	F	63	Torino	lower eyelid
3	1995	Stemberger H. (cit. by Auer et al. 1997; surgery in Austria)	M	adult	Italy n.s.	leg
4	1996	Garaffini et al. (surgery in France)	F	72	Locality n.s.	periocular region
5	1996	Braun et al. (surgery in Austria)	F	61	Locality n.s.	retrobulbar region
6	1996	Cancrini et al.	F	42	Augusta (Siracusa)	leg
7	1996	Cancrini et al.	F	26	Augusta (Siracusa)	ankle
8	1996	Cancrini et al.	F	48	Sortino (Siracusa)	gluteal region
9	1996	Molet, p.c.	F	35	Venice	scalp
10	1996	Jelinek et al. (surgery in Germany)	F	30	Piedmont?Tuscany?	lung*
* The diagnosis was *D. immitis* but probably it was *D. repens*.						
11	1996	Jelinek et al. (surgery in Germany)	n.s.	n.s.	n.s.	glabella
12	1996	Jelinek et al. (surgery in Germany)	n.s.	n.s.	n.s.	abdominal wall
13	1996	Jelinek et al. (surgery in Germany)	n.s.	n.s.	n.s.	shoulder
0	1996c	Pampiglione et al.	F	43	Sciacca (Agrigento)	temporal region
0	1996e	Pampiglione et al.	F	66	Pegognaga (Mantova)	lung
0	1996e	Pampiglione et al.	M	69	Concordia (Modena)	lung
0	1996a	Pampiglione et al.	M	55	Rivalta S (Alessandria)	thigh
0	1996b	Pampiglione et al.	F	44	Asti	iliac region
0	1996b	Pampiglione et al.	M	42	Gavorrano (Grosseto)	leg
0	1996b	Pampiglione et al.	M	68	Surbo (Lecce)	neck

continued

No.	Date	Author(s)	Sex	Age (yrs)	Locality	Location
0	1996a	Pampiglione et al.	M	38	Oristano	zygomatic region
0	1996a	Pampiglione et al.	M	54	Oristano	forehead
0	1996b	Pampiglione et al.	F	66	Staranzano (Gorizia)	leg
0	1996b	Pampiglione et al.	M	40	Asti	upper eyelid
14	1996	Pampiglione et al.	M	58	Arbatax (Nuoro)	subconjunctival
15	1996	Pampiglione et al.	F	35	Olbia (Sassari)	leg
16	1996	Vitullo, p.c.	F	45	Isernia	breast
17	1996	Vitullo, p.c.	M	57	Venafro (Isernia)	neck
0	1997	Pampiglione et al.	M	52	Ravenna province	spermatic chord
0	1997	Giannetto and Ubaldino	F	38	Mazara del Vallo (Trapani)	subconjunctival
18	1997	Auer et al. (surgery in Austria)	M	35	Udine province, or Corsica, or Southern France	epididymis
19	1997	Zardi et al.	M	45	Gaeta	thoracic wall
20	1997	Mastinu et al.	F	42	Asti province	arm
21	1997	Mastinu et al.	M	36	Asti province	subconjunctival
22	1997	Mastinu et al.	F	45	Asti province	periocular region
23	1997	Heep, p.c., Auer 1997 (surgery in Austria)	n.s.	n.s.	n.s. or Greece	parotid gland
24	1997	Garavelli, n.p.	M	61	Bergamasco(Alessandria)	scalp
25	1998	Mastinu et al.	M	50	Asti province	temporal region
26	1998	Mastinu et al.	F	19	Asti province	hand
27	1998	Mastinu et al.	M	39	Asti province	breast
28	1998	Mastinu et al.	F	62	Asti province	mesenteron
29	1998	Pampiglione et al.	F	46	Milano	breast
0	1998	Pampiglione et al.	F	52	Garlasco (Pavia)	breast
30	1998	Auer, p.c. (surgery in Austria)	M	43	Sardinia? Malta?	sacral region
31	1998	Cancrini et al.	M	59	Grado (Gorizia)	submandibular lymphnode
32	1998	Cancrini et al.	M	45	Augusta (Siracusa)	finger
33	1998	Cancrini et al.	F	48	Augusta (Siracusa)	leg
34	1998	Cancrini et al.	F	60	Elba Island	breast
35	1998	Cancrini et al.	F	20	Mazara del Vallo (Trapani)	eyelid
36	1998	Cancrini et al.	M	74	Augusta (Siracusa)	subconjunctival
37	1998	Cancrini et al.	F	42	Siracusa	subconjunctival
38	1998	Cancrini et al.	F	56	Torino	subconjunctival
39	1998	Cancrini et al.	M	42	Siracusa	eyelid
40	1998	Strianese et al.	F	adult	Napoli province	orbital region
41	1998	Strianese et al.	F	47	Napoli province	orbital region
42	1999	Pampiglione et al. (surgery in Hungary)	M	37	Northern Italy or Hungary n.s	spermatic chord
43	1999	Pampiglione et al.	M	57	Odalengo (Alessandria)	axilla
44	1999	Pampiglione et al.	M	39	Novara province	zigomatic region
45	1999	Pampiglione et al.	F	n.s.	Novara province	popliteal region
46	1999	Pampiglione et al.	M	57	Novara province	thoracic wall
47	1999	Pampiglione et al.	F	83	Viarigi (Asti)	abdominal wall
48	1999	Pampiglione et al.	F	41	Castagneto Carducci (Livorno)	abdominal wall
49	1999	Pampiglione et al.	F	50	Piombino (Livorno)	thigh
50	1999	Pampiglione et al.	F	57	Crescentino (Vercelli)	leg
51	1999	Pampiglione et al.	F	50	Casale Monferrato (Alessandria)	arm
52	1999	Pampiglione et al.	M	25	Novara province	thoracic wall
53	1999	Pampiglione et al.	F	100	Casale Monferrato (Alessandria)	thoracic wall
54	1999	Pampiglione et al.	F	30	Novara province	forearm
55	1999	Pampiglione et al.	F	69	Novara province	axilla

continued

No.	Date	Author(s)	Sex	Age (yrs)	Locality	Location
56	1999	Pampiglione et al.	M	59	Casorzo (Asti)	scalp
57	1999	Pampiglione et al.	F	34	Trino (Vercelli)	thoracic wall
58	1999	Pampiglione et al.	F	64	Fontanetto Po (Vercelli)	knee
59	1999	Pampiglione et al.	M	32	Casale Monferrato (Alessandria)	scalp
60	1999	Pampiglione et al.	F	69	Casale Monferrato (Alessandria)	thoracic wall
61	1999	Pampiglione et al.	F	40	Trino (Vercelli)	neck
62	1999	Pampiglione et al.	F	61	Moncalvo (Asti)	scalp
63	1999	Pampiglione et al.	F	41	Lecce	upper eyelid
64	1999	Pampiglione et al.	F	29	Asti	hand back
65	1999	Pampiglione et al.	M	39	Roatto (Asti)	breast
66	1999	Pampiglione et al.	F	27	Gela (Agrigento)	cheek
67	1999	Pampiglione et al.	M	50	Ravenna province	subconjunctival
68	1999	Pampiglione et al.	M	70	Novara province	hand palm
69	1999	Pampiglione et al.	F	52	Novara province	axilla
70	1999	Pampiglione et al.	F	56	Novara province	abdominal wall
71	1999	Pampiglione et al.	M	52	Saturnia (Grosseto) or Livorno	epididymis
72	1999	Pampiglione et al.	M	61	Alessandria	scalp
73	1999	Pampiglione et al.	F	69	Alfiano Natta (Alessandria)	foot back
74	1999	Pampiglione et al.	M	adult	Asti province	n.s.
75	1999	Pampiglione et al.	F	73	Asti province	omentum
76	1999	Pampiglione et al.	F	56	Asti province	orbital region
77	1999	Pampiglione et al.	F	56	Vercelli or Mazara del Vallo (Trapani)	lung
78	1999	Pampiglione et al.	M	42	Novara province	arm
79	1999	Pampiglione et al.	M	29	Asti province	temporal region
80	1999	Pampiglione et al.	M	49	Asti province	lung
81	1999	Pampiglione et al.	M	45	Asti province	arm
82	1999	Pampiglione et al.	F	66	Asti province	breast
83	1999	Pampiglione et al.	F	54	Baratili S.P. (Oristano)	groin
84	1999	Pampiglione et al.	M	37	Cantalupo (Alessandria)	lower eyelid
85	1999	Pampiglione et al.	M	64	Valenza Po (Alessandria)	neck
86	1999	Pampiglione et al.	F	68	Mirabello (Alessandria)	nose region
87	1999	Pampiglione et al.	M	adult	Ravenna province	leg
88	1999	Pampiglione et al.	M	adult	Ravenna province	spermatic chord
89	1999	Pampiglione et al.	F	68	Vercelli	knee
90	1999	Pampiglione et al.	M	4	Milano	cheek
91	1999	Pampiglione et al.	M	44	Novara province	forehead
92	1999	Pampiglione et al.	F	42	Novara	forearm
93	1999	Pampiglione et al.	M	76	Castellammare del Golfo (Trapani)	abdominal wall
94	1999	Pampiglione et al.	F	59	Castelleone (Cremona) or Ischia Island	shoulder
95	1999	Pampiglione et al.	M	36	Scano M. (Oristano)	epididymis
96	1999	Pampiglione et al.	F	34	Pontestura (Alessandria)	abdominal wall
97	1999	Pampiglione et al.	M	28	Novara province	omentum
98	1999	Pampiglione et al.	M	44	Crescentino (Vercelli)	spermatic chord
99	1999	Pampiglione et al.	F	40	Vignale M.(Alessandria)	forearm
100	1999	Pampiglione et al.	F	65	Villanova (Alessandria)	forearm
101	1999	Pampiglione et al.	F	63	Valenza Po (Alessandria)	thigh
102	1999	Pampiglione et al.	M	24	Reggio Emilia	abdominal wall
103	2000	Giansanti, p.c.	F	69	Petrelle (Perugia)	breast
103	2000	Gobbo and Bisoffi, p.c.	M	39	Venezia	forearm
104	2000	Pastormerlo, p.c.	F	49	Valenza Po (Alessandria)	abdominal wall
105	2000	Pastormerlo, p.c.	F	35	Motta di Conti (Vercelli)	thigh

continued

No.	Date	Author(s)	Sex	Age (yrs)	Locality	Location
106	2000	Pastormerlo, p.c.	F	74	Casale M.(Alessandria)	knee
107	2000	Pastormerlo, p.c.	F	62	Vignale M. (Alessandria)	abdominal wall
108	2000	Speranza p.c	M	35	Modena province	groin
109	2000	Feyles, p.c	F	74	Asti	breast
110	2000	Feyles, p.c	M	28	Asti province	epididymis
111	2000	Feyles, p.c	M	76	Asti province	subcutaneous n.s.
112	2000	Pavesi, p.c.	F	49	Valenza Po (Alessandria)	abdominal wall
113	2000	Pavesi, p.c.	F	47	Moncalvo (Asti)	axillary region
114	2000	Pavesi, p.c.	F	74	Casale Monferrato (Alessandria)	knee
115	2000	Pavesi, p.c.	F	36	Motta di Conti (Vercelli)	thigh
116	2000	Pavesi, p.c.	F	62	Vignale Monferrato (Alessandria)	abdominal wall
117	2000	Elek et al. (surgery in Hungary)	F	71	Rome province	lower eyelid
Romania						
1	1997	Olteanu	n.s.	n.s.	n.s.	n.s.
2	2000	Panaitescu et. al.	n.s.	n.s.	n.s.	n.s.
3	2000	Panaitescu et. al.	n.s.	n.s.	n.s.	n.s.
Serbian Republic						
1	1996	Misic et al.	F	adult	Smederevo	head*
* Both female and male nematode were recovered in the same nodule.						
2	1996	Misic et al.	n.s.	n.s.	Smederevo	head
3	1996	Misic et al.	n.s.	n.s.	Beograd	shoulder
Slovak Republic						
1	1992	Vasilkova et al.	M	18	Bardeiov	vitreous body*
* From the description of the nematode it seems to be *D. immitis*.						
Slovenia						
1	1998	Auer (surgery in Austria)	M	24	n.s. or Albania?	groin lymphnode
Spain						
1	1998	Ruiz-Moreno et al.	M	43	Elche (Alicante)	subconjunctival
Ukraine						
1	1971	Melnicenko and Prosvetova	F	55	Poltava	subconjunctival
2	1971	Melnicenko and Prosvetova	M	29	Poltava province	subconjunctival
3	1977	Kondrazkyi and Parkomienko	F	46	Kiev	upper eyelid
4 to 11	1996	Davydov et al.	n.s.	n.s.	Kiev province	eye region (n.s)
12	1996	Pogolciuk	M	60	Odessa province	lower eyelid
13 to 16	1997	Dorofeev et al.	n.s.	n.s.	n.s.	n.s
17	1997	Dorofeiev et al.	F	67	Crimea	knee

continued

No.	Date	Author(s)	Sex	Age (yrs)	Locality	Location
18	1997	Dorofeiev et al.	n.s.	n.s.	n.s.	mesenteron
19	1997	Avdiukhina et al.	F	27	Simferopol	lower eyelid
20	1997	Avdiukhina et al.	M	60	Odessa province	lower eyelid.
21	1997	Postnova et al.	M	55	Soci	neck
22	1997	Postnova et al.	M	60	Belgorod Dniestrovski	lower eyelid
23	1997	Postnova et al.	F	adult	Belgorod Dniestrovski	groin
			AFRICA			
Kenya or Senegal						
1	1997	Orihel et al. (surgery in the States)	M	42	n.s.	periocular region*
* Two worms were recovered at ten months apart between each other.						
Tunisia						
1	1990	Chaabouni et al.	M	55	Kairouan	subconjunctival
2	1995	Ben Said et al.	F	39	Gabès	axillary region
3	1995	Ben Said et al.	F	48	Sousse	forehead
4	1999	Mrad et al.	F	32	n.s.	breast
			ASIA			
Georgia						
1	1967	Kamalov	M	n.s.	Grusiya	upper eyelid
2	1967	Kamalov	F	36	Vostocnaya Grusyia	upper eyelid
India						
1	1999	Senthilvel & Pillai	F	39	Ottapalan (Kerala)	lip
Iran						
1	1996	Degardin & Simonart (surgery in Belgium)	M	26	n.s.	leg*
* Long persistance (5 years) 3 worms in one nodule						
Israel						
1	1999	Stayerman et al.	M	35	Safed?	penis
Kazakhstan						
1	1961	Mizkevi and Leontieva	F	25	n.s.	lower eyelid
2	1970	Lubova	F	52	Ksil Orda	lower eyelid
3	1970	Lubova	F	57	Ksil Orda	thoracic wall
4	1985	Tasberghenova and Anbakirova	M	13	Alma Ata	subconjunctival
5	1992	Glinciuk et. al.	F	43	n.s.	eye, preretinic*
* Long persistance (5 years) 3 worms in one nodule						

continued

No.	Date	Author(s)	Sex	Age (yrs)	Locality	Location
Malaysia						
1	1996	Shekhar et al.	M	48	Penang	inguinal lymphonode*
* Both male and female worms present in the nodule.						
2	1996	Shekhar et al.	M	39	Melaka	cervical lymphonode
Russia (Siberia inclusive)						
1	1954	Koroiev	F	23	Northern Caucasus	upper eyelid
2	1971	Prosvetova et al.	n.s.	n.s.	n.s.	n.s.
3	1977	Kondrazkii and Parkomienko	F	46	Voronez province	lower eyelid
4	1981	Merkusceva et al.	M	46	Astrakhan province	lower eyelid
5	1991	Maximova et al.	F	41	Tula region	zygomatic region
6	1993	Plotnikov et al.	n.s.	n.s.	Tomsk (Siberia)	subconjunctival
7	1993	Plotnikov et al.	n.s.	n.s.	Saratov province	subconjunctival
8	1993	Avdiukhina et al.	M	35	Vladivostock (Siberia)	submandibular
9	1995	Asarova et al.	F	37	Barnaul (Siberia)	upper eyelid
10	1995	Asarova et al.	n.s.	n.s.	Barnaul (Siberia)	eye region (n.s.)
11	1995	Asarova et al.	n.s.	n.s.	Barnaul (Siberia)	eye region (n.s.)
12	1995	Asarova et al.	n.s.	n.s.	Barnaul (Siberia)	eye region (n.s.)
13	1996	Artamonova and Nagornyi	n.s.	n.s.	Northern Caucasus	n.s.
14	1996	Avdiukhina et al.	F	13	Astrakhan province	upper eyelid
15	1996	Avdiukhina et al.	F	36	Astrakhan province	upper eyelid
16	1996	Avdiukhina et al.	F	50	Astrakhan province	subconjunctival
17	1996	Avdiukhina et al.	F	42	Astrakhan province	subconjunctival
18	1996	Avdiukhina et al.	F	61	Astrakhan province	subconjunctival
19	1996	Avdiukhina et al.	F	49	Astrakhan province	periocular region
20	1996	Avdiukhina et al.	F	26	Astrakhan province	lower eyelid
21	1996	Avdiukhina et al.	F	33	Astrakhan province	periocular region
22	1996	Avdiukhina et al.	M	23	Krasnodar	upper eyelid
23	1996	Avdiukhina et al.	F	49	Astrakhan province	periocular region
24	1996	Avdiukhina et al.	F	26	Astrakhan province	lower eyelid
25	1996	Avdiukhina et al.	F	33	Astrakhan province	periocular region
26	1996	Avdiukhina et al.	F	37	Barnaul (Siberia)	upper eyelid
27	1996	Avdiukhina et al.	M	23	Krasnodar province	upper eyelid
28	1997	Avdiukhina et al.	F	55	Krasnodar province	lower lip
29	1997	Avdiukhina et al.	F	58	Krasnodar province	subconjunctival
30	1997	Avdiukhina et al.	F	57	Krasnodar province	nape
31	1997	Avdiukhina et al.	M	38	Krasnodar province	thoracic wall
32	1997	Avdiukhina et al.	M	23	Krasnodar	upper eyelid
33	1997.	Avdiukhina et al.	M	50	Volgograd	hip
34	1997	Avdiukhina et al.	F	adult	Barnaul (Siberia)	breast
35	1997	Avdiukhina et al.	F	adult	Barnaul (Siberia)	breast
36	1997	Avdiukhina et al.	M	38	Volgograd	thoracic wall
37	1997	Avdiukhina et al.	M	n.s.	Barnaul (Siberia)	shoulder
38	1997	Avdiukhina et al.	M	n.s.	Barnaul (Siberia)	hip
39	1997	Postnova et al.	F	55	Soci	neck
40	1997	Postnova et al.	F	27	Moskow province	lower eyelid
41	1997	Postnova et al.	F	46	Astrakhan province	elbow
42	1997	Postnova et al.	F	41	Astrakhan province	hand

continued

No.	Date	Author(s)	Sex	Age (yrs)	Locality	Location
43	1997	Postnova et al.	F	56	Astrakhan province	shoulder
44	1997	Postnova et al.	F	58	Astrakhan province	knee
45	1997	Postnova et al.	M	58	Astrakhan province	hand
46	1997	Postnova et al.	F	39	Astrakhan province	forearm
47	1997	Postnova et al.	M	31	Astrakhan province	cheek
48	1997	Postnova et al.	F	13	Astrakhan province	upper eyelid
49	1997	Postnova et al.	F	50	Astrakhan province	subconjunctival
50	1997	Postnova et al.	F	36	Krasnodar or Soci	subconjunctival
51	1997	Postnova et al.	F	34	Astrakhan province	forearm
52	1997	Postnova et al.	F	36	Astrakhan province	shoulder
53	1997	Postnova et al.	F	42	Astrakhan province	upper eyelid
54	1997	Postnova et al.	F	61	Astrakhan province	subconjunctival
55	1997	Postnova et al.	F	30	Astrakhan province	thoracic wall
56	1997	Postnova et al.	F	18	Astrakhan province	cheek
57	1997	Postnova et al.	M	23	Astrakhan province	upper eyelid
58	1997	Postnova et al.	F	49	Astrakhan province	upper eyelid
59	1997	Postnova et al.	F	26	Astrakhan province	upper eyelid
60	1997	Postnova et al.	F	35	Astrakhan province	soft palate
61	1997	Postnova et al.	F	33	Astrakhan province	cheek

Sri Lanka

[abbreviations: CP = Central Province; EP = Eastern Province; NP = Northern Province; NCP = North Central; Province; NWP = North Western; Province; SP = Southern Province; SabP = Sabaragamuwa Province; UP = Uva Province; WP = Western province]

0	1976	Wijesundera	F	32	Moragolla (CP)	forearm
0	1976	Wijesundera	F	n.s	Kandy (CP)	eye region (n.s.)
0	1980	Wijesundera	M	19	n.s.	scrotum
0	1980	Wijesundera	F	63	Wattala (WP)	breast
0	1988	Wijesundera	M	1.6	Waharaka (SabP)	scrotum
1	1997	Dissanaike et al.	M	4 m	Pannipityia (WP)	scrotum
2	1997	Dissanaike et al.	M	5	Angola (WP)	subconjunctival
3	1997	Dissanaike et al.	M	27	Karainagar (NP)	forearm
4	1997	Dissanaike et al.	M	38	Colombo (WP)	forearm
5	1997	Dissanaike et al.	M	43	Batticaloa (EP)	subconjunctival
6	1997	Dissanaike et al.	F	1	Colombo (WP)	foot
7	1997	Dissanaike et al.	M	n.s.	SP	wrist
8	1997	Dissanaike et al.	M	6	Waharaka (SabP)	perianal region
9	1997	Dissanaike et al.	F	22	Embilipitiya (Sab. P)	lower eyelid
10	1997	Dissanaike et al.	F	2	Moratuwa (WP)	upper eyelid
11	1997	Dissanaike et al.	F	72	Colombo (WP)	subconjunctival
12	1997	Dissanaike et al.	F	54	n.s.	subconjunctival
13	1997	Dissanaike et al.	M	10	Gandara (SP)	abdominal wall
14	1997	Dissanaike et al.	M	3	Lunuwila (NWP)	cheek
15	1997	Dissanaike et al.	M	60	Kolonnawa (WP)	subconjunctival
16	1997	Dissanaike et al.	M	4	Mirigama (WP)	scrotum
17	1997	Dissanaike et al.	F	10 m	Kuliyapitiya (NWP)	cheek
18	1997	Dissanaike et al.	M	28	Tissamaharama (SP)	cheek
19	1997	Dissanaike et al.	M	9 m	Ambalangoda (SP)	scrotum
20	1997	Dissanaike et al.	M	1.4	Galle (SP)	scrotum
21	1997	Dissanaike et al.	M	6 m	Wanduramba (SP)	penis
22	1997	Dissanaike et al.	n.s.	n.s.	Hikkaduwa (SP)	subconjunctival
23	1997	Dissanaike et al.	M	40	Kandy (CP)	thumb

continued

No.	Date	Author(s)	Sex	Age (yrs)	Locality	Location
24	1997	Dissanaike et al.	M	5 m	n.s.	subconjunctival
25	1997	Dissanaike et al.	M	5 m	Kandy (CP)	subconjunctival
26	1997	Dissanaike et al.	F	6	CP	eye region (n.s.)
27	1997	Dissanaike et al.	n.s.	n.s.	CP	eye region (n.s.)
28	1997	Dissanaike et al.	F	52	Kandy (CP)	breast
29	1997	Dissanaike et al.	M	3	Ragama (WP)	scrotum
30	1997	Dissanaike et al.	M	40	CP	lower eyelid
31	1997	Dissanaike et al.	M	2.2	Kadugannawa (CP)	scrotum
32	1997	Dissanaike et al.	M	36	CP	groin
33	1997	Dissanaike et al.	M	40	Undugoda (Sab.P)	eye (n.s.)
34	1997	Dissanaike et al.	F	43	Gambola (CP)	eye (n.s.)
35	1997	Dissanaike et al.	M	1.6	Batapola (SP)	scrotum
36	1997	Dissanaike et al.	M	1.2	Panadura (WP).	penis
37	1997	Dissanaike et al.	M	11	SP	face
38	1997	Dissanaike et al.	M	11	Deraniyagala (Sab.P)	neck
39	1997	Dissanaike et al.	F	33	n.s.	peritoneum
40	1998	Kumarasimghe et al.	F	21	Jaffna (NP)	forearm
41	1998	Dissanaike p.c.	F	18	Rajagiriya (WP)	subcutaneous n.s.
42	1998	Dissanaike p.c.	F	7 m	Apura (NCP)	sclera
43	1998	Dissanaike p.c.	M	11	Agalawatte (WP)	neck
44	1998	Dissanaike p.c.	F	65	Katunayake (WP)	subconjunctival
45	1998	Dissanaike p.c.	M	1.6	Negombe (WP)	n.s.
46	1998	Dissanaike p.c.	M	1	n.s.	scrotum
47	1998	Dissanaike p.c.	M	1.3	n.s.	spermatic chord
48	1998	Dissanaike p.c.	M	1.4	Ambalantota (SP)	spermatic chord
49	1998	Dissanaike p.c.	M	50	Negombo (WP)	ankle
50	1998	Dissanaike p.c.	F	30	Agalawatte (WP)	forearm
51	1999	Dissanaike p.c.	M	21	Kekirawa (NCP)	scalp
52	1999	Dissanaike p.c.	F	53	Kandy (CP)	eyelid
53	1999	Dissanaike p.c.	n.s.	n.s.	n.s.	n.s.
54	1999	Dissanaike p.c.	M	2.6	WP	n.s.
55	1999	Dissanaike p.c.	F	11 m	Homagama (WP)	scapular region
56	1999	Dissanaike p.c.	F	1.7	Negombo (WP)	scapular region
57	1999	Dissanaike p.c.	n.s.	n.s.	n.s.	n.s.
58	1999	Dissanaike p.c.	F	9	n.s.	hand
59	1999	Dissanaike p.c.	F	41	Badulla (UP)	neck
60	1999	Dissanaike p.c.	M	1.1	WP	scrotum
61	1999	Dissanaike p.c.	F	1.7	Negombo (WP)	scapular region
62	1999	Dissanaike p.c.	M	2.3	n.s.	scrotum
63	1999	Dissanaike p.c.	M	48	Walasmulla (SP)	knee
64	1999	Dissanaike p.c.	M	7 m	WP	scrotum
65	1999	Dissanaike p.c.	M	4.6	WP	scrotum
66	1999	Dissanaike p.c.	F	2.3	Kandy (CP)	thumb
67	1999	Dissanaike p.c.	F	55	Kandy (CP)	subconjunctival
68	1999	Dissanaike p.c.	F	30	Kandy (CP)	subconjunctival
69	1999	Dissanaike p.c.	F	30	Kandy (CP)	hip
70	1999	Dissanaike p.c.	M	35	Kandy (CP)	forehead
71	1999	Dissanaike p.c.	M	1,8	WP	scrotum
72	1999	Dissanaike p.c.	M	4.5	Matugama (WP)	hip
73	1999	Ratnatunga and Wijesundera	M	16	n.s.	abdominal wall
74	1999	Ratnatunga and Wijesundera	F	41	n.s.	thigh
75	1999	Ratnatunga and Wijesundera	F	65	n.s.	cheek
76	1999	Ratnatunga and Wijesundera	M	48	n.s.	thoracic wall

continued

No.	Date	Author(s)	Sex	Age (yrs)	Locality	Location
77	1999	Ratnatunga and Wijesundera	F	45	n.s.	breast
78	1999	Ratnatunga and Wijesundera	M	5	n.s.	subconjunctival
79	1999	Ratnatunga and Wijesundera	M	16	n.s.	forearm
80	1999	Ratnatunga and Wijesundera	M	30	n.s.	thoracic wall
81	1999	Ratnatunga and Wijesundera	M	40	n.s.	temporal region
82	1999	Ratnatunga and Wijesundera	F	34	n.s.	forehead
83	1999	Ratnatunga and Wijesundera	F	22	n.s.	thoracic wall
84	1999	Ratnatunga and Wijesundera	M	1.6	n.s.	scrotum
85	1999	Ratnatunga and Wijesundera	M	45	n.s.	cheek
86	1999	Ratnatunga and Wijesundera	M	1.6	n.s.	scrotum
87	1999	Ratnatunga and Wijesundera	n.s.	n.s.	n.s.	n.s.
88	1999	Ratnatunga and Wijesundera	n.s.	n.s.	n.s.	n.s.
89	1999	Fernando et al.	M	3.4	Laxapana (CP)	scrotum*
* Two nodules with a male and female worms each.						
90	1999	Pitakotuwage et al.	F	80	Ampara (EP)	cheek
91	1999	Dissanaike, p.c.	F	35	Galle (SP)	spermatic chord
92	2000	Dissanaike, p.c.	M	7m	n.s.	n.s.
93	2000	Dissanaike, p.c.	n.s.	n.s.	n.s.	n.s.
94	2000	Dissanaike, p.c.	n.s.	n.s.	n.s.	n.s.
95	2000	Dissanaike, p.c.	M	1	n.s.	forearm
96	2000	Dissanaike, p.c.	F	n.s.	Kandy (CP)	n.s.
97	2000	Dissanaike, p.c.	F	8	Ampara (EP)	n.s.
98	2000	Dissanaike, p.c.	M	1.4	Homagama (WP)	eye region (n.s.)
99	2000	Dissanaike, p.c.	M	n.s.	EP	testis*
* As Professor Dissanaike claims, the location of this case is doubtful, being probably into the epidydimis.						
100	2000	Dissanaike, p.c.	F	50	Narahenpita (WP)	subconjunctival
101	2000	Dissanaike, p.c.	F	50	Maharagama (WP)	subconjunctival
Turkey						
1	1997	Otkun et al.	F	44	Edirne	Edirne
Turkmenistan						
1	1970	Nurliyev	n.s.	n.s.	n.s.	n.s.
Uzbekistan						
1	1961	Mizkievic & Leontieva	F	35	Tashkent	crystalline*
* The diagnosis of *D. repens* was only presumed.						

Histopathology

In an important work on parasites affecting human tissues, Orihel and Ash (1995) present a wide range of histological sections illustrating the various Dirofilariae including *D. repens*, that can infect man. Ratnatunga and Mijesundera (1999) have described the histopathologic features observed in Sri Lanka in 14 subcutaneous nodules due to *D. repens*. Two fully illustrated works on the histopathologic features of the lesions caused by the parasite in man have been recently published by Pampiglione et al. (1999e, 2000b): the first is particularly concerned with the difficulties a histopathologist may encounter in his attempt to arrive at a correct diagnosis of the species when the nematode is in a more or less advanced stage of decomposition; the second takes 60 new cases and classifies the histopathological features into four basic groups, which appear to depend on the length of time spent by the nematode in the host's tissues and on the defence reaction triggered by it. In the first group, which includes the majority of cases, the phlogistic reaction is of an abscess-like type due to the presence of necrotic matter containing neutrophil and eosinophil leukocytes and less numerous chronic inflammatory cells surrounding the nematode.

A reactive granulation tissue is around, while acute and chronic inflammatory cells infiltrate the surrounding soft tissues. In the second group, the central zone containing the nematode is delimited by an actual vallum consisting of epithelioid cells, histiocytes and occasional plurinucleate giant cells as of a foreign body. In the third, less numerous, group the nematode is in a state of more or less advanced decomposition and is surrounded by scant mixed necrotic matter and occasional inflammatory cells. This area is delimited by reactive tissue forming a dense fibrous ring of mainly fibroblastic tissue. As in the previous cases, the surrounding soft tissues display an aspecific acute and chronic phlogistic reaction. In the fourth group, comprising just a few cases, the histological pictures feature a dense inflammatory infiltrate consisting almost entirely of lymphoid elements, sometimes massing together to form germinative centres. This reactive tissue diffuses throughout the surrounding soft tissues. In the cases affecting the lungs, the nematode is almost always in an advanced state of decomposition inside a thrombosed arteriole giving rise to a small, roundish infarctual zone. The adjacent pulmonary parenchyma displays mainly acute inflammatory infiltrates with a redominance of eosinophils. Flieder and Moran (1999), in a documented article on human pulmonary dirofilariasis, report on a clinicopathological study of 41 infarctual nodules observed in the USA. Even though the cases are all due to *D. immitis*, the study is very pertinent to *D. repens*, for the clinical and histopathological pictures of the two species do not differ greatly, apart from the morphology of the parasites. Following on from the research of previous authors (Schaub and Rawlings, 1979; Kaiser et al., 1992), it has recently been shown that filarial parasites alter pulmonary artery endothelial cell relaxation. Thus, vasoconstriction may play an important role in tissue necrosis in pulmonary

human dirofilariasis (Mupanamunda et al., 1997).

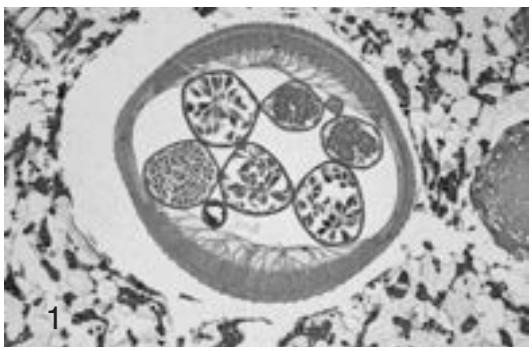
The histological findings relating to nodules localised in the breast, epididymis, spermatic chord, omentum and mesentery overlap: the nematode is immersed in fibrin-leukocyte necrotic material surrounded by demarcation tissue consisting of fibroblast elements together with lymphocytes, plasma cells and eosinophils.

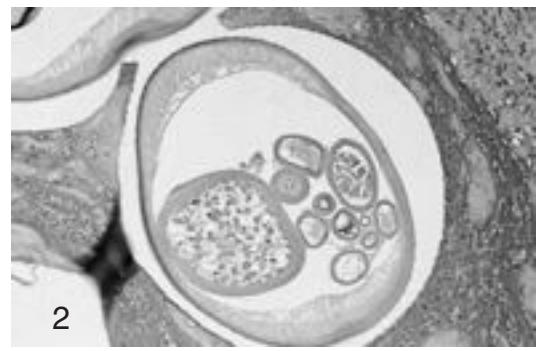
Parasitology

The samples comprised mostly immature females from a few centimetres to 150 mm in length and with a maximum thickness of μm 620. At times, the samples were already dead on surgical removal; at others, they had been partially destroyed by the inflammatory reaction of the host. Often, however, they were still alive, as could be seen from their lively movements; indeed, Dissanaike et al. (1997) found this to be so in 42 out of 70 cases observed.

In Italy, the ratio between living and dead *D. repens* observed by the authors was practically the same, although it was not always possible to be sure judging by the histological preparation. In 2 cases, a male and a female were found together in the same nodule (Misic et al., 1996; Mrad et al., 1999), and in another case a male and a female were found in 2 separate nodules but close to each other in the skin of the scrotum (Fernando et al., 2000). The presence of 3 nematodes in the same nodule was reported by Degardin and Simonart (1996) in a patient from Iran and by Avdiukhina et al. (1997) in another patient in the Russian Federation. It has been known that, after being extracted from the nodule, *D. repens* is capable of surviving for up to 48 hours in physiological solution at 4°C (Thérizol-Ferly et al., 1996). In some rare cases it was noted that the histopathological transverse sections of females of *D. repens* inexplicably revealed an unusual number (up to ten or more) of sex tubules (Figs. 1 and 2) (Pampiglione et al., 1992, 1996). Orihel et al. (1997) have now provided an explanation. They were examining 2 females of *D. repens* extracted from the same site in the same patient, but at an interval of 10 months.

They deduced correctly that the two





Figs. 1 and 2. Paratransverse sections of *D. repens* where numerous sections of female sex tubules are visible (Haematoxylin/Eosin, 250). [Fig. 1: courtesy by Blackwell Ltd, Histopathology (in press)].

parasites must have been introduced by the carrier at the same time and that therefore one was older than the other. They explain that, as a female matures, her vagina lengthens, becomes coiled and looped and extends towards the head beyond the vulva and oesophagus. If the nematode is sectioned above that point, the sex tubules can thus appear to be very numerous. Orihel and Eberhard (1998) have helped to clarify another important morphological aspect for the recognition of *D. repens* in histological section: whereas Gutierrez (1990) stated that "key figures useful in identification of the species include longitudinal ridges separated by a distance wider than the ridge itself, 95-105 ridges on the circumference of the body", Orihel and Eberhard point out that "the shape, height and interridge distances are actually quite variable at different levels of the body in the same worm or even within a single transverse section and hence do not constitute reliable criteria".

Another morphological feature whose relative value needs to be reassessed is that of the diameter of the nematode's body in transverse section. Apart from the possible variation in diameter depending on the stage of development reached, on eventual regressive alterations and on shrinkage due to the worm being killed by the fixative while still alive inside the nodule (as can often be seen from the empty space created around it) (Fig. 3), the diameter will normally vary by many microns in the same individual, depending on the point at which the section is taken (Fig. 4).

From a review of the different species of *Dirofilaria* existing worldwide (Canestri Trotti et al., 1997) it appears that, of the 27 species considered valid, only 5 have been reported as having infected man, apart from *D. immitis* and *D. repens*, namely: *D. (N.) tenuis*, a parasite of the racoon in North America; *D. (N.) ursi*, a parasite of the brown bear in Canada, the northern USA, Siberia and Japan; *D. (D.) spectans*, a parasite of Mustelidae in Brazil; *D. (N.) magnilarvatum*, a parasite of catarrhine monkeys in Asia; *D. (N.) striata*, a parasite of the puma and other American carnivores. The specimens of *D. ursi* found in human cases

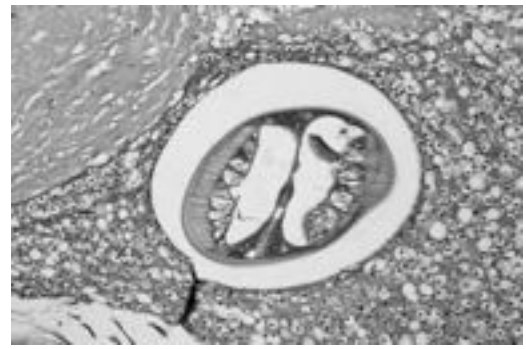


Fig. 3. Transverse section of *D. repens*. The empty space around the nematode is due to shrinkage of its body diameter (Haematoxylin/Eosin, 250).

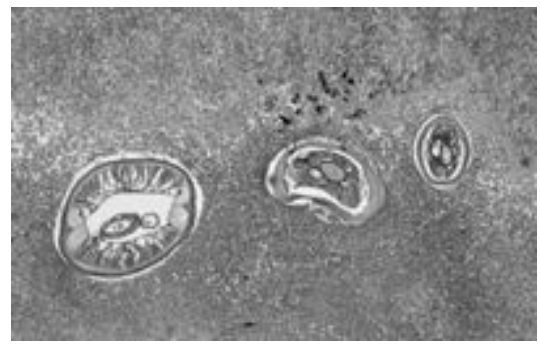


Fig. 4. Three transverse sections of *D. repens*. Note the difference in diameter in the same specimen sectioned at different points of the body (Haematoxylin/Eosin, 150).

have been termed *D. ursi*-like, since there is no absolute certainty that we are dealing with a single species, either for bears or for man, even though in histological section they appear to be practically identical from a morphological point of view. Since the zoonotic aspects of dirofilariasis in Africa are little known, future research into the study of findings from that continent will bear in mind *D. (N.) corynodes* (a frequent parasite of African monkeys), given its great similarity to *D. repens* (Orihel, 1969; Orihel and Eberhard, 1998).

On examining the peripheral blood of a 64-year old patient living on a Greek Island in the Ionian sea (western Greece) Petrocheilou et al. (1998) discovered microfilariae measuring 210-230 μm in length (and therefore not matching either *D. repens* or *D. immitis*), which they attribute to the *Dirofilaria*-like genus, but without being able to find the adults. In Sri Lanka, rare cases of subcutaneous dirofilariasis due to a different species but still belonging to the sub-genus *Nochtiella*, *D. linstowi* (Dissanaike, 1972), have been reported in man in jungle areas, home to the monkeys *Presbytis entellus* and *Macaca sinica* which are its natural reservoir. Since it is difficult to differentiate between the two species, some cases due to this latter species could have been interpreted as being due to *D. repens* (Abeyewickreme et al., 1997).

Studies of great interest have recently shown the presence in *D. repens* of *Wolbachia* sp., intracellular bacteria already reported in insects and filariae, including *D. immitis* (McLaren et al., 1975), and transmitted transovarially. They are held to play an important role in the embryogenesis of filariae (Genchi et al., 1998), and so their suppression with tetracycline is supposed to inhibit the development of the microfilariae.

Biomolecular techniques have recently been employed (Casiraghi et al., 2000; Favia et al., 2000) for filogenetic analysis. These authors have compared 10 different species of Onchocercidae, among which *D. repens*, and confirm the clustering of the species of the genus *Onchocerca* with those of the genus *Dirofilaria*.

Diagnosis

The clinical diagnosis of the parasitosis is almost always wrong, except for some subconjunctival cases where the oculist can see the nematode, given that the conjunctiva is transparent and so is able to diagnose a "helminth parasitosis", mistaken sometimes for onchocerciasis. In cases of pulmonary infection, clinical diagnosis has been that of cancer or sarcoidosis (Jelinek et al., 1996) and thoracotomy was always carried out; a spermatic chord location, erroneously interpreted as a tumour or testicular tuberculosis, was treated with orchiectomy (Pampiglione et al., 1999a); in breast infections, diagnosis of cancer or fibrous dysplasia of the breast was also frequently suspected. In a case of retroocular infection, the doctors diagnosed tumour of the orbit (Braun et al., 1996). In another case of localisation in the vitreous body, the clinical diagnosis was that of disseminated chorioretinitis of probable parasitic origin (Vasilkova et al., 1992). In subcutaneous forms the most frequent diagnosis has been that of sebaceous

cyst; other diagnoses have included lipoma, dermoid cyst, fibroadenoma, dental abscess, muscular haematoma, trauma-induced detachment of the *temporalis* muscle, collagen disease, angio-oedema, atypical Beliset syndrome, periodical disease, *larva migrans*, recurrent thrombophlebitis, rheumatic disease, herpetic keratitis, tendinous cyst, neuroma, neurofibroma, inguinal lymphadenitis, cervical lymphadenitis, filariasis due to *Wuchereria bancrofti*, onchocerciasis, loiasis, tumour of the parotid gland and, in two cases, acute psychosis (Jelinek et al., 1996).

With regard to the differential diagnosis of the parasite, there is an article by Orihel and Eberhard (1998) that reports, with photographic illustrations, the morphological data of the various zoonotic filariae capable of infecting man by targeting the subcutaneous tissues and subconjunctiva, the heart and pulmonary vessels, the lymphatic and nervous systems.

In this same context, there are two very recent reports of other zoonotic filariae: *Brugia* sp., probably *ceylonensis*, localised under the conjunctiva of a 53-year-old patient in Sri Lanka (Dissanaike et al., 2000) and *Macacanema formosana*, also localised under the conjunctiva in a 23-year-old woman in Taiwan (Lin-Ing Lau, personal communication), neither of which species has ever previously been reported in that site in man and which can be borne in mind in the diagnostic differentiation of *D. repens*. Fine Needle Aspiration Biopsy has proved successful in only 2 cases, one affecting the breast (Bertoli et al., 1997), the other subcutaneous tissue.

In the latter case, however, the nematode revealed itself only because it emerged spontaneously through the hole made by the needle (Kumarasinghe et al., 1997). But generally it is not recommended in dirofilariasis, as demonstrated by the American statistics for lung infections due to *D. immitis*, in which the outcome has invariably been negative (Flieder and Moran, 1999).

In the majority of cases, diagnosis is based on histological examination of the nodule with identification of the morphological features of the nematode.

The presence of the external longitudinal cuticular ridges has proved essential for the diagnosis of the subgenus *Nochtiella* and for its differentiation from *D. immitis*, which is the most common of the other zoonotic Dirofilariae. The most useful stains, apart from the common haematoxylin/eosin, for the purpose of highlighting the morphological details have proved to be PAS and Masson Goldner's trichrome. In cases in which the parasite had been dead for many months or years, histological diagnosis was more difficult; only a careful analysis of the remains of the nematode's body and the existence at the same time of other recognisable sections of it enabled a diagnosis to be made (Pampiglione et al., 1999e).

There have been numerous studies carried out by various groups of researchers aimed at perfecting a reliable and practical diagnostic reaction on histological sections both of the parasite and the carriers (Chandrasekharan et al., 1994; Favia et al., 1996a,b, 1997, 1998, 2000a,b,c; Cancrini et al., 1998a,b, 2000; Favia, 1999; Ricci et al., 2000).

Although they have succeeded in

developing a PCR on fresh tissues or those fixed in ethyl alcohol, they have not been able to reproduce the test on tissues fixed in formalin. This is a serious drawback, for biopsies are normally sent to the histologist already fixed in 10% formalin. And since the diagnosis of dirofilariasis is hardly ever suspected beforehand, it is virtually impossible to get the surgeon doing the biopsy to fix the excised nodule in methyl alcohol or send it fresh to the laboratory without any form of fixation. However, Vakalis et al. (1999) have developed a PCR technique which, in their hands, seems to have given good results on tissues fixed in formalin as well.

As regards serological diagnosis, progress has undoubtedly been made by research groups of various nationalities, notably Spanish and Italian (Perera et al., 1994, 1998; Favia et al., 1996, 1997, 2000; Simon et al., 1997; Cancrini et al., 1998, 1999), but they have not yet been able to perfect a reaction that is reliable, quick and simple to perform.

However, Ruiz Moreno et al. (1998) did succeed in obtaining confirmation of recovery in a case of human subconjunctival dirofilariasis by measuring the reduction in the level of anti-*D. repens* serous antibodies by immunoenzymatic means for 3-6 months from the time of surgery.

Prognosis

In the majority of subcutaneous cases the prognosis can be considered benign, the lesion healing in a few days following the removal of the nodule. Sometimes, healing occurs spontaneously with the worm emerging from the nodule without any surgical intervention. In cases affecting the ocular region, however, Avdiukhina et al. (1996) report a 10% incidence of complications of a permanent nature, such as detached retina, glaucoma, opacity of the vitreous body or the crystalline lens, or other deterioration in visual acuity. In cases affecting the visceral organs (lungs, mesentery, omentum) or the sexual organs (epididymis, spermatic chord, scrotum, female breast), surgery (open-chest, laparotomy) can have relatively serious consequences or cause pointless mutilation, such as pulmonary lobectomy (Jelinek et al., 1996; Pampiglione et al., 2000d) or the removal of a breast, epididymus or spermatic chord.

Prophylaxis

Effective prevention of parasitosis had already been achieved in dogs exposed to natural infection in endemic zones of central Italy by treatment with ivermectine per os (>6 mcg/kg) once a month for 7 consecutive months from May to November (Marconcini et al., 1993). At present, the use of ewable tablets of ivermectine/pirantel pamoate is yielding good results in dogs exposed to natural infection (Pollono et al., 1998).

In man, however, it would appear that no experiments in prevention against dirofilariasis due to *D. repens* or other Dirofilariae have been undertaken. Since the presence of a large number of mosquitoes, which are known carriers of the parasite, and the high incidence of the infection in dogs, which are the natural reservoir of the parasitosis, are considered to be risk factors for man, it can be supposed that a reduction in the incidence of canine infection together

with a drive to eliminate the carriers might considerably reduce the number of human cases in endemic areas. This implies a preliminary study both of the prevalence and distribution of the parasitosis in dogs and of the density and distribution of the carriers.

Therapy

The therapy of choice remains surgery. Whereas both diethylcarbamazine and ivermectine have often been used in cases of canine dirofilariasis, they have only occasionally been used in man (van den Ende et al., 1995; Jelinek et al., 1996; Avdiukhina et al., 1997; Petrocheilou et al., 1998) and not always to any apparent effect. A patient with a nodule on one hand and who had been treated with the two drugs had a second nodule appear on his thigh 4 weeks after the end of treatment (Jelinek et al., 1996). Other drugs used have been levamisole (Dorofeiev et al., 1997), albendazole (van den Ende et al., 1995), thiabendazole and cortisones (Delage et al., 1995), again with doubtful results. That drugs are not in current use is partly due to the fact that the clinical diagnosis of the cases is generally wrong and appropriate therapy is not prescribed. In any case, the use of filaricides with a certain level of toxicity does not seem appropriate in all those cases of subcutaneous infection in which a minor surgical operation performed in outpatients can resolve the problem. The same holds for subconjunctival or periocular infections, where possible violent allergic reactions induced by the drug and/or by the death of the nematode could cause permanent damage to the visual organ.

Discussion

In view of the number of publications that have appeared in the last 20 years, by comparison with previous decades, it is evident that the medical world has gradually become increasingly interested in this zoonosis. This can be attributed not only to the clear increase in the incidence of the disease but also in part to the reports of the parasite infecting the viscera (lungs, mesentery) as well as the female breast and the male genitalia (scrotum, verga, spermatic chord, epididymis). The involvement of these locations has almost always led to diagnoses of forms of malignant tumour requiring drastic surgery and has thus highlighted the importance of the parasite in human pathology. It could be argued that the increase in the number of cases, which until half a century ago were considered exceptional and have been reported with increasing frequency in the last few decades, is only an apparent increase resulting from a more careful examination of subjects affected and from more refined diagnostic techniques. Yet the increase does seem to be in part real, particularly in view of the enhancement in the temperate zones of the climatic conditions (temperature, relative humidity, rainfall, evaporation) that favour not only the growth of the carrier Culicidae (Martin and Lefebvre, 1995) but also the development of the larval phase of the nematode inside the carrier. That this is so is proved by the contemporaneous

increase in the number of dogs infected, at least in endemic zones of Piedmont, as emerges from careful surveys carried out in recent years (Rossi et al., 1996). A similar increase in the number of cases in recent decades has been observed in some republics of the Russian Federation (Avdiukhina et al., 1997). Parasitosis due to *D. repens* can therefore be considered an emergent zoonosis in many geographical areas of the Old World. The number of cases reported worldwide since 1885 has reached 782 spread over 37 different countries, all of which are in the Old World (Fig. 5).

To this must be added a fair number of cases not diagnosed, and therefore not published, others that recovered spontaneously, others occurring in highly endemic zones as in some provinces of Italy (Piedmont), Sri Lanka (Western province) and Russia (Caucasus) and which, being considered by now an everyday complaint, are no longer reported and, finally, those occurring in the developing countries and which, given the lack of facilities for histological diagnosis, are not even screened by the histopathologist. Taking into account the 1995 figures, Italy is still in number one position as regards the number of cases reported (298) between 1885 and 2000, followed by Sri Lanka (132 cases), Russia (Siberia inclusive) (83 cases), France (76 cases), Ukraine (51 cases), Greece (27 cases), Turkey (18 cases), Hungary (11 cases), and then the other countries with less than 10 cases each. However, if we relate these figures to the number of inhabitants and territorial area, Sri Lanka, with a population of 18,300,000 inhabitants and a surface area of 65,610

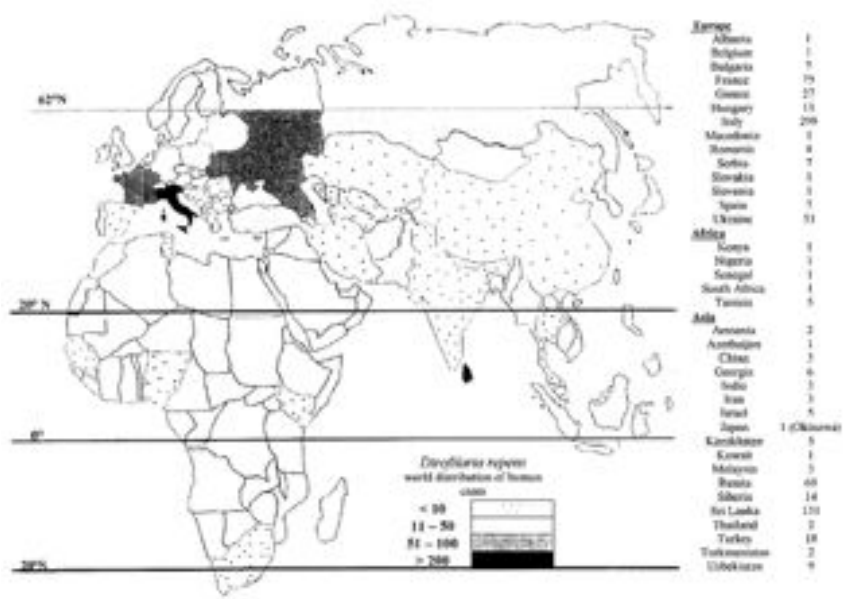


Fig. 5. World distribution of human dirofilariasis due to *D. repens* (cases recorded from 1885 to 2000).

km² (FAO, 1997) is the country most severely affected, while Italy, with a population of 57,200,000 inhabitants and a surface area of 301,278 km², moves down to second place.

The age group most commonly affected comprises adults, mainly between 40 and 49 years of age (Fig. 6), as also reported in the 1995 review. The high number of children affected is due above all to those reported from Sri Lanka, where 33.6% (44 out of 132) referred to children below the age of 10.

Women are more commonly affected than men, even if not statistically significant, as appeared in the previous review, accounting for 55.4% of all cases reported between 1885 and the present day (Fig. 7). Again with reference to overall figures published between 1995 and 2000, the distribution of sites of infection in the human body (Fig. 8) remains much the same as reported in 1995. The majority of cases (75.8%) affect the upper half of the body, particularly the ocular region, which alone accounts for 30.5% of the total; the female breast (5.4%), the male genital organs (6.5%), the lungs (2.6%) and the abdominal viscera, comprising mesentery and omentum (1.3%), also remain relatively important. The marked prevalence of cases reported from Sri Lanka affecting the genitalia, particularly of children (Dissanaike et al., 1997), may be due to the clothing and sleeping habits of the local child population as well as to the special tropisms of the carrier mosquitoes, that are perhaps attracted by ammonia smells and other odours in subjects with low levels of personal hygiene. Of the various dirofilariases in the world that can affect man, that due

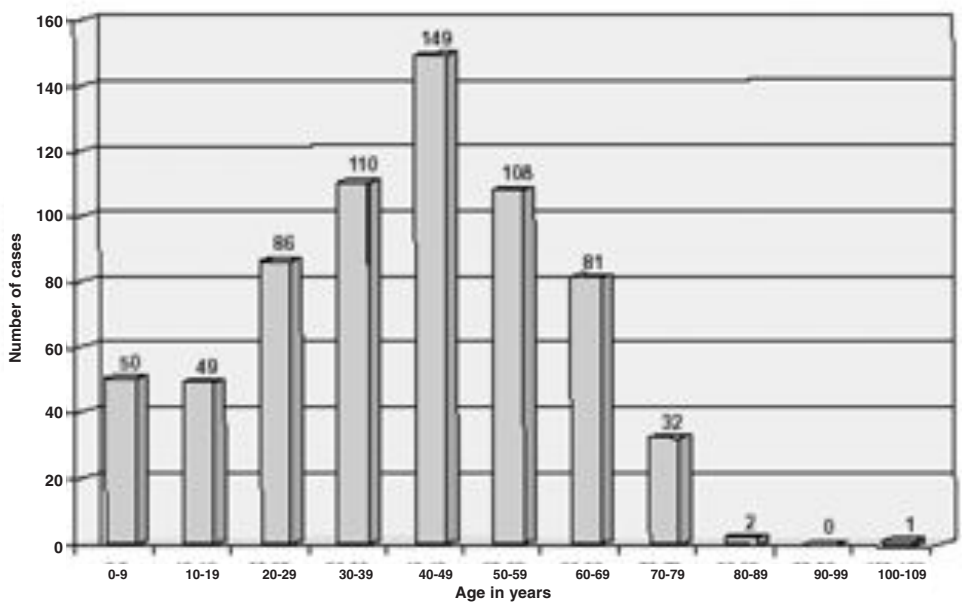


Fig. 6. Distribution of human dirofilariasis due to *D. repens* according to age groups, when specified, from 1885 to 2000.

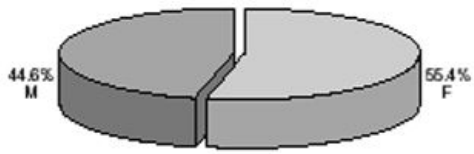


Fig. 7. Distribution of human dirofilariasis due to D. repens according to sex, when specified, from 1885 to 2000.

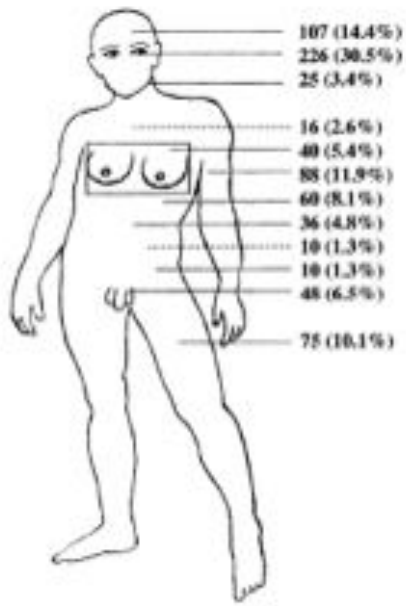


Fig. 8. Locations of *D. repens* in the human body, when specified, from 1885 to 2000 (the dashed lines refer to internal sites).

to *D. repens* is the most common in terms of frequency, diffusion and variety of localisation in the body; the others account overall for fewer than 300 cases. The great variety of organs affected by dirofilariasis due to *D. repens* justifies the current interest among medical practitioners in this zoonosis: specialists in various health sectors, from dermatology to ophthalmology, from urology to pneumology, from internal medicine to surgery, from immunology to molecular biology – they are all involved. But those most directly involved in the study of its aspects and as yet unresolved problems are, of course, the parasitologists and histopathologists. At present, the two sectors in which research teams need to conduct a thoroughgoing investigative inquiry are diagnostics – with a view to developing practical techniques for histological and serological diagnosis of the parasitosis – and therapeutics, in particular to avoid drastic surgery in cases with involvement of lung, breast, male sexual organs or orbital cavity. Diagnostic research will also lead to improved analysis of the prevalence of current cases of human infection, apparent or not, in a given population and thus make for a clear understanding of the mechanisms underlying the relationship between parasites and man.

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8

Heartworm (*Dirofilaria immitis*) disease in dogs

Luigi Venco

Introduction

Despite the name "heartworm" suggests a primitive cardiac involvement, the main localization of the worms and the first damages are in pulmonary arteries and heartworm disease should be considered as a pulmonary disease that in the last stage only involves the right cardiac chambers.

Few days after heartworms reach caudal pulmonary arteries, endothelial cells become swollen with wide intercellular junctions and disoriented longitudinal axes, as response to the trauma. Activates neutrophiles adhere to the endothelium surface and enter the space between endothelial cells. Furthermore, as linear areas of sub endothelium are exposed, platelet adhesion and activation is greatly stimulated. Damaged arterial surface allows albumin, plasma fluid and blood cells to reach the perivascular space.

After the endothelial changes, the intima thickens with fluid, leukocytes invade the wall and smooth muscles cells multiply within tunica media and migrate toward the endovascular surface as response to growth factor released by platelets. The multiplication and migration of smooth muscle cells cause the presence, on the internal arterial surface, of villi which are made by smooth muscle cells and collagen and covered by endothelial like-cells (Rawlings, 1986; Calvert and Rawlings, 1988). The gravity of villous proliferation is directly related to duration of infection and worm burden. The arterial surface of heavily infected dogs and cats appear rough and velvety, and both the lumen and the compliance of the pulmonary arteries are reduced.

Lung disease occurs secondary to vascular changes. Fluid and protein leaking through the vessel wall of affected arteries produce oedema in the parenchyma. Spontaneous death of some worms can produce thromboembolism and severe inflammatory reactions.

The reduction of compliance and gauge of pulmonary arteries, that can be also occluded by either thromboembolism or severe villous proliferation (Fig. 1), results in a hypertensive pulmonary state and, as a consequence, in an increased after load for the right ventricle which can induce "cor pulmonare" and right cardiac congestive heart failure. Protein and fluid leaking through the vessel wall of affected arteries produce further oedema and inflammation in the parenchyma (Dillon et al., 1995).

Based on the pathogenesis the clinical evolution of heartworm disease in dogs is usually chronic. Most infected dogs do not show any symptoms of the disease for a long time, months or years, depending on worm burden, individual reactivity and exercise, as arterial damages are more severe in dogs with

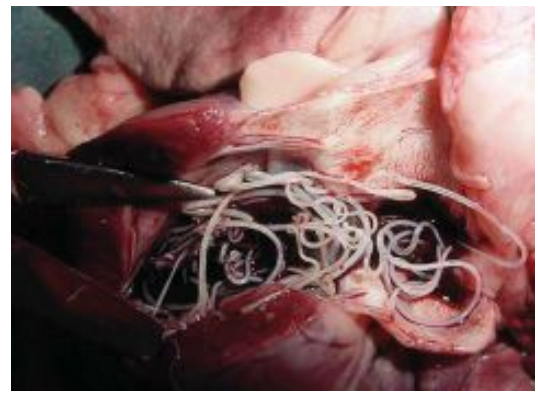

Fig. 1. Right ventricle outflow tract and pulmonary artery of a dog with severe heartworm infections. Note the large worm burden and the villous proliferation of the pulmonary artery endothelium.

intensive exercise than in dogs at rest (Dillon et al., 1995). Signs of the disease develop gradually and may begin with a chronic cough.

Coughing may be followed by dyspnoea, from moderate to severe, weakness, and sometimes lipothymias after exercise or excitement. At this time abnormal pulmonary sounds (crackles) over the caudal lung lobes and second heart sound splitting can be often heard. Later, when right cardiac congestive failure is developing, swelling of the abdomen and sometimes legs from fluid accumulation, anorexia, weight loss, dehydration, is usually noted. At this stage, cardiac murmur over the right side of the thorax due to tricuspid valve insufficiency and abnormal cardiac rhythm due to atrial fibrillation are common findings. Sudden death rarely occurs and usually it happens following respiratory distress or cachexia.

In the chronic pathway of the disease sometimes acute symptomatology may occur. After severe spontaneous thromboembolism following the natural death of many heartworms, dogs may show acute life threatening dyspnoea and haemoptysis.

In small sized dogs is furthermore a common event the displacement of adult worms from pulmonary arteries to right cardiac chambers due to pulmonary hypertension and sudden fall in right cardiac output. In this case dogs affected shows the so called "caval syndrome". Dyspnoea, tricuspid cardiac murmur and emoglobinuria (due to mechanical haemolysis in right cardiac chambers) are the most typical signs and fatal outcome is usual (Kitagawa et al., 1987; Atwel and Buoro, 1988; Venco, 1993).

Diagnosis

Diagnosis of heartworm infection can be made in dogs by blood test detecting circulating microfilariae or adult antigens but further diagnostic procedures are usually required to determine the severity of disease and which is the best treatment (Knight, 1995).

Blood test for microfilariae

Blood sample is examined after concentration (Knott or Difill test) for the presence of microfilariae. If microfilariae are seen and identified as *D. immitis,* based on morphology that is considered a definitive proof of infection (specificity 100 %). However up to 30% of dogs do not have circulating microfilariae even though they harbour adult worms, due to the presence of only worms of the same sex (quite unusual in dogs), immune reactivity of the host to microfilariae or administration of microfilaricidal drugs. The sensitivity of test for microfilariae is not therefore considered sufficient to rule out the infection in case of negative test.

Blood test for adult female antigens

Tests designed to detect heartworm adult antigens based on ELISA or colloidal gold staining techniques are considered highly specific as cross reactivity with other dogs parasites (i.e. *D. repens, Dipetalonema* sp.) does not occur.

These tests allow detection of adult heartworm antigens produced only by female worms and may provide information about worm burden (Knight, 1995; Venco et al., 2003). The sensitiv-

ity is actually very high, but false negative results may occur in prepatent or very light infections or when only male worms are present (McCall, 1992).

Thoracic radiographs

Thoracic radiographs may show, in the advanced stage, enlargement of the pulmonary arteries, abnormal pulmonary patterns and in the worst cases right-sided cardiomegaly. If congestive right heart failure is present peritoneal and pleural effusion can be noted (Rawlings, 1986; Calvert and Rawlings, 1988). They are useful to assess the severity of the pulmonary lesions but not for evaluating worm burden (Venco et al., 2003). Since radiographic signs of advanced pulmonary vascular disease may persist long after an infection has run its course, some of the most severely diseased dogs may have disproportionately low worm burden (Fig. 2). On the contrary, some inactive dogs may have large worm burdens and be clinically asymptomatic with no or trivial radiographic lesions (Fig. 3).

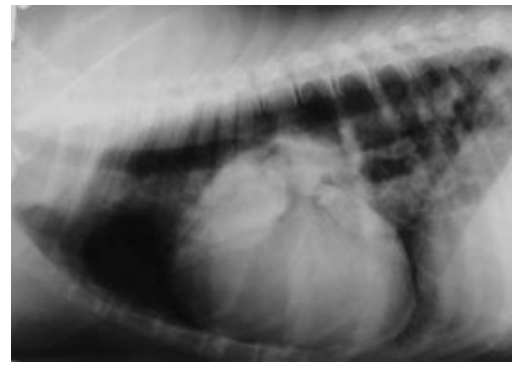

Fig. 2. Thoracic radiograph of a 12-year-old infected dog. With right sided cardiomegaly and severe pulmonary vascular disease. The level of circulating antigens were very low.

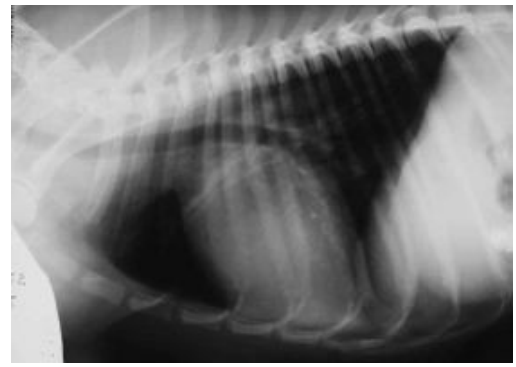

Fig. 3. Thoracic radiograph of a 3 year old infected dog with just a trivial cranial pulmonary artery enlargement. From this dog 53 adult worms were surgically removed.

Electrocardiography

As electrocardiogram displays the electrical activity of the heart, abnormalities, (electrical axis right deviation, atrial fibrillation) are usually found only in the last stage of the disease, when right cardiac chambers present severe damages.

Echocardiography

Echocardiography allows a direct visualization of cardiac chambers and connected vessels (Moise, 1988). It also allows the visualization of parasites in right cardiac chambers, caudal vena cava, main pulmonary artery and proximal tract of both caudal pulmonary arteries (Fig. 4). The heartworms are visualized as double, linear parallel objects floating in the right cardiac chambers or into the lumen of vessels (Moise, 1988; Badertscher et al., 1988). It is performed mainly in cases where clinical and radiographic findings suggest severe disease. Cardiac ultrasound can increase the accuracy in staging the

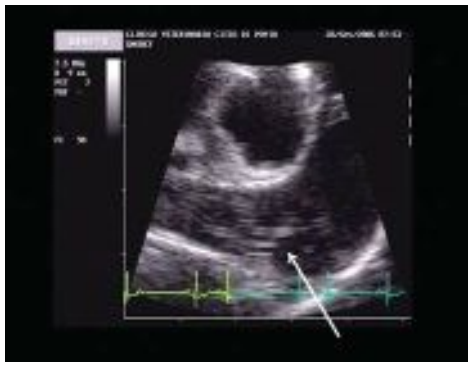

Fig. 4. Echocardiogram of the same dog of Fig. 3. Heartworms are visualized as double, linear parallel objects (arrow) floating into the lumen of the right pulmonary artery.

disease and estimating the worm burden, both of which affect the treatment program and the prognosis.

Therapy

It has been said that the treatment of heartworm infection is difficult. There are several strategies that can be used including the option of not treating at all. The important concept to realize is that treating for heartworm infection is neither simple nor safe in itself.

Prior to therapy, the heartworm patient is assessed and rated for risk of developing post-adulticide thromboembolism.

Previously 4 classes were described in order to prevent the post-adulticide thromboembolism and give a correct prognosis (from class 1, low risk to class 4, very high risk) (Di Sacco and Vezzoni, 1992) but at present a more simple classification is usually preferred and the patient is included into one of two categories (low and high risk). Important factors include: how many worms are thought to be present based upon the ELISA tests performed and ultrasound examination (Venco et al., 2004), the size of the dog, the age of the dog (dogs ranging from 5 to 7 years are at high risk to harbour the largest worm burden; Venco et al., 2004), concurrent health factors, severity of the pulmonary disease, and the degree to which exercise can be restricted in the recovery period.

The categories into which patients are grouped are as follows:

Low risk of thromboembolic complications (low worm burden and no parenchymal and/or pulmonary vascular lesions)

Dogs included in this group must satisfy all this conditions:

- No symptoms
- Normal thoracic radiographs
- Low level of circulating antigens or a negative antigen test with circulating microfilariae
- No worms visualized by echocardiography
- No concurrent diseases
- Permission of exercise restriction

High risk of thromboembolic complications

In this group should be included all the dog that do not satisfy one or more of these conditions:

- Symptoms related to the disease (coughing, lipotimias, swelling of the abdomen)
- Abnormal thoracic radiographs
- High level of circulating antigens
- Worms visualized by echocardiography
- Concurrent diseases
- No permission of exercise restriction.

Symptomatic therapy includes drugs and measures that can improve cardiopulmonary circulation and lung inflation in order to relief symptoms in dogs that cannot undergo causal therapy or to prepare them for a adulticide or surgical therapy.

Restriction of exercise and, in selected cases, cage rest seems to be the most important measure to improve cardiopulmonary circulation and to reduce pulmonary hypertension (Dillon et al., 1995).

Anti-inflammatory doses of glucocorticosteroid (prednisolone 2 mg/kg s.i.d. for four or five days) given at diminishing rate can control pulmonary inflammation and thromboembolism.

Diuretics (furosemide 1 mg/kg b.i.d.) are useful when right congestive heart failure is present to reduce fluid effusions. Digoxin may be administered only to control atrial fibrillation. The use of aspirin is debatable and as not secure proofs of beneficial antithrombotic effect have been reported, for this reason the empiric use of aspirin is not advised (Knight, 1995).

The organical arsenical melarsomine dihydocloride is the only available compound to be used in the adulticide heartworm therapy in dogs.

Two intramuscular injections of 2.5 mg/kg 24 hours apart is the standard regimen, but to reduce the risk of pulmonary thromboembolism a more gradual two step treatment is strongly advised by giving one injection and then administering the standard pair of injections at least 50 days later (Keister et al., 1992; Rawlings and McCall, 1996). In fact, one administration of melarsomine at the dose of 2.5 mg/kg kills about 90% of male worms and 10% of female worms resulting therefore in 50% reduction of the worm burden (which is safer in terms of embolism and shock). For this reasons, the three-injection alternative protocol is the treatment of choice of the American Heartworm Society and several university teaching hospitals, regardless of stage of disease.

Pulmonary thromboembolism is an inevitable consequence of a successful adulticide therapy. If several worms die widespread pulmonary thrombosis frequently develops. Mild thromboembolism may be clinically unapparent, but in severe cases life threatening respiratory distress can occur.

These complication can be reduced by restriction of exercise (no walks, no running around; the dog must stay indoors or, in selected cases, a cage rest) during the 30-40 days following the treatment and by administration of calcium heparin and anti-inflammatory doses of glucocorticosteroid to control clinical signs of thromboembolism (Di Sacco and Vezzoni, 1992; Vezzoni et al., 1992; Rawlings and McCall, 1996).

It is now known that certain macrocyclic lactones have adulticidal properties (McCall et al., 2001).

Experimental studies have shown ivermectin to have partial adulticidal properties when used continuously for at least 16 months at preventative doses (6-12 mcg/kg/month) and 100% adulticidal efficacy if administered continuously for over 30 months (McCall et al., 2001).

While there may be a role for this therapeutic strategy in few and selected cases in which patient age, or concurrent medical problems prohibit melarsomine therapy, the current recommen-

dations are that ivermectin is not adapted as the primary adulticidal approach, and that this kind of therapy should be used carefully. In fact, the adulticide effect of ivermectin generally requires long time span before heartworms are eliminated completely. Furthermore, the older are worms when first exposed to ivermectin, the slower they are to die. In the meantime, the infection persists and continues to cause disease. Clinical observations suggest that heartworm-positive active dogs in prolonged ivermectin treatment may worsen if ivermectin is given monthly for 2 years (Venco et al., 2004).

Surgical therapy is advised when several worm displacement in the right cardiac chambers produces the sudden onset of severe symptoms (caval syndrome). It can be accomplished under general anaesthesia with Flexible Alligator Forceps introduced via jugular vein.

Flexible Alligator Forceps aided by fluoroscopic guidance can access not only right cardiac chambers but also pulmonary arteries. The main pulmonary artery and lobar branches can be accessed with flexible alligator forceps, aided by fluoroscopic guidance (Ishihara et al., 1990). Intra-operative mortality with this technique is very low. Overall, survival and rate of recovery of dogs at high risk of pulmonary thromboembolism is improved significantly by physically removing as many worms as possible. When the facilities are available, worm extraction is the procedure of choice for the most heavily infected and high risk dogs. Before electing this method of treatment, echocardiographic visualization of the pulmonary arteries should be performed to determine if a sufficient number of worms are in accessible locations.

Surgical removal of heartworm can avoid pulmonary thromboembolism, as compared to pharmacologic adulticides, such as melarsomine (Morini et al., 1998). This procedure, however, requires specialized training and instrumentation, including fluoroscopic imaging capabilities. Nevertheless, it remains a very good and a safe alternative for the management of high risk patients and the best choice in dogs harbouring a large worm burden.

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9

Heartworm (*Dirofilaria immitis*) disease in cats

Luigi Venco

Cat is considered a susceptible but not ideal host for *Dirofilaria immitis*. Increased host resistance is reflected by the relatively low adult worm burden in natural infections (cats generally harbour 1 to 8 worms with 2 to 4 worms being the usual burden, Genchi et al., 1992), the low number of heartworms that develop after experimental inoculation with infective larvae, the prolonged pre-patent period (7-8 months), the low level and short duration of microfilaremia, and the short life span of adult worms (2-3 years) (Dillon, 1984; Calvert, 1989; McCall et al., 1992; Atkins et al., 1995).

In cats, changes in pulmonary arteries and lungs after infection seem to be similar to those found in dogs, but right cardiac chambers well bear pulmonary hypertension and right cardiac heart failure is an unusual finding (Dillon et al., 1995; Atkins et al., 1995).

In cats, the clinical presentation is quite different than in the canine counterpart. Most cats seem to well bear the infection for long time. These cats may have a spontaneous self-cure due to the natural death of parasites without any kind of clinical signs or may suddenly show dramatic acute symptoms. Respiratory signs as coughing, dyspnoea, haemoptysis are usually seen but also vomiting frequently occurs. Sudden death in apparently healthy cats is furthermore not rarely observed (McCall et al., 1994; Holmes, 1995; Atkins et al., 1995) (Fig. 1). Chronic symptoms including coughing, vomiting, diarrhoea, weight loss can be less frequently observed.

On the contrary than in dogs, symptomatology related to right ventricular heart failure is not considered consistent with heartworm infection in cats.

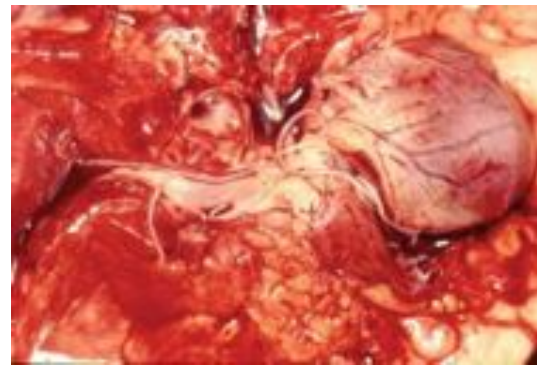

Fig. 1. Two adult female heartworms in a cat that experienced sudden death.

The onset of symptoms in most cases seems to be related to the natural death of parasites or to the first arriving of L5 heartworms in the pulmonary arteries.

Diagnosis

Blood test for microfilariae

As microfilaremia in cats is unlikely, sensitivity of test for detection of circulating microfilariae is very low despite specificity is considered 100% as in dogs (Atkins et al., 1995).

Blood test for adult antigens

Test detecting adult female heartworm antigens can provide a definitive proof of infections in cats because of the very high specificity. Nevertheless because the worm burden is usually very light in cats, infections caused only by male heartworms are not infrequent and symptomatology may be frequently due to immature worms, these test yield false negative result in a large average. A negative test can not therefore considered sufficient to rule out the infection (Atkins et al., 1995).

Blood test for antibodies to adult heartworm

Due to the low sensitivity of tests for circulating microfilariae and adult antigens in cats, test for detection of antibodies to adult heartworm can be useful used (Atkins et al., 1995; Prieto et al., 1997; Genchi et al., 1998). This kind of test has high sensitivity but not complete specificity because of cross reactivity with other parasites or antibodies to abortive infections. Consequently antibody tests should be interpreted carefully, taking other relevant clinical information into consideration.

Thoracic radiographs

Thoracic radiography is an important tool for the diagnosis of feline heartworm disease. Despite thoracic abnormalities in few cases are absent or transient (Selcer et al., 1996), typical findings as enlarged peripheral branches of the pulmonary arteries accompanied by varying degrees of pulmonary parenchymal disease are strongly consistent with heartworm infection (Fig. 2). Enlargement of the main pulmonary artery cannot be observed because this tract of artery is obscured by cardiac silhouette. Right-sided cardiomegaly is not considered a typical finding in cat.

Non selective angiocardiography

Non selective angiocardiography is useful in visualizing the gross morphology of the pulmonary arteries. Seldom the heartworms can be seen as negative filling defects within opacified arteries (Atkins et al., 1995).

Electrocardiography

Heartworm infection does not involve right cardiac chambers. Consequently electrocardiography cannot provide useful information in infected cats.

Echocardiography

Cardiac ultrasound allows the direct visualization of the parasites in right atrium and ventricle, main pulmonary artery and proximal tract of both its peripheral branches (Fig. 3).
Specificity is virtually 100% and sensi-

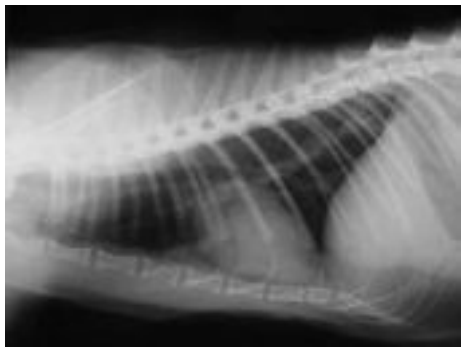

Fig. 2. Thoracic radiograph of a heartworm infected cat with of pulmonary parenchymal disease (caudal lung lobes).

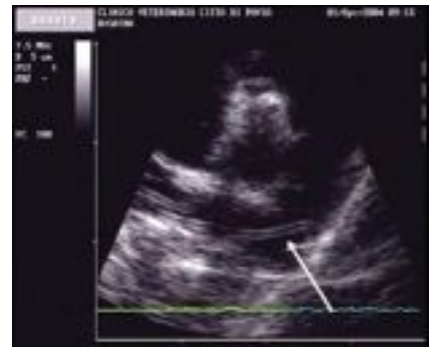

Fig. 3. Echocardiogram of a heartworm infected cat. A worm is visualized as double, linear parallel lines (arrow) into the lumen of the right pulmonary artery.

tivity in cats seems to be very high (Venco et al., 1998, 1999b) because the portion of caudal pulmonary arteries that can not be thoroughly interrogate because of the acoustic impedance of the air inflated lungs is very short when compared with the length of the adult parasite. Based on these considerations, cardiac ultrasonography should be always performed when heartworm infection is suspected.

Transtracheal lavage

The presence of eosinophiles in a tracheal wash, with or without eosinophilia, may be noted 4 to 7 months after infection but this findings is not specific and infection with other pulmonary parasites (*Paragonimus kellicotti*, *Aelurostrongylus abstrusus*) and allergic pneumonitis should be ruled out (Atkins et al., 1995).

Therapy

Diminishing doses of prednisone are advised in cats in order to relief respiratory distress. The dosage is 2 mg/kg daily initially, then declining to 0.5 mg/kg every other day for two weeks, and then discontinuing treatment after an additional two weeks (Atkins et al., 1995).

If crisis is due to embolization of dead worms high doses of prednisone (1-2 mg/kg 3 times a day) are recommended (Dillon, 1986).

In previous studies, when thiacetarsamide was the only arsenical available compound, some treatment regimen was attempt against adult worms. The same dosage and regimen used in treating dogs, 2.2 mg/kg twice daily for two days, was used for cats. The results were debatable. Turner et al. (1989) reported some toxicity in heartworm naive cats, but later reports showed that thiacetarsamide delivered to normal cats produced no respiratory distress or altered the body temperature (Dillon et al., 1992). However a large average heartworm infected cats develop acute respiratory distress or sudden death in the post-treatment period. These effects seem to be due to embolization associated with worm death (Dillon et al., 1992).

The organical arsenical melarsomine dihydocloride is the only available compound now on the market, but there is insufficient experience about its use in cats until now. Furthermore, few data suggests that melarsomine is toxic to cats at dosages >3.5 mg/kg. Ivermectin at 24 µg/kg monthly given for 2 years has been reported to reduce worm burdens as compared to untreated cats. Since cats usually harbour low worm burdens and the main problem is the reaction that arise when the worms die (and not the worm mass by itself) this reaction could probably occur even when the ivermectin-treated worms die, but its intensity is unknown.

Anyway, there are no studies suggesting that medical adulticidal therapy increases the survival rate of heartworm naturally infected cats (Knight et al., 2002). Due to these reasons, macrofilaricide treatment is not advised in cats unless in selected cases.

In case of caval syndrome or when a heavy worm burden is visualized by echocardiography in right cardiac chambers, surgery may be attempted. Worm can be extracted via jugular vein using thin alligator forceps, horse hair brush or basket catheters (Glaus et al., 1995; Borgarelli et al., 1997; Venco et

al., 1999b). Because of the small size of the feline heart pulmonary, arteries cannot be accessed. Special care must be taken during the heartworm removal because traumatic dissection of a worm may result in circulatory collapse and death (Venco et al., 1999b).

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10

Dirofilaria (Nochtiella) repens infection in dogs and cats

Luigi Venco

Dirofilaria (Nochtiella) repens, is the causative agent of canine and feline subcutaneous dirofilariosis, a mosquito borne disease that has become increasingly recognized in several countries in southern and central Europe, Africa and Asia.

D. repens life cycle, like that of *Dirofilaria immitis*, consists of 5 larval stages which develop both in a vertebrate host and an arthropod (mosquito), intermediate host and vector. Adult female worms produce thousands of embryos (microfilariae) that are ingested by a blood-feeding insect.

Microfilariae have a unique circadian periodicity in the peripheral circulation over a 24-hour period. The arthropod vectors also have a circadian rhythm in which they obtain blood meals. The highest concentration of microfilariae usually occurs when the local vector is most actively feeding. Microfilariae then undergo 2 developmental moults in the insect. During feeding, the infected mosquito deposits third-stage larvae throughout a drop of haemolymph in the proximity of bite wound from where larvae actively migrate to subcutis. Larvae develop to the adult stage through moults in the vertebrate hosts. Prepatency lasts 6 1/2 to 9 months (Webber and Hawking, 1955; Cancrini et al., 1989). The adults reside in the subcutaneous tissues of dogs and cats and may cause mild clinical signs such as pruritus, dermal swelling, subcutaneous nodules or no symptoms at all (Baneth et al., 2002; Živičnjak et al., 2006).

Despite the usual hosts of *D. repens* are domestic and wild carnivores, human beings may act as accidental and dead-end hosts and a big concern about zoonotic human cases is arising. Human infection manifests with either subcutaneous nodules, ocular or lung parenchymal nodules mostly asymptomatic. The significance of infection in humans is that pulmonary and some subcutaneous lesions are commonly labelled as malignant tumours requiring invasive investigation and surgery before a correct diagnosis is made. The pathology of the condition is associated with aberrant localization of immature worms that do not reach adulthood; therefore, microfilariae are almost always absent (Pampiglione et al., 1995).

Diagnosis

Blood test for microfilariae

Detection of circulating microfilariae using the method developed by Knott is the best way for doing an *in vivo* diagnosis but collection of hystopatological cutaneous specimens.

Larvae species determination is made on the basis of morphological or hystochemical method or by using PCR.

Blood test for adult antigens

Tests detecting adult heartworm (*D. immitis*) do not detect *D. repens* antigens and no cross reactivity is described.

Therapy

No adulticide treatment for *D. repens* is registered and a off-label use of melarsomine has only recently been described on the basis of a case report

where combined therapy with the arsenic adulticide melarsomine and the avermectin microfilaricidal doramectin was effective in clearing infection with *D. repens* in a dog (Baneth et al., 2002), although the death of the patients does not allow conclusive evidences.

Symptomatic therapy of canine dirofilariosis due to *D. repens* is indicated for dogs suffering from clinical signs of this disease, such as dermal swelling, sub-cutaneous nodules and pruritus. Steroids and/or antibiotics administration and nodules surgical removal may be suggested in these cases for relieving symptoms.

Some macrocyclic lactones (ivermectin, moxidectin, selamectin) are claimed to be effective for the prevention of *D. repens* infection in dogs (Genchi et al., 2002; Rossi et al., 2002) and labelled for this use in some country (Italy) on the basis of field study.

While there is no doubt that they are able to prevent mirofilaraemia in dogs (and this is important for zoonotic implications), as most of the performed studies were not based on necropsy confirmation some concerns about the ability of completely preventing infection in dogs still remain (Cancrini et al., 1989).

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11

Guideline for the laboratory diagnosis of canine and feline *Dirofilaria* infections

Claudio Genchi, Luigi Venco, Marco Genchi

Microfilariae

Fresh blood smear (not advised)

A drop of fresh venous blood is placed on a clean microscopic slide and covered with a coverslip and examined under low microscopic power.

Microfilariae are seen throughout the movement they cause to the blood red cell layer.

To note that the intensity of microfilaremia is not correlated to the adult worm burden: in general, high microfilaremic dogs harbour few worms.

When dogs are monthly treated with preventive drugs during heartworm transmission season, re-testing each year before starting again the preventive treatment is advisable.

Advantage
Rapid and inexpensive

Disadvantages
Very low sensitivity, frequent false negative, no species diagnosis (it is not possible to differentiate microfilariae)
Not useful in cats

Concentration methods

Several methods can be used to concentrate circulating microfilariae from the blood. These methods are sensitive and make possible to differentiate microfilariae throughout morphological criteria (see Table 1 and Fig. 1).

Microfilariae can be concentrated by the modified Knott test or by a filter test.

To note that intensity of microfilaremia is not correlated to the adult worm burden: in general, high microfilaremic dogs harbour few worms.

Modified Knott test

One ml of venous blood is mixed with approximately 10 ml of 2% buffered formalin and the mixture is centrifuged for 3-5 minutes at 1500 rpm.

The supernatant is decanted from the centrifuge tube and the sediment is mixed with equal parts of a 1:1000 methylene blue stain. The stained sediment is placed on a slide, covered with a coverslip and examined under a microscope.

Advantage
Sensitive in dogs and specific: microfilariae belonging to different species can be differentiate

Disadvantages
Time consuming, need of a skill operator with good knowledge of microfilarial morphology
Specific but of low sensitivity in cats

Filter test

One ml of venous blood with anticoagulant of either EDTA or heparin is added to approximately 10 ml of lysate solution. The mixture is injected through a filter (Millipore) chamber. The filter is removed from the chamber, placed on a glass slide, stained, and examined under a microscope.

To note that intensity of microfilaremia is not correlated to the adult worm bur-

Table 1. Morphological features of microfilariae[1] from filarial worms of dogs and cats.

Species	Length µm	Width µm	Features
Dirofilaria immitis	290-330	5-7	No sheath, cephalic end pointed, tail straight with the end pointed
Dirofilaria repens	300-360	6-8	No sheath, cephalic end obtuse, tail sharp and filiform often ending as an umbrella handing
Acanthocheilonema reconditum	260-283	4	No sheath, cephalic end obtuse with a prominent cephalic hook, tail button hooked and curved
Acantocheilonema dracunculoides[2]	190-247	4-6.5	Sheath, cephalic end obtuse, caudal end sharp and extended
Cercopithifilaria grassii	567	12-25	Sheath, caudal end slightly curved

[1] microfilariae measure by Knott test
[2] microfilariae from the uterus

den: in general, high microfilaremic dogs harbour few worms.

Advantage
Rapid and sensitive in dogs
No need for a centrifuge apparatus

Disadvantages
Expensive (tests are sold as kit, Difil Test Evsco); the lysate solution shrinks the microfilariae and new measurement standards are required to differentiate species
Low sensitivity in cats

Histochemical stain

One ml of venous blood collected in EDTA is injected into 10 ml of deionized water and centrifuged at 1500 rpm for 15 minutes. The supernatant is discarded and the sediment placed on a slide and air-dried. The smear is then fixed with absolute acetone, air dried, and covered with acid phosphatase substrate. The substrate needs to be either made fresh, as described by Chalifoux and Hunt (1971), or frozen at -80°C in aliquot portions. After 2 hours at room temperature, the slide is air dried and covered with a coverslip.

- *D. immitis* microfilariae show 2 acid phosphatase activity spots localized around the anal and the excretory pores, respectively.
- *D. repens* microfilariae show only 1 acid phosphatase activity spot localized around the anal pore.
- *Acantocheilonema* spp. microfilariae show acid phosphatase activity throughout the body.

Peribáñez et al. (2001) have described an alternative method using a commercial kit with similar results.

Advantage
Very specific

Disadvantages
Costly, time consuming, need for a skilled laboratory technician

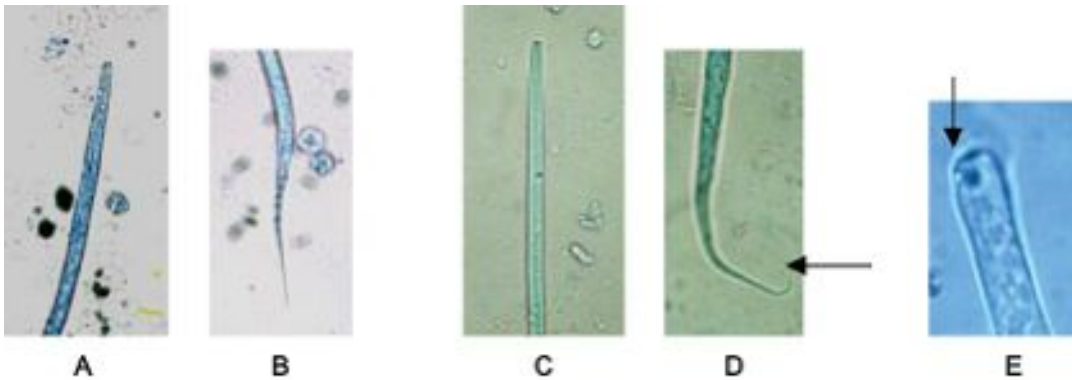

Fig. 1. A and B: *Dirofilaria immitis*: cephalic(x400) and caudal end (x1000)
C and D: *Dirofilaria repens*: cephalic (x400) and caudal end (x1000)
E: cephalic end of *Acantocheilonema reconditum* (detail showing cephalic hook) (x1000)

ELISA and immunochromatographic tests for adult female heartworm circulating antigens

Several ELISA and immunochromatographic kits are commercially available to detect the presence of adult female circulating antigens in serum, plasma and whole blood of dogs and cats. Most are very specific, quite sensitive, rapid and easy to be performed. Most are in-clinic test kits for single diagnosis but ELISA plates for multitest are also available. Manufacturers claim for a positive result when 1 adult female worm is infecting the animal, though many factors can affect the sensitivity of the tests such as age of worms, number of female worms versus number of males and the dog size, and reliable and reproducible results can be obtained from 2-3 or more adult female worms. Male worms are not detectable by antigen tests. In dogs, detectable antigenemia develops about 5 to 6.5 months post infection.

Because the clearance of antigens is quite rapid after the death of worms, these techniques can be used to assess the efficacy of an adulticide therapy. However, the newest tests are quite sensitive and for definitive diagnosis to confirm the success of the adulticide therapy dogs have to be retested 5 and 9 months later. If the test at 5 months is negative, testing at 9 months can be avoided.

Because unisex infections consisting of only male worms or symptomatic immature infections are not infrequent in cats, none of the presently available antigen tests can be relied upon to rule out heartworm disease in cats. In cats with heavy infections, detectable antigenemia develops at about 5.5 to 8 months post infection.

To note that semi-quantitative and laboratory ELISA tests (not immunochromatographic tests) have a direct, but imprecise, relationship to the adult female worm burden. The utility of the ELISAs for assessing the degree of parasitism can be limited by the transient increase in antigenemia as a consequence of recent worm death.

When animals are monthly treated with preventive drugs during heartworm transmission season, re-testing each year

before starting the preventive treatment in the following season is advisable.

If the chemoprophylactic treatment is performed with a sustained release drug injection (only for dogs), periodic testing (each 2 or 3 years) will ensure there have been no efficacy breaks (Nelson et al., 2005b).

Advantage
Very specific and sensitive for heartworm diagnosis [gold standard: when positive, the test is the definitive prove of heartworm infection in dogs and cats]

Disadvantages
Costly, not available for other filarial infections

Antibody tests

Several antibody test kits are available for the diagnosis of feline heartworm disease. These tests cannot be used in dogs.

Antibody tests have the advantage to being able to detect the exposure of cats to the infection of both male and female adult worms and larvae, and the immune response in detectable as early as 2 months post infection. However, their interpretation is complicate because positive results can be found both in aborted infections (developing larvae are destroyed by the host immune response and will not be able to develop to adult worms) and in patent infections. Furthermore, no proved data is available on whether the antibody level will decrease over the expected two-to-three year lifespan of an adult worm (Nelson et al., 2005a).

Advantage
Very sensitive
Able to detect the cat exposure to heartworm infection
Suitable to asses the infection risk in cats and for epidemiological survey

Disadvantages
Costly
Not fully specific
Difficult to be interpreted

PCR

PCR (polymerase chain reaction) is a sensitive and accurate tool to discriminate microfilariae from the different filarial worms able to infect dogs and cats. Its use is advisable in case of morphological abnormalities of microfilariae, not infrequent in dogs treated incorrectly with preventive drugs or when multiple infections with more than one species of filarial worm makes difficult to differentiate microfilariae (Favia et al., 1996; Mar et al., 2002; Casiraghi et al., 2006; Rishniw et al., 2006).

Advantage
Very sensitive, specific and accurate
Able to discriminate all the filarial species

Disadvantages
Costly, time consuming
Need for specialized laboratory and skilled technicians

Guideline for the diagnosis of filarial infections in dogs.

Mf Knott	Ag test	Interpretation	Comment
Positive	Positive	Definitive diagnosis of HW	ThR can help to manage the disease Clinical signs and the results of semiquantitative ELISA tests can help in discriminating between low and high risk of thromboembolism
Positive	Negative	Definitive diagnosis of HW: very low HW burden if *D. immitis* Mf are present Filarial infection caused by other species than *D. immitis*	Normal ThR patterns Low/very low risk of thromboembolic complications Histochemical stain or PCR can be used to differentiate Mf
Negative	Positive	Definitive diagnosis of HW **occult infection**	Dogs were previously treated incorrectly with preventive drugs or with macrocyclic lactone injectable formulations ThR and ECHO can help to manage the disease Clinical signs and the results of semiquantitative ELISA tests can help in discriminating between low and high risk of thromboembolism

Mf: microfilariae HW: heartworm TR: thoracic radiography ECHO: echocardiography

Guidelines for the diagnosis of filarial infections in cats.

Mf Knott	Ag test	Ab test	Interpretation	Comment
Positive	Positive	Positive	Definitive diagnosis of HW	ThR and ECHO can help to manage the disease
Positive	Negative	Positive	Definitive diagnosis of HW if *D. immitis* Mf are present	ThR and ECHO can help to manage the disease
Positive	Negative	Negative	Filarial infection due to other species than *D. immitis*	Histochemical stain or PCR for differentiate Mf and give specific diagnosis
Negative	Positive	Positive	Definitive diagnosis of HW	ThR and ECHO can help to manage the disease
Negative	Negative	Positive	Low adult female worm burden Immature worms Aborted infection Immune response to previous patent infection, but worms are already died	ThR and ECHO are useful to confirm the suspicion of HW infection Re-testing after 4-8 months can help to confirm the suspicion

Mf: microfilariae HW: heartworm TR: thoracic radiography ECHO: echocardiography

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12

Epidemiology and prevention of *Dirofilaria* infections in dogs and cats

Claudio Genchi, Jorge Guerrero,
John W. McCall, Luigi Venco

Heartworm infection (*Dirofilaria immitis*)

Dirofilaria immitis is a parasite that can be potentially fatal to a variety of animal species. Dirofilariosis, however, is a fully preventable disease due to the availability of highly effective preventive drugs that are safe, effective, convenient and easy to administer. According to the recently published American Heartworm Society guidelines, all animals that are at risk for contracting the disease should routinely receive heartworm preventive medications (Nelson et al., 2005a,b).

Chemoprophylatic drugs for heartworm infection fall into two basic classes, the macrocyclic lactones or macrolides (avermectins and milbemycins) and diethylcarbamazine (DEC). In heartworm-endemic areas, puppies and kittens that are born during the transmission season should be given their first dose of macrocyclic lactones between 2 to 8 weeks of age and 6 to 8 weeks of age, respectively. The required daily administration of DEC is inconvenient, more than one missed dose can result in a breakdown in protection and the overall prevention program is difficult to manage. The use of DEC is fairly limited in the United States and the drug is no longer available in Europe.

The present chapter will briefly review current information on the epidemiology of *D. immitis* infections in dogs and cats and the use of macrocyclic lactones in the prevention of heartworm infection, as well as their effect on other important gastrointestinal nematode parasites.

Epidemiology

More than 70 species of mosquitoes have been shown to be capable of developing microfilariae (first stage larvae) to the infective, third-stage larvae (L3), but fewer than a dozen of these species are believed to be major vectors (Otto et al., 1981). Although the susceptibility of different geographical strains may vary, the likelihood of the presence of at least one susceptible vector species in a geographic area that is conducive to the propagation of mosquitoes is high, and once the ubiquitous heartworm parasite is introduced into an area, its transmission is virtually insured.

Many countries are now endemic for heartworm infection. Furthermore, in spite of efforts aimed at prevention and control, particularly in dogs, infection appears to be spreading into areas previously considered to be free of the disease (Genchi et al., 2005). The prevalence and distribution is better known for dogs, but gradually more information on the frequency of diagnosis of infection in cats is becoming available. It is now generally accepted that heartworm disease may occur in cats in any area where dogs are infected (Genchi et al., 1992b, 1998; Guerrero et al., 1992b; McCall et al., 1994; Kramer and Genchi, 2002) but the geographical distribution and level of infection are less predictable in cats than in dogs. In highly endemic areas, with sufficient rainfall, essentially every unprotected dog becomes infected (McTier et al., 1992a). In contrast to dogs, about 75% of cats can be infected experimentally with *D. immitis* L3

(McCall et al., 1992). However, the prevalence rate of natural infections in cats is between 5% and 20% of that for dogs in the same geographical area (Ryan and Newcomb, 1995).

Cats with naturally acquired infections usually harbor fewer adult worms than dogs. Because of their small body size and exaggerated pulmonary vascular and parenchymal response to infection, cats with low worm burden can be considered to be relatively heavily infected in terms of parasite biomass (Genchi et al., 1998). Microfilaremia, when present, is low and transient, even in cats with experimentally induced infections. Mosquitoes fed on heartworm microfilaremic cats develop L3, which are capable of producing an infection in dogs (Donhaoe, 1975), but circulating microfilariae are seldom found in cats. Thus, cats generally become infected via mosquitoes that have fed on microfilaremic dogs.

It is not currently possible to determine which cats are resistant to heartworm infection and which are susceptible and will permit the infection to develop to the adult stage. Such a distinction between resistant and susceptible animals would require constant monitoring at very high costs.

Furthermore, environmental measures taken to reduce the risk of infection seem to be of little consequence in the cat. In fact, keeping animals indoors, one of the most important measures in reducing infection rates in dogs, seems to be ineffective in protecting cats from disease. Outdoor cats and strays who are seemingly exposed to high numbers of bites from infective mosquitoes may be able to mount an effective immune response that could be partially protective (Dillon et al., 1996; Prieto et al., 2001); however, studies to determine levels of susceptibility have not been performed. For indoor cats, it seems that even one encounter with an infective vector may lead to the development of a large proportion of transmitted larvae to the adult stage, causing severe illness (Genchi et al., 1992a). It has recently been reported that between 9 and 27 percent of cats were seropositive for *D. immitis* in northern Italy, 19 percent of which were apartment-dwelling cats (Kramer and Genchi, 2002).

Chemoprophylactic treatment, therefore, is a viable option for cats residing in any area where heartworm is considered endemic in dogs, even cats living more sheltered lives. As suggested by Atkins (1997), it seems rational to recommend chemoprophylaxis for feline heartworm infection, given that the disease has a higher incidence than both Feline Leukemia Virus (FeLV) and Feline Immunodeficiency Virus (FIV), infections for which vaccination protocols are increasingly advocated.

Heartworm transmission to dogs and cats is influenced by several well-known epidemiological factors, among the most important of which is environmental heat. Development of *D. immitis* to L3 in mosquitoes occurs at a rate that is dependent on ambient temperature, and development may not occur at a threshold temperature of about 14 °C (Fortin and Slocombe, 1981). The effects of heat on larval development is assumed to be cumulative and may be calculated in terms of degree-days above the developmental threshold, or heartworm development units (HDU). One model of heartworm

seasonality assumes a requirement of 130 HDU (°C) for complete development and a maximum life expectancy of 30 days for common vector mosquitoes (Slocombe et al., 1989). Using this laboratory-derived model, which requires numerous inherent assumptions and was designed to study only the influence of macroenvironmental temperature on the heartworm development period, investigators have predicted the seasonal limits of transmission in Canada (Slocombe et al., 1995), the USA (Knight and Lok, 1995) and Europe (Genchi et al., 2005) and formulated recommendations for timing of heartworm chemoprophylaxis and scheduling of diagnostic testing. While these model-based predictions are academically appealing, they ignore several potentially important factors, such as influence of microclimate and the unique biological habits and adaptations of the numerous mosquito vectors, on larval development.

Prevalence

North America

Heartworm infection in dogs has now been diagnosed in all of the 50 states of the USA. Although transmission of infection has not been clearly documented for Alaska, the disease is considered to be endemic in all of the remaining 49 states. Heartworm is enzootic along the Atlantic Seaboard and Gulf Coast areas, with the southeastern states generally showing the highest prevalence values. Infection continues to be diagnosed at a high frequency in the Mississippi River basin and in states along the Ohio and Missouri Rivers. New foci have been detected in northern California and Oregon, and autochthonous infections in dogs in the states of Wyoming, Utah, Idaho, and Washington have been documented in recent years (Zimmerman et al., 1992).

There is a high probability that the introduction of the tree-hole breeding mosquito, *Aedes sierrensis*, in Salt Lake City, Utah, during the past decade or so is associated with the establishment of enzootic heartworm transmission in the area (Scoles and Dickson, 1995). The tiger mosquito, *A. albopictus*, a known vector of heartworm in Japan, has spread rapidly throughout much of the USA and Europe since it was introduced from Asia in 1985. It breeds mainly in piles of discarded tires and is spread within the country by movement of tires from place to place. This mosquito readily feeds on dogs and other mammals, and laboratory strains have shown it to be an excellent host for *D. immitis*.

Two recent surveys conducted by Merial in cooperation with the American Heartworm Society showed that the number of canine heartworm cases diagnosed in the US were close to a quarter of a million. The first survey conducted in 2002 requesting diagnostic data for 2001 had the participation of 15,366 clinics which reported diagnosing heartworm infections in 244,291 dogs. The second survey conducted in 2005 reviewing data for 2004 had the participation of 12,173 clinics (out of a total of 25,000), which reported 250,000 cases of canine heartworm infections diagnosed that year (Guerrero et al., 2006). Interestingly, 8,800 of the responders in the second

survey had also responded in the first survey. Analyzing the data obtained in the clinics that participated in both surveys the following was seen: number of canine heartworm cases decreased in 17 states, in 3 states the numbers were the same and in 30 states plus Washington, DC the number of cases increased. Nationally, the reported positive cases of heartworm infections in dogs increased slightly in those clinics reporting in both 2002 and 2005.

In a parallel study, the national prevalence of heartworm infections in dogs was performed by evaluating medical records of more than 500 Banfield Pet Hospitals that see approximately 80,000 pets on a weekly basis. Data collected from January 1st 2002 to December 31st 2005 was evaluated. Results of this study show that 1.46% of the 871,839 dogs examined tested positive for circulating antigen of *D. immitis*. Based on this data, the estimates of pet dogs in the USA and the proportion of them probably on heartworm prevention, the investigators (Apotheker et al., 2006) estimated that 509,932 dogs in the USA had heartworm infections, a figure that is almost exactly double the number of cases reported in the Merial-AHS surveys of 2002 and 2005. Keep in mind that in the 2005 Merial-AHS survey close to half of the total number of Companion animal clinics in the USA responded to the survey.

In Canada, the overall canine prevalence rate is 0.24% (Slocombe, 1992). Prevalence is higher (8.4%) in endemic areas of southwestern Ontario. The most significant reports of heartworm infections in British Columbia are from the Okanagan Valley area, which represents a classic instance of recent introduction of the parasite and a resulting local pocket of infection (Zimmerman et al., 1992). Hunters, in previous years, had transported hunting dogs from Texas to this area, setting up new foci of infection.

South America

The prevalence and spread of heartworm infection in South America has been recently reviewed by Labarthe and Guerrero (2005). Surveys indicate that heartworm is endemic in several countries of South America. In Brazil, the overall prevalence of canine heartworm infection in the state of Rio de Janeiro was 21.3% (Guerrero et al., 1992a), with the highest rate for dogs in the northern beaches (49%), followed by dogs from the mountain towns near the cities of Rio de Janeiro (27.4%) and Niteroi (26.4%). The rate in the suburbs of Rio de Janeiro was around 33% (Labarte et al., 1992). Labarthe and co-workers (1997 a,b) confirmed and extended these findings, and also reported heartworm infection in random-source cats in the city. Alves and co-workers (1999) found a prevalence rate, as determined by necropsy of dogs, in the city of Recife in northeastern Brazil of 2.3%. In the same state (Pernambuco), prevalence in dogs on Itamaracá Island was found to be as high as 43%. Labarthe (1997) reviewed the literature on prevalence in Brazil and found reports of prevalence rates as high as 45% in the state of São Paulo. In Argentina, endemic areas have been identified, with prevalence in dogs ranging from 5.0% in the greater Buenos Aires area to 34.2% in the northeastern province of Formosa (Guerrero et al., 1992a). A rate of

10.9% was recorded for Corrientes. More recently, Peteta and co-workers (1998) reported a prevalence, determined by microfilarial and antigen testing, of 13.6% for dogs in Villa La Nàta, which is located near the Parana Delta and surrounded by the Lujan River and Villanueva and LaRioja Channels in the Tigre district of the province of Buenos Aires. In Argentina the prevalence ranges from 0% to 71% (Vezzani et al., 2006). In Venezuela, examination of canine blood samples submitted to the School of Veterinary Medicine, Central University of Venezuela in Aragua, revealed that 2.3% were positive for microfilariae of *D. immitis* (Perez and Arlett, 1998). Recent studies performed in Lima, Peru, reported 4.35% of the blood samples from 140 randomly selected dogs were positive for circulating antigen of *D. immitis*. These samples were examined by the ELISA Snap 3Dx test (Gonzales, 2002).

Central America and the Caribbean

Kozek and co-workers (1995) reviewed the prevalence of canine filariae in the Caribbean islands and conducted a thorough epidemiological survey in Puerto Rico. Prevalence values for heartworm infection in Puerto Rico ranged from 3.1% to 20.4%, with the highest rate recorded for the city of Ponce, on the southern coast. In Cuba, prevalence for Havana ranged from 7% to 19% and from 37% to 63% on the Isla de Juventud. For Curacão, it ranged from 9% to 11% and was 53% for the Grand Bahamas and 18% for the Dominican Republic.

In a survey covering 15 cities in Mexico (Guerrero et al., 1992a), the overall heartworm prevalence in dogs was found to be 7.5%, with the highest rates (20-42%) observed for dogs from the Gulf Coast cities of Tuxpan, Tampico, and Ciudad Madero.

Australia

Heartworm infection is enzootic along the northern coastal areas of Western Australia and the eastern states of Queensland, New South Wales, and Victoria, where prevalence rates generally mimic those for the southeastern states of the USA. The prevalence of heartworm infection in dogs in Sidney was as high as 30% in the late 1980s, and cats were also found to be infected (Kendall et al., 1991). More recently, the rate for dogs in this area was reported to be only 11.4% (Bidgood and Collins, 1996).

Asia and the South Pacific

Heartworm disease is well established in most of the Islands of the Pacific and in many countries of Asia, but survey results are not readily available for every country. A prevalence of 11.3% for dogs from the Fars province of Iran has been reported (Jafari et al., 1996). Heartworm is enzootic on the islands of Japan, where the disease is well known and prevalence is well documented. A survey conducted on stray dogs and cats in the Kanto region of Japan in 1985 revealed a prevalence rate of 59% for dogs and 2% for cats (Tanaka et al., 1985). More recently, Roncalli and co-workers (1998) reported that prevalence rates for feline heartworm infection in Japan ranges from 0.5% to 9.5% in stray cats and

from 3.0% to 5.2% in house cats. A survey of German shepherds in five areas of South Korea revealed an overall prevalence of 28.3%, by an antigen test (Lee et al., 1996). Prevalence was highest in Hoengsong-gun (84.4%), while Yechon-gun and Chungwon-gun areas had rates of 20.0% and 14.3%, respectively. None of the dogs in the Kimhae-shi and Kwanju areas was positive. Kuo and co-workers (1995) reported a 53.8% prevalence for dogs in the Taipei province of Taiwan, and Wu and Fan (2003) an overall prevalence of 57% in stray dogs in Taiwan.

Europe

The prevalence and spread of heartworm infection in Europe has been comprehensively reviewed by Genchi and co-workers (2005). The disease is diagnosed mainly in the southern European countries of Spain, Italy, Portugal, and France, with scattered reports from Greece, Turkey, and some Eastern European countries. An increasing number of cases are now being diagnosed in northern countries such as Austria, Germany, and The Netherlands in dogs that were either imported from the Mediterranean area or had accompanied their owners to the area. One possible exception is a heartworm-positive dog from the Canton of Tessin (Switzerland), which appears to have acquired an autochthonous infection.

For Europe, the area of highest prevalence values for dogs and cats is along the Po River Valley in northern Italy. The prevalence rate for cats in this area is high (up to 24%), and the rate for dogs ranges from 35% to 80% in animals not treated with preventive drugs. The disease has recently spread northward into the provinces of Friuli-Venezia-Giulia. Furthermore, the spreading of *A. albopictus* in Italy and the evidence that this mosquito species can act as a natural vector for *D. immitis* can enhance the risk of transmission from animals to humans, considering the aggressive anthropophylic behavior of the species (30-48 bites/hour) (Cancrini et al., 2003).

The highest rates for dogs in Spain are reported for the southern provinces of Huelva (37%), Cadiz (12%), and Badajoz (8%) (Guerrero et al., 1992 a). During the past few years, *D. immitis* infection appears to be spreading into other regions of Cataluña (Catalonia). A recent survey of Barcelona showed that 12.8% of the dogs were infected. The Canary Islands of Tenerife (20.0%) and Las Palmas (36.0%) are highly endemic, and a recent report suggests that about 59% of the dogs on Gran Canaria Island are infected with heartworms. In Portugal, infection is diagnosed mainly in the southern regions, with prevalence values ranging from 12% (Algarve) to 30% (Island of Madeira). Although limited survey data are available, prevalence values for dogs range from 2% to 17% for Slovenia, Bulgaria, Greece, and Turkey and up to 65% for Romania, and some of these areas are considered to be endemic. In Croatia, canine heartworm infection has been reported from Istrian pensula (about 16% prevalence) and the sutest regions (Dubrovnik; about 8%) (Živičnjak et al., 2006).

Africa

Heartworm is found in dogs from various regions of Africa, but no infor-

mation is available regarding infections in cats. Infection in dogs appears to be common throughout western Africa and in eastern parts, extending from the Republic of South Africa and Mozambique (Schwan and Durand, 2002) to the Republic of Sudan. Matola (1991) reported a prevalence of 10.2% for dogs in Tanzania.

Chemoprophylactic treatment of canine heartworm disease

Since the discovery of ivermectin and the initial description of activity against developmental stages of *D. immitis* a large number of publications have appeared reporting on their attributes.

Monthly oral administrations of ivermectin at 6-12 mcg/kg, milbemycin oxime at 500-999 mcg/kg, or oral moxidectin at 3 mcg/kg provide effective protection against heartworm infections in dogs.

An ivermectin/pyrantel chewable formulation is also available in Europe and in the United States to expand the indications to include treatment and control of certain gastrointestinal parasites, including *Toxocara canis, Toxascaris leonina*, *Ancylostoma caninum* and *A. braziliense.* Milbemycin oxime at the recommended dose for heartworm prophylaxis is also effective against *T. canis, T. leonina*, *A. caninum* and *Trichuris vulpis.* Treatment with any one of these macrolide compounds should begin within a month after the beginning of the transmission season and the final dose should be administered within one month after the end of mosquito activity although, at present time the most accepted recommendation is to treat year-round (Nelson et al., 2005b). All three drugs have a wide range of efficacy, which allows them to be administered every 30 days. This provides a safeguard in the case of omission or delay of a monthly treatment, or when the chemoprophylactic history cannot be verified. Ivermectin and milbemycin oxime have both been found to provide a high degree of protection when administered on a regular basis, beginning 3 months after infection. In fact, monthly treatment with ivermectin over a one-year period has been shown to be >95 percent effective in preventing development of *D. immitis* larvae that were 4 months old; however, under the same conditions, milbemycin oxime was only 41.5 to 49.3 percent effective as a clinical prophylactic agent (McCall et al., 1992). This retroactive or "reachback" effect has not been reported for moxidectin, although products with this compound do have a label claim for efficacy of 2 months duration. This so-called "safe net" or "reachback" effect of macrocyclic lactones is very useful to compensate for missed or delayed treatments, but should not be considered as justification to modify the recommended monthly interval for treatment.

A newly developed avermectin, selamectin, has recently been approved in Europe and in the United States for the prevention of heartworm infections in dogs and cats. The drug was also 100% effective in preventing the development of heartworm infection in dogs when administered as a single topical dose of 3 or 6 mg/kg given at 30 or 45 days after inoculation with L3 or a single topical dose of 6 mg/kg given 60 days PI (Clemence et al., 2000; McTier

et al., 2000; Dzimianski et al., 2001; McCall et al., 2001). The drug was also safe when given to dogs and cats with existing heartworm infections. There are several important characteristics unique to selamectin: the drug is given as a topical formulation, thereby avoiding problems associated with oral administration. At the dose recommended for heartworm prevention in dogs and cats (6 mg/kg) selamectin is also effective in preventing and controlling flea infestations (*Ctenocephalides felis*) and for treating and controlling ear mites (*Otodectes cynotis*) and biting lice (*Trichodectes canis* and *Felicola subrostratus*) in dogs and cats; sarcoptic mange in dogs *(Sarcoptes scabiei*); and hookworm (*Ancylostoma* spp) and roundworm (*Toxocara* spp) in both dogs and cats.

An injectable formulation of moxidectin to be solely utilized by veterinarians in dogs for prevention of heartworm infections is sold in Italy and Australia. The commercial formulation (moxidectin sustained release injectable for dogs) has been approved for use in dogs six months of age and older, but not growing dogs. It is able to protect dogs for an entire heartworm transmission season (Genchi et al., 2002) and also treats infections with *A. caninum*.

Control testing

Retesting for *D. immitis* infection should always be carried out after the first season of preventive treatment and must include testing for circulating adult antigens as well as for microfilariae. The reason for this is that regular chemoprophylactic use of the macrolides will usually clear any microfilariae from the blood, so that even if an infection has become patent during the therapy, it would not be detected by screening for microfilariae. However, some cases of microfilaraemic dogs can be observed, mainly when some treatments are inadvertent omitted towards the end of the transmission season. Testing should be performed a minimum of 6 months after the last administration of one of the macrolide compounds. It is also advisable to repeat testing at the beginning of each transmission season before the start of treatment in order to exclude the possibility of infection due to poor owner compliance during the preceding season, or to verify that there was no pre-existing infection. Annual retesting should be advisable even if chemoprophylactic treatment has been utilized continuously (year-round) due mainly to the overall low owner compliance (Nelson et al., 2005b).

Chemoprophylactic treatment of feline heartworm disease

Although the general guidelines and criteria for the use of preventive drugs in dogs may be generally applied to heartworm infection in cats, several specific features of feline heartworm disease must first be considered when choosing the correct prevention regimen. *D. immitis* infection in cats can cause an unpredictable, and often fatal, disease. Cats are known to be susceptible hosts for heartworm, but are extremely resistant to infection (Genchi et al., 1992a; McCall et al., 1992).

Preventive treatment in the cat follows the same regimen established for

the dog, i.e., monthly dosing should begin within one month from the start of the transmission season and the last dose should be given within one month from the end of the risk period. Ivermectin is marketed for use as a prophylactic agent in cats given monthly at the dose of 24 mcg/kg (McTier et al., 1992b). This oral dosage is also highly effective for treatment and control of *A. tubaeforme* and *A. braziliense*. The oral chewable formulation of ivermectin has been found to be 100 percent effective in preventing development of *D. immitis* larvae when administered 30 or 45 days after challenge with infective larvae.

Furthermore, McCall and co-workers (2000, unpublished data) demonstrated that the recommended prophylactic dosage of ivermectin administered monthly for a maximum of 12 months was 66.5 percent effective in clearing 7-month-old *D. immitis* that had been transplanted from an infected dog. A dramatic decrease in circulating antigen levels also was detected in ivermectin-treated cats. These findings are highly noteworthy for the cat veterinarian since adulticide treatments are not considered a viable option for cats.

Milbemycin oxime is also known to be effective in cats for heartworm prophylaxis at a rate of 2 mg/kg (Genchi et al., 2004). In Europe, milbemycin oxime is available in combination with praziquantel (5 mg/kg) and the product is efficacious for preventing heartworm infection and for the treatment and control of *A. tubeforme*, *T. cati*, *Dipylidium caninum*, *Taenia* spp and *Echinococcus multilocularis*. Selamectin is also available for use in cats as a heartworm preventative.

Using the same dosage as in the dog, 6 mg/kg by topical application, selamectin is also active against *T. cati*, *A. tubaeforme, C. felis, Felicola subrostratus* and *O. cynotis* (Guerrero et al., 2002).

As with the dog, pre-treatment testing is advisable in the cat, utilizing both an antibody and an antigen test for cats, to verify the absence of *D. immitis* infection; however, it is not mandatory. Retesting of cats should be considered after the first season of preventive treatment and is advisable at the beginning of each new transmission season before preventive therapy is to be initiated, unless the clinician has wisely chosen to utilize chemoprophylaxis year-round. The longer life cycle of the parasite in the cat, as well as the difficulty in accurately diagnosing infection, increase the risk of inadvertently treating an infected animal; however, based on data obtained by McCall et al. (2000, unpublished data), monthly administration of preventive drugs to cats infected with adult worms did not precipitate any negative reactions.

Subcutaneous filarial infection (*Dirofilaria repens*)

As for heartworm infections, subcutaneous filarisosis can be safely and effectively prevented by chemoprophylactic treatment of both in dogs and cats.

Although the disease is less severe than heartworm infection and dogs can show cutaneous disorders of different severity, such as pruritus, dermal swelling and subcutanous nodules containing the parasites (Baneth et al., 2002; Bredal et al., 1998), very severe infec-

tions have been reported (Restani et al., 1962; Mandelli and Mantovani, 1966), with allergic reactions probably due to microfilariae. However, the main concerrn about this *Dirofilaria* species is its ability to cause infections in humans in Europe. The number of zoonotic infections has dramatically increased in the last few decades (Pampiglione et al., 1995) and the infection now can be included in the list of emerging zoonoses (Pampiglione et al., 2001). The infection is spreading in many southern (Giannetto et al., 1997) and eastern European countries (Mazurkevich et al., 2004; Fok, 2007), probably as a consequence of the movement of infected dogs and global warming that increase the transmission season. Recently, Živičnjak et al. (2006) reported a prevalence ranging from 7-18% in dogs from several regions of Croatia. Cats, as well as dogs can be infected, but the prevalence seems quite low (0.2-0.5%; Genchi et al., 1993).

Ivermectin, selamectin and moxidectin (both tablets and subcutaneous injectable) have been found to be effective in preventing this subcutaneous infection in dogs naturally exposed to infective mosquitoes, by treating monthly (oral formulations) or once a year (moxidectin injectable formulation) and at the same doses that are effective against *D. immitis* (Marconcini et al., 1993; Genchi et al., 2002c; Rossi et al., 2002, 2004).

Domestic ferrets

The domestic ferret (*Mustela putorius furo*) has been reported to be susceptible to naturally acquired and experimentally induced infections of *D. immitis* (McCall, 1998). Laboratory studies have shown the ferret to be highly susceptible, with infection and recovery rates similar to those achieved in the dog and higher than those seen in cats (Supakorndej et al., 1994). The life span of adult heartworms in ferrets is thought to cover the entire life time of the ferret. Heartworms are somewhat smaller in ferrets than in dogs, with mean lengths of male and female worms recovered from the heart and associated vessels of 118 mm and 144 mm, respectively, 140 days after infection (Supakorndej et al., 1994).

Microfilaremia is characteristically of low concentration and transient in nature, similar to that seen in heartworm-infected cats. A definitive diagnosis can be made from ELISA-based antigen tests, echocardiography, and angiography, but suggestive radiographic findings require additional supportive information to confirm a tentative diagnosis (McCall, 1998; Sasai et al., 2000). Clinical signs upon presentation include lethargy, inappetence, exercise intolerance, pleura effusion, cyanosis and dyspnea (Antinoff, 2001).

Supakorndej and co-workers (2001) found that adulticide treatment of infected ferrets with melarsomine dihydrochloride enhanced cardiomegaly and alveolar infiltrates and increased the severity of interstitial disease. The drug at the dosage of 3.25 mg/kg was equally effective (80.6-83.3%) when given twice, 24 hours apart or one injection followed one month later by 2 injections, 24 hours apart, but several animals in the treated and control groups died during the study.

Prevention has been shown to be

effective with currently used canine prophylactic compounds such as monthly tretament with ivermectin at 6 mcg/kg (McCall, 1998) or cat chewable ivermectin formulation (24 mcg/kg) (Kemmerer, 1998), but effective treatment of adult heartworms in ferrets has not yet been confirmed by controlled studies. There is currently no approved drug for prevention or treatment of *D. immitis* in ferrets.

Guideline for the chemoprophylactic treatment of *Dirofilaria* infections in dogs

1. Test the dog for circulating microfilae (*D. immitis* and *D. repens*) and adult female antigens (*D. immitis*) when the preventive treatment is administered for the first time. Testing cats is not necessary but it is advised; drugs are safe even in cats with patent infection.

2. When a ≥ 1 year-old dog with an unknown history, living in an endemic area (risk area) is prophylactically treated for the first time, check for microfilariae and antigen before starting treatment and recheck after 6 months to exclude a possible prepatent infection.

3. Retesting should always be carried out after the first season of preventive treatment and must include testing for circulating antigens as well as for microfilariae. It is also advisable to repeat testing at the beginning of each transmission season, before the start of treatment in order to exclude possible infections due to poor owner compliance or to verify that there was no pre-existing infection. In endemic areas where year-round treatments are utilizad, yearly testing is recommended.

4. Treatment must be given montly at the recommended dosage, but for moxidectin in the injectable formulation, one injection is able to protect dogs against *Dirofilaria* infection for at least six months. The commercial formulations are sold at dosages that are able to cover different ranges of body weight. The minimum effective dosage of macrocyclic lactones for prevention of *Dirofilaria* infection in dogs and cats and the indications against other parasites are shown in the table below.

ML	Presentation	Species	Dose	Indications	Minimum age of treatment
IVM	Tablets/chewables	Dog	6 mcg/kg	Di; Dr	6 weeks
	Chewables	Cat	24 mcg/kg	Di, At, Ab	6 weeks
IVM/PYR	Chewables	Dog	6 mcg/kg	Dog: Di, Dr, Tc, Tl, Ac, Us	6 weeks
MBO	Flavour tablets	Dog	0.5 mcg/kg	Di, Tc, Tl, Ac, Tv	2 weeks or 0.5 kg
MBO/PZQ	Tablets	Dog	0.5 mcg/kg	Di, Tc, Tl, Ac, Tv, Dc, Tae, Eg,Ms	6 weeks
		Cat	2 mg/kg	Di, Tct, At, Dc, Tae, Em	6 weeks
MBO/LFN	Tablets	Dog	0.5 mcg/kg	Di, Tc, Ac, Tv, Cf	2 weeks

continued

ML	Presentation	Species	Dose	Indications	Minimum age of treatment
MOX	Tablets	Dog	3 mcg/kg	Di, Dr	6 weeks
	Injectable		0.17 mg/kg	Di, Dr, Ac	adult
SLM	Topical	Dog	6 mg/kg	Di, Dr, Tc, Cf, Ss, Oc, Trc	6 weeks
		Cat	6 mg/kg	Di, Tct, At, Cf, Oc, Fs	6 weeks

VM: ivermectin
PYR: pyrantel pamoate
MBO: milbemycine oxime
MOX: moxidectin
SLM: selamectin

Di: *Dirofilaria immitis*; Dr: *D. repens*; Ac: *Ancylostoma caninum*; At: *A. tubaeforme*; Ab: *A. braziliense*, Tc: *Toxocara canis*; Tct: *T. cati*; Us: *Uncinaria stenocephala*; Tv: *Trichuris vulpis*; Dc: *Dipylidium caninum*; Ms: *Mesocestoides* sp; Tae: *Taenia* spp, Eg: *Echinococcus granulosus*; Em: *Echinococcus multilocularis*; Cf: *Ctenocephalides felis*; Trc: *Trichodectes canis*; Fs: *Felicola subrostrata*; Oc: *Otodectes cynotis*; Ss: *Sarcoptes scabiei*

Note: All of the compounds are intended for monthly administration except moxidectin injectable.

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13

Safety and efficacy of selamectin in dogs with *Dirofilaria immitis* infection

John W. McCall, Michael T. Dzimianski, W.L. Steffens, Nonglak Supakorndej, Prasit Supakorndej, Abdelmoneim E. Mansour, Mary B. Ard, Scott D. McCall, Richard Hack, Dan Domingo

In both laboratory (McTier et al., 1998, 2000) and field studies (Clemence et al., 2000; Boy et al., 2000), selamectin has proven to be highly effective in the prevention of *Dirofilaria immitis* (heartworm) infections in dogs and cats following monthly prophylactic administrations at a minimum dose rate of 6 mg/kg. In studies in which administration occurred 45 days (simulated delayed treatment) or 60 days (simulated delayed or missed treatment) after inoculation with infective third-stage larvae (L3), a single treatment with selamectin was completely effective (McTier et al., 2000). It has also been demonstrated that no adverse effects are likely if selamectin is inadvertently administered to animals with existing infections of adult *D. immitis* (Novotny et al., 2000) In heartworm endemic areas, there are several potential scenarios for compliance failure in heartworm prevention, which a clinician may encounter.

These include dogs that have been exposed to infection for various periods prior to initiation of their heartworm prophylaxis, situations of missed monthly dosing, and circumstances in which dogs with patent infections and various degrees of microfilaremia are presented for treatment. The series of studies presented here was designed to assess the performance of selamectin in 3 situations, which hitherto had not been investigated. The studies included an assessment of the safety and efficacy of selamectin in heartworm-positive dogs with high microfilaremia (study A); the microfilaricidal and adulticidal efficacy of selamectin after long-term monthly administration to dogs with patent *D. immitis* infections (study B); and the "reachback" or clinical prophylactic effect in dogs inoculated with heartworm L3 3 months prior to initiation of long-term monthly prophylaxis (study C).

Materials and methods

Treatments

Selamectin in the commercial formulation[a] was administered as a unit dose to provide a minimum dose rate of 6 mg/kg of body weight. The placebo (vehicle only) was administered at the rate of 0.05 ml/kg of body weight to provide an equivalent volume.

Treatments were applied to the skin at a single site on each dog's back at the base of the neck and cranial to the scapulae. Treatments were administered at 30-day intervals beginning on study day 0.

Animals

Equal numbers of colony-bred male and female Beagles were used. The initial age and weight ranges for the dogs were 5 to 7 months and 6.45 to 15.4 kg, respectively. Dogs were housed individually in mosquito-proof indoor pens inside purpose-built accommodations, with controlled temperature and ventilation systems. They were fed once daily with an appropriate quantity of maintenance diet and water was supplied *ad libitum*. Animals were main-

[a] Stronghold®/Revolution®, Pfizer Inc., New York, New York, USA.

tained with due regard for their welfare and in accordance with applicable legislation and guidelines.

Parasite challenge

For dogs in studies A and B, mature adult *D. immitis* worms aged approximately 10 to12 months were obtained from donor animals having experimentally induced infections. Twenty worms (10 males, 10 females) were introduced surgically into the cranial vena cava via the external jugular vein while the dogs were under general anaesthesia. For study C, *D. immitis* L3 were obtained from experimentally infected *Aedes agypti,* and each dog was inoculated with approximately 50 L3 by subcutaneous injection into the inguinal region 3 months prior to initiation of treatment.

Dirofilaria immitis *antigen testing and microfilarial counting*

Dogs were tested for the presence of *D. immitis* antigen by using a commercial test kit[b]. A modified Knott concentration technique was used to count microfilariae.

Adult worm counts

After euthanasia, dogs in studies B and C were necropsied for recovery of adult *D. immitis*. The pleural and peritoneal cavities were examined for adult worms, and the cranial and caudal vena cavaee were clamped before removal of the heart and lungs (and liver in study C). The pre-cava, right atrium, right ventricle, and pulmonary arteries (and caudal vena cava as far as the liver in study C) were dissected and the worms removed, recorded as male or female, recorded as alive or dead, and fixed in formalin.

Design

Study A. Safety and efficacy in dogs with high microfilarial counts

Eighteen dogs (9 males, 9 females) that tested negative for heartworm antigen and microfilariae were inoculated with 20 adult *D. immitis* on day –222. Sixteen dogs (8 males, 8 females) with the highest *D. immitis* microfilarial counts (mean, >10,000 mf/ml on day –5) were selected, weighed, and randomly allocated to treatment with selamectin or a placebo. Treatments were administered on days 0, 30, and 60. Clinical observations were made prior to each treatment, at 4 and 8 hours after treatment, and once daily for the next 7 days. General health observations were made daily on all other days throughout the study. Blood was collected by venipuncture for microfilarial counting on the day of treatment (days 0, 30, and 60) and on days 1, 7, and 14 after each of the 3 treatments.

Study B. Efficacy of long-term treatment against adult **D. immitis** *and circulating microfilariae*

Eighteen dogs (9 males, 9 females), with negative results for blood microfilariae and heartworm antigen on day

[b] DiroChek® Canine Heartworm Antigen Test Kit, Synbiotics Corporation, San Diego, California, USA.

32, were each inoculated with 20 adult *D. immitis* worms on day 30. On day 3, 16 dogs (8 males, 8 females) with the highest levels of microfilariae and antigen were selected, weighed, and randomly allocated to treatment with selamectin or a placebo. A total of 18 monthly treatments were administered on days 0, 31, 60, 90, 122, 152, 182, 213, 243, 273, 304, 334, 364, 395, 425, 455, 486, and 516. Clinical observations were made prior to each treatment, and then within 10 minutes, and at 2, 4, 8, and 24 hours after treatment. General health observations were made daily on all other days throughout the study. Blood was collected by venipuncture for microfilarial counting and antigen testing approximately every 30 days. All dogs were euthanized on day 546 for necropsy and recovery of adult *D. immitis*.

Study C. Long-term monthly prophylaxis beginning at 3 months after heartworm inoculation ("Reachback" efficacy)

Seventeen dogs that had tested negative for microfilariae and *D. immitis* antigen on day –92 were each inoculated with approximately 50 *D. immitis* L3 on day –90. On day –3, blood samples were obtained for microfilariae counting and antigen testing. On day –1, 16 dogs were selected and allocated to treatment with selamectin or a placebo (8 dogs/treatment). Monthly treatments were administered beginning on day 0 (90 days after infection), and continuing thereafter for 11 months (total of 12 monthly treatments). Clinical observations were made immediately prior to each treatment, and then at 4, 8, and 24 hours after treatment. General health observations were made daily on all other days throughout the study. Blood samples for microfilarial counts and antigen tests were collected at approximately 30-day intervals and the dogs were euthanized on day 363 (15 months after inoculation) for necropsy and recovery of adult *D. immitis*.

Data analysis

Parasite counts were log transformed {ln(x+1)} prior to analysis by using a repeated- measures model. *A priori* contrasts among least squares means of the log transformed data were used to assess the treatment differences on each counting day at the 5% level of significance ($P \leq 0.05$). Geometric mean parasite counts for each treatment were calculated from least squares means of the log transformed data, and these means were used to estimate percentage reductions in parasite burden at each time point for those animals treated with selamectin, compared with those receiving the placebo, using the following formula:
(Geometric mean count for placebo) – (geometric mean count for selamectin)/(Geometric mean count for placebo) x 100 = % reduction

Results

Study A. Safety and efficacy in dogs with high microfilarial counts

There were no adverse clinical observations related either directly to treat-

ment or to the effects of treatment on parasites within heartworm-positive dogs during the study. Geometric mean microfilarial counts for placebo-treated dogs on days 0, 1, 30, 60, and 74 were 9,882, 7,117, 3,780, 6,322, and 2,523 mf/ml, respectively, compared with selamectin-treated dogs, which had counts on the same days of 11,568, 10,462, 459, 606, and 223 mf/ml, respectively (Table 1). Thus, the microfilarial count for the selamectin-treated dogs gradually decreased during the month after the first treatment (87.5% reduction on day 30), compared with the placebo-treated controls, and generally remained at approximately that value for the remainder of the study. The microfilarial counts were significantly ($P \leq 0.05$) lower for selamectin-treated dogs, compared with placebo-treated dogs, on days 30, 31, 67, and 74.

Study B. Efficacy of long-term treatment against adult **D. immitis** *and circulating microfilariae*

At necropsy, the geometric mean adult *D. immitis* counts were 10 for selamectin-treated dogs and 15 for placebo-treated dogs (controls). There was no significant difference between the treatments, even though the reduction in worm count for the selamectin-treated dogs was 36.4%. All of the live worms recovered from the selamectin-treated dogs and placebo-treated dogs were normal in motility. Only 1 live worm recovered from the control dogs was abnormal in appearance (i.e., 1 female worm was opaque white), whereas 6 of 8 selamectin-treated dogs had abnormal appearing worms. Twenty-one of the 51 worms from selamectin-treated dogs were

Table 1. Geometric mean counts of *Dirofilaria immitis* microfilariae following monthly treatment of dogs with an initial high microfilaremia. Treatment began 222 days after experimentally acquired infection with adult worms.

Study A

	Geometric mean *D. immitis* microfilarial counts (mf/ml)											
Treatment*	Day 0	Day 1	Day 7	Day 14	Day 30	Day 31	Day 37	Day 44	Day 60	Day 61	Day 67	Day 74
Placebo (n=8)	9,882	7,117	6,776	917	3,780	3,653	4,401	4,938	6,322	5,228	3,878	2,523
Selamectin (n=8)	11,568	10,462	2,620	432	459	496	654	796	606	458	336	223
% reduction**	0.0%	0.0%	61.3%	52.9%	87.9%	86.4%	85.1%	83.9%	90.4%	91.2%	91.3%	91.2%
P-value†	NS	NS	NS	NS	≤0.05	≤0.05	NS	NS	NS	NS	≤0.05	≤0.05

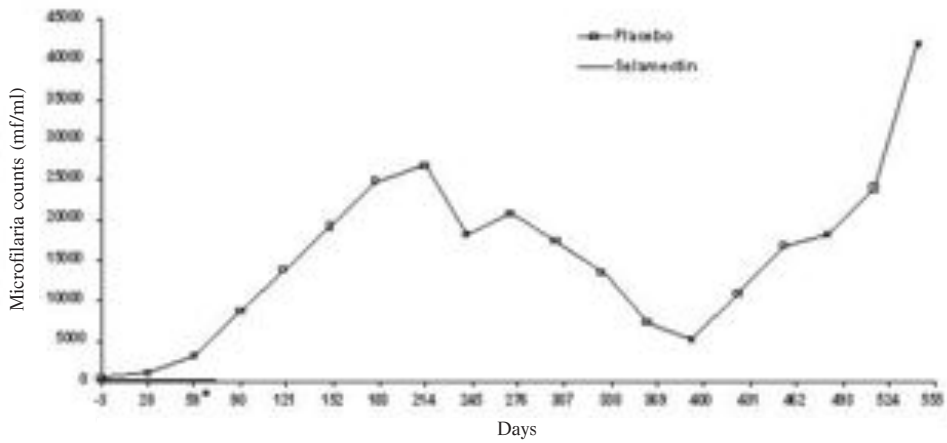
* Dogs were treated on days 0, 30, and 60.
** For selamectin treatment compared with placebo.
† Significance of difference between treatments.
NS = Not significant.

abnormal (i.e., opaque white with or without a darkened anterior end). All dogs in both treatments remained positive for heartworm antigen throughout the study, except for 1 selamectin-treated dog that tested negative on 1 occasion (day 483).

On day –3 (prior to the first treatment), the mean microfilarial counts were 382 and 315 mf/ml for the placebo- and selamectin-treatment groups, respectively (Fig. 1). For dogs treated with the placebo, the count increased to >1,000 mf/ml on day 28 (1 month after the first dose) and to >3,000 mf/ml by day 59. The count was >10,000 mf/ml from day 119 through the end of the study, except on days 362 (12 months) and 392 when counts were 7,284 and 5,207 mf/ml respectively. For dogs treated with selamectin, the mean microfilarial count showed no increase form day 3 to day 28 (348 mf/ml), and then declined to reach 0 at day 180. From day 180 through the end of the study (day 544, after 18 monthly treatments), the count remained 0 except on days 271, 301, and 332 when there was a mean count of 1 mf/ml. From day 59 to the end of the study, selamectin-treated dogs had significantly ($P \leq 0.05$) fewer microfilariae than dogs treated with the placebo. The reductions in geometric mean microfilarial count for dogs treated with selamectin, compared with those treated with the placebo, were 66.9% on day 28, 95.8% on day 59, 99.7% on day 90, 99.9% on day 119, and approximately 100% from day 150 to study completion.

No adverse reactions attributed to selamectin treatment were observed in this study.

Study B



* Treatment differences were significant (P ≤ 0.05) from day 59 through completion (day 544).

Fig. 1. Geometric mean counts of *Dirofilaria immitis* microfilariae in dogs treated monthly, beginning 30 days after experimentally induced infection with adult worms.

Table 2. Geometric mean counts of adult *D. immitis* at necropsy following monthly treatment that began 90 days after inoculation with L3 in dogs.

Study C

	Geometric mean counts of adult *D. immitis* worms recovered at necropsy on day 363	
Treatment*	Live worms	Dead worms
Placebo (n=8)	19.8	0.4
Selamectin (n=8)	0.3	0.0
% reduction**	98.5%	100%
P-value†	≤0.05	NS

* Dogs were treated on days 0, 30, 60, 90, 120,150, 180, 210, 240, 270, 300, and 330.
** For selamectin treatment compared with placebo.
† Significance of difference between treatments.
NS = Not significant.

Study C. Long-term monthly prophylaxis beginning at 3 months after heartworm inoculation ("Reachback" efficacy)

At necropsy (15 months after inoculation), the geometric mean counts of live adult *D. immitis* from placebo- and selamectin-treated dogs were 19.8 and 0.3, respectively (Table 2). There was a significant ($P \leq 0.05$) difference between the treatments, and the reduction in the heartworm burden for selamectin-treated animals, compared with that for the control group, was 98.5%. Dead heartworms were recovered at necropsy from the placebo-treated dogs only, but there was no significant difference between treatments for the number of dead heartworms. Adult *D. immitis* antigen tests were negative for all 16 dogs (both placebo- and selamectin-treated) from day –94 until day 57 (Fig. 2). From day 118 through day 361, the percentage of antigen-positive dogs for the placebo-treatment group remained above 85%, whereas for selamectin-treated dogs, the percentage never exceeded 38% and decreased over time, with 12.5% of dogs antigen-positive on day 361. Microfilarial counts were negative for all 16 dogs until day 118, and then only dogs treated with the placebo had positive counts. The selamectin-treated dogs remained negative throughout the study (Table 3).

Discussion

Results of the first study presented here (study A) found that no adverse effects were observed when selamectin was administered monthly for 3 months to dogs with high microfilaremia (mean, >10,000 mf/ml) prior to the first application. Furthermore, just 3 treatments with selamectin at monthly intervals was shown to have a substantial microfilaricidal effect, as demonstrated by reductions in microfilaria count of >90%, compared with those for the control group (study A). The microfilaricidal activity was con-

Study C

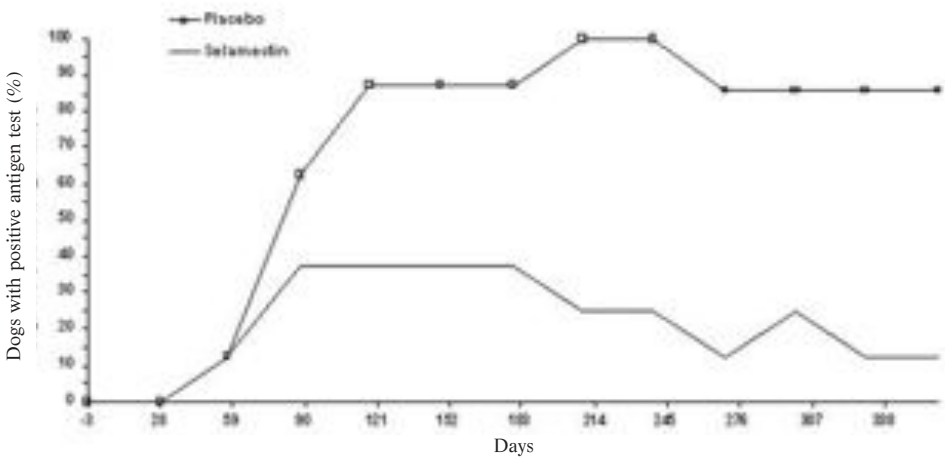


Fig. 2. Percentages of dogs positive for *D. immitis* antigen in dogs treated monthly, beginning 90 days after inoculation with (L3) larvae.

firmed in dogs with lower initial levels of microfilariae (study B), where a reduction of 95% in microfilarial counts was observed by the third month after commencing monthly treatment and 100% by the sixth month. It is generally considered that if microfilaremic dogs are treated with an effective microfilaricide, there is an increased risk of adverse effects occurring during the first 4 to 8 hours after administration (Knigth, 1998). Such reactions following the rapid death of large numbers of microfilariae can occur with microfilaremias of at least 5,000 mf/ml, but are more usually associated with burdens greater than 10,000 mf/ml. Reactions include lethargy, inappetance, ptyalism, vomiting, tachycardia, pallor, and acute circulatory collapse (Knigth, 1998). From previous studies with other macrolides,

Table 3. Geometric mean counts of *D. immitis* microfilariae following monthly treatment that began 90 days after inoculation with L3 in dogs.

Study C

	Geometric mean *D. immitis* microfilarial counts (mf/ml)											
Treatment*	Day 28	Day 57	Day 88	Day 118	Day 148	Day 179	Day 209	Day 239	Day 270	Day 300	Day 330	Day 361
Placebo (n=8)	0	0	0	2	80	986	1,205	3,325	3,353	13,131	2,554	2,219
Selamectin (n=8)	0	0	0	0	0	0	0	0	0	0	0	0
% reduction**	-	-	-	100%	100%	100%	100%	100%	100%	100%	100%	100%

* Dogs were treated on days 0, 30, 60, 90, 120,150, 180, 210, 240, 270, 300, and 330.
** For selamectin treatment compared with placebo.

notably ivermectin (20-50 mg/kg, ie, higher than prophylactic dosages) and milbemycin oxime, (prophylactic dosage) treatment-induced reductions in the levels of circulating microfilariae in dogs with patent infections have been demonstrated (Courtney et al., 1998). Milbemycin oxime causes a more rapid reduction in microfilaremia than ivermectin (Courtney et al., 1998). Thus, it is possible to see adverse effects due to dead microfilariae, particularly following the use of milbemycin oxime (prophylactic/microfilaricidal dosage), and it is recommended that microfilaremic dogs be carefully monitored for at least 8 hours after treatment with microfilaricidal dosages of these drugs (Knigth, 1998). It has been shown previously that selamectin can be safely administered to dogs with mature adult heartworms and low levels of circulating microfilariae (Novotny et al., 2000).

The efficacy of selamectin in gradually reducing levels of microfilariae (90-100%) over time offers benefits in terms of reducing transmission potential within a population. This effect indicates the potential for selamectin to be used following adulticidal therapy to eliminate a residual microfilaremia in dogs with patent heartworm infections. In common with other macrolides, such as ivermectin and milbemycin, when selamectin is administered to dogs with patent infections, the dogs may become amicrofilaremic resulting in "occult" infections. Hence, in a dog with an unknown history or when it is possible that there has been exposure to infection, particularly in young dogs or strays, it is important to establish the heartworm status of the dog prior to commencement of a prophylactic regimen. However, if for some reason this is omitted, the majority of infected amicrofilaremic dogs should be identifiable by using an adult antigen test.

It is not known at present whether microfilarial counts will increase following cessation of monthly administration of selamectin, as has been reported for milbemycin and ivermectin (Courtney et al., 1998). The other macrolides have been shown to effect embryogenesis in female heartworms (Lok and Knight, 1995); however, at this time, the effects of selamectin on adult worms, particularly female worms, have not been fully characterized.

Historically, as a class of compounds, the macrolides have not been considered to have activity against mature adult heartworms. In study B, when treatment was initiated against mature adult heartworms (11 months old) and treatment was administered monthly for 18 consecutive months, there were 36.4% fewer adult heartworms present in selamectin-treated dogs than in the controls, however, there was no statistical difference between the worm counts in the 2 treatment groups. In addition, many of the live selamectin-treated worms were abnormal in appearance. It is possible that additional monthly treatments with selamectin would increase the efficacy against adult heartworms. When ivermectin was administered monthly for 16 consecutive months to dogs harbouring mature adult heartworms, efficacy was 56.3% (McCall et al., 1998), and when treatment was administered for 29 months, efficacy improved to 94.9% (McCall et al.,

2001). It has also been suggested that if an effective regimen could be devised, ivermectin might offer some clinical advantages in heartworm-infected dogs, because the slow killing effect may reduce the risk of adverse effects (particularly those associated with pulmonary thromboembolism) from dead worms or worm fragments, compared with the risk of using adulticidal agents (melarsomine, thiacetarsamide), which have a much more rapid killing effect on adult heartworms, which leads to the death of some dogs (McCall et al., 1998).

These studies demonstrated that selamectin, when administered as 12 monthly treatments beginning 3 months after L3 infection (reachback), is also highly effective in preventing the maturation of heartworms, with a reduction of 98.5%.

Previously it has been shown that monthly prophylaxis for 13 months with ivermectin or milbemycin, beginning 3 months after L3 infection, significantly reduces the number of adult worms that develop by 97.7% and 96.8% for ivermectin and milbemycin, respectively (McCall et al., 1996). It has also been found that monthly prophylaxis for 12 months beginning 4 months after L3–induced infection leads to 95.1% and 41.4% reductions in maturation to adult worms for ivermectin and milbemycin, respectively, indicating the potent reachback effects of ivermectin and the limits of reachback activity for milbemycin. The effects of long-term monthly prophylactic treatment with selamectin, commencing 6 months after inoculation, is currently under investigation, and results will be forthcoming.

Conclusions

Selamectin is microfilaricidal but kills microfilariae relatively slowly, even in dogs with high microfilaremia. This gradual reduction of microfilariae virtually eliminates, or at least greatly minimizes potential adverse reactions due to large numbers of dead or dying microfilariae. Selamectin appears to have an adverse effect on mature adult heartworms, but the effect is gradual during long-term monthly prophylaxis. This slow killing effect on adult heartworms should minimize the potentially severe adverse effects seen with a rapid kill of adult heartworms. In addition, selamectin has a 3-month reachback effect that is similar to the other macrolide preventives. Thus, selamectin has demonstrated exceptional safety in heartworm-positive dogs and, together with robust reachback activity, increases the confidence with which this product can be used in the fight against heartworm disease in dogs.

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14

Heartworm (*Dirofilaria immitis*) infection in dogs: current update in Spain

J.A. Montoya, M. Morales, M.C. Juste, J.A. Corbera

Heartworm infection in dogs has been diagnosed around the globe. Relocation of infected, microfilaremic dogs appears to be the most important factor contributing to further dissemination of the parasite. The ubiquitous presence of one or more species of vector competent mosquitoes makes transmission possible wherever a reservoir of infection and favourable climatic conditions co-exist.

A climate that provides adequate temperature and humidity to support a viable mosquito population, and also sustain sufficient heat to allow maturation of ingested microfilariae to infective, third-stage larvae (L3) within this intermediate host is a pivotal prerequisite for heartworm transmission to occur. The length of the heartworm transmission season in the temperate latitudes is critically dependent on the accumulation sufficient heat to incubate larvae to the infective stage in the mosquito. The peak months for heartworm transmission in the Northern Hemisphere are July and August.

Heartworm prevalence has increased dramatically during the latest years. The disease has extended from tropical or subtropical countries to others with more temperate climate. The more frequent animal international travelling could contribute to dissemination of the parasite. Heartworm transmission is related to the dog's life style, because outdoor permanence increases the possibility for mosquito contact. However, breed or hair length has not been correlated with the risk of heartworm infection.

Heartworm infection is entirely preventable in dogs, despite their high susceptibility. Since most dogs living in heartworm endemic areas are at risk, chemoprophylaxis is a priority. Furthermore, some evidence strongly suggests that by reducing the reservoir population through increasing the number of dogs receiving chemoprophylaxis, a disproportionate decrease in the prevalence of infection among unprotected dogs may occur relative to the percentage of additional dogs receiving chemoprophylaxis. This collateral protection spreads the umbrella of chemoprophylaxis most effectively in communities where heartworm prevalence and dog population density are both relatively low.

During the latest years several epidemiological surveys on heartworm have been developed in many countries. The disease is distributed, mainly, in temperate climates in the World. The more prevalent countries are in America, Africa, Polynesia, Australia and Japan. In Europe the more prevalence (>10%) occurs in the Mediterranean countries. We must point out that several studies in the north of Italy, south of France and Spain have elucidated prevalence over 20%. Therefore, Spain is an endemic area of Heartworm (Genchi et al., 2005).

Inside Spain, the prevalence of the disease is higher in the southern coastal areas. Also, Canary Islands are considered an endemic area of the disease. Specifically, in the Canaries, sited in front of the north-western African coast, the available data has emphasized the highest prevalence of the disease in Spain (Valladares et al., 1987; Guerrero et al., 1989). Veterinary Surgeons in Canary Island include Heartworm Disease as the first differ-

Table 1. Prevalence of heartworm in Spain.

AUTONOMOUS COMMUNITY Province	Prevalence	References
ANDALUCIA	8.5%	Guerrero et al., 1989
Cádiz	12%	Guerrero et al., 1989; Ortega Mora et al., 1991
Cádiz-Málaga	5.5%	Rojo-Vázquez et al., 1990
Córdoba	18.0%	Anguiano et al., 1985
Córdoba	4%	Guerrero et al., 1989
Huelva	36.7%	Guerrero et al., 1989; Ortega Mora et al., 1991
Jaén	2.1%	Guerrero et al., 1989
Málaga	2%	Guerrero et al., 1989
Sevilla	1.5%	Guerrero et al., 1989
ARAGON	4.3%	Guerrero et al., 1989
Zaragoza	13.5%	Castillo et al., 1989
Zaragoza	8 %	Rodes, 2006
ASTURIAS	0 %	Guerrero et al., 1989
CANTABRIA	0%	Guerrero et al., 1989
CASTILLA-LA MANCHA	0%	Guerrero et al., 1989
CASTILLA-LEON	0%	Guerrero et al., 1989
Salamanca	12.0%	Pérez-Sanchez et al., 1989
Salamanca (ribera Tormes)	> 30%	Pérez-Sanchez et al., 1989
CATALUÑA	2.17%	Gutiérrez et al., 1995
CATALUÑA	0,6%	Solano-Gallego et al., 2006
Barcelona	1.2%	Rojo-Vázquez et al., 1990
Barcelona (bajo Llobregat)	12.8%	Aranda et al., 1998
Barcelona	2%	Solano-Gallego et al., 2006
Tarragona	0.85	Solano-Gallego et al., 2006
Tarragona (delta Ebro)	35.8 %	Anguera, 1995
Tarragona (delta Ebro)	26 %	Rodes, 2006
COMUNIDAD VALENCIANA		
Alicante	13%	Guerrero et al., 1989
Alicante	1.6%	Rojo-Vázquez et al., 1990
Alicante	18%	Rodes, 2006
Elche	2.6%	Cancrini et al., 2000
Valencia	4.1%	Guerrero et al., 1989
EUSKADI	0%	Guerrero et al., 1989
EXTREMADURA	6.7%	Guerrero et al., 1989
Badajoz	8%	Ortega Mora et al., 1991
GALICIA	0%	Guerrero et al., 1989
ISLAS BALEARES	6.3%	Guerrero et al., 1989
Mallorca	0.3%	Solano-Gallego et al., 2006
Ibiza	39%	Rodes, 2006
MADRID	2%	Ortega Mora et al., 1988
Madrid	1.1%	Guerrero et al., 1989
Madrid	1.9	Rojo-Vázquez et al., 1990
MURCIA	6.3%	Guerrero et al., 1989
MURCIA	9%	Rodes, 2006
NAVARRA	0%	Guerrero et al., 1989

Table 2. Prevalence of heartworm disease in Canary Islands.

Canary Island	Prevalence	Reference
CANARIAS (total)	28%	Guerrero et al., 1989
Gran Canaria	36%	Guerrero et al., 1989
Gran Canaria	58.89%	Montoya et al., 1998
Gran Canaria	23.87%	Sosa et al., 2002
Tenerife	34.4%	Valladares et al., 1987
Tenerife	20%	Guerrero et al., 1989
Tenerife	23%	Stenzenberger & Gothe, 1999
Tenerife	22.3%	Morales et al., 2001
Tenerife	21%	Montoya et al., 2006

ential diagnosis in dogs with cardiopulmonary signs.

Previous studies in Spain (Guerrero et al., 1989) showed that Huelva has the highest prevalence in Spain (36.7%). This study determined that the prevalence of *Dirofilaria immitis* in Gran Canaria was 36%. Later studies revealed prevalence over 30% in Salamanca (Muro et al., 1993) and the delta area of the river Ebro (Anguera, 1995). More recently, the highest prevalence (58%) of *D. immitis* in Spain has been described in Gran Canaria (Montoya et al., 1998). The prevalence in Gran Canaria is one of the highest described in the World; similar prevalence has been published in Cuba (Dumenigo et al., 1988) or Japan (Tanaka et al., 1985; Suenaga et al., 1978; Hatsushika et al., 1992). Otherwise, the highest prevalence ever described (86%) is in Papua-New Guinea (Hamir et al., 1986).

However, it is encouraging that the prevalence of *D. immitis* in Gran Canaria is decreasing: more recent surveys demonstrated prevalence under 23% (Table 2). In our opinion, this data could due to the increasing use of effectiveness drugs to prevent the disease, and mainly a very important veterinarian task in order to make aware of the disease to their clients. Also, it is more frequent that clients demand the use of the newest drugs for the treatment of their infected pets; this increasing demand is extending to rural areas where population is not aware as far as pet cares are concerned.

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15

The importance of dirofilariosis in carnivores and humans in Hungary, past and present

Éva Fok

Introduction

Several worm species of Superfamily Filarioidea develop in human and animal hosts and many may cause zoonotic infections. Two families are recognized: Filariidae and Onchocercidae (Anderson, 2000). These filarioid nematodes are transmitted by haematophagous arthropod vectors, including mosquitoes (Culicidae), blackflies (*Simulium* spp.), biting midges (*Culicoides* spp.), ticks (*Rhipicephalus* sp.), fleas and flies (Kassai, 1999, 2003). Filariidae elicit skin lesions and release eggs and/or larvae which attract arthropods, mainly flies (Muscidae). The Onchocercidae, on the other hand, have evolved blood or skin-inhabiting microfilariae and are transmitted by arthropod vectors which create lesions or pierce the skin and then suck blood. In the recent decade the importance of some zoonotic worms of carnivores from Onchocercidae, such as *Dirofilaria* and *Onchocerca* spp. is increasing (Pampiglione and Rivasi, 2000; Eberhard et al., 2000; Pampiglione et al., 2001; Sréter et al., 2002; Genchi, 2003; Salló et al., 2005).

The climate changes and travelling with pets may help both the spreading of vectors and the appearance of worm infection at risk for human health in non-endemic or previously free areas (Trotz-Williams and Trees, 2003). Two species of genus *Dirofilaria*, *D. immitis* and *D. repens* mostly are important agents of zoonotic infections in the Mediterranian area (Muro et al., 1999).

For both the parasites, the most important natural host is the dog, which has a high prevalent and persistent microfilaremia. In the middle part of the continent, *D. repens* is mostly widespread and nowadays dirofilariosis is considered as an emerging zoonosis (Irwin, 2002).

Dirofilariosis in carnivores

Cardiovascular-dirofilariosis

The first recorded case of *Dirofilaria*-infestation of carnivores in Hungary was published more than 20 years ago (Boros et al., 1982). In this report it was shown *D. immitis* infection in two female beagle dogs imported from the United States. The adult worms were detected in the right cavity of the heart. These dogs, with the other beagles, were kept for experimental purpose in closed recovers, so it was clear that the infection was imported.

The next reported filarial infection in a dog was in 2000 (Vörös et al., 2000). The dog lived in the USA for years with the owner and they returned to Hungary some months before the date of admission into clinic. This five-year-old male dog of mixed breed was recovered with a two-days long history of depression, exercise intolerance, dyspnoea, anorexia, vomiting and voiding of reddish urine. The animal, in spite of the intensive therapy died within some hours after admission. Necropsy revealed several adult *D. immitis* in the blood clots within the caudal vena cava and within the dilated right ventricle. Microfilariae were found within the capillaries of the myocardium, interstitial pulmonary capillaries and in other organs, including spleen and liver and in the peripheral blood smears. Vena cava syndrome due to the occlusion of

the caudal vena cava by adult worms together with blood clots was diagnosed by necropsy and this complication was stated as the cause of the sudden crisis and the death of the patient. In spite of that, no newer imported case of canine heartworm infection has been published in the scientific literature in Hungary, we have got some information from personal communications conducted with small animal practitioners about some cases (e. g. imported Mastino Napoletano dogs from Canary Islands, Cane Corso puppies from Italy) where *D. immitis* infection was suspected.

An interesting case was detected in 1999, when a 3-year-old mongrel dog living in Tisza river area was dissected after an accident and the veterinarian found worms in the left ventricle of the heart. These worms were identified as *D. immitis.* This dog was living together with his mother who was housed six years earlier by an owner from the street. The dog was living at the river shore, not far from a parking place of international trucks. However, it was not possible to clarify if the infection was autochthonous or acquired through the biting of an infected mosquito transported by one of the trucks.

Although proved autochthonous dog heartworm infection has not so far recorded in Hungary, the described cases demonstrate the risk of introduction of this disease with infected animals from endemic countries (Farkas, 2003).

Cutaneous-dirofilariosis

In 1997, Zahler et al. published the results of an epidemiological survey (Zahler et al., 1997) carried out between 1993-1996 in München. Out of five dogs found infected with *D. repens*, two dogs were imported from Italy or Greece, and the third had travelled with its owner in Hungary and former Yugoslavia. The infection was diagnosed by blood examinations with Knott's method. The origin of the infection was not clear and it cannot be excluded that the infection was really acquired in Hungary.

The first three autochthonous *D. repens* cases recorded in dogs were published in Hungary in 1998/1999 (Fok et al., 1998; Széll et al., 1999). In the very first case the worm was found in a verruca-like nodule removed in autumn of 1995 from the subcutaneous tissue of the neck region of a 4-year-old dog, which had spend the summer at river Tisza (Fok et al., 1998). In the other two cases, the worms were found in a granuloma removed from the thorax region and from the subcutaneous tissue of the leg, respectively (Széll et al., 1999). One of them had spend some days at Lake Balaton and the other one was living in the Tolna County, near the southern boarder of the country. On the basis of the morphological features, the worms were identified as gravid females in all cases. Furthermore, 101 dogs from Tolna County were examined for microfilariaemia by Knott's method. The prevalence of *D. repens* microfilaremic dogs was 9%. Considering that these dogs were never abroad, it was stated that there are endemic areas for *D. repens* in Hungary.

In the recent years, according to the personal communications of colleagues from the main veterinary diagnostic laboratories, more and more microfilar-

ia positive blood samples of dogs as accessory findings are detected in the praxis. Most of these cases are suspected *D. repens* infection, although the detailed epidemiological background is missing. Moreover, reports on human infection (see later) are frequently detected in Hungary.

Why is the number of cases increasing in our country? What can be the epidemiological background? What is the prevalence of infection in dogs and cats in Hungary? How it is possible (or weather it is necessary) to treat *D. repens* positive animals in Hungary? Which mosquitoes species might be the vector of this worm in our country? The cutaneous dirofilariosis can cause problems in the diagnostic differentiations, as well. What kind of connections might be between the infection of the animals and the increasing number of the human cases in our country? Because of such a questions and the lacking of *D. repens* prevalence data in domestic carnivores in Hungary, in 2005 we decided to start with a research work in our Department of Parasitology and Zoology, including a PhD programme. In parallel with the study, we plan to monitor the human cases of *Dirofilaria* infection to obtain more information on the epidemiological background in our country. The aim of this work is to survey the infection of the most frequent final hosts, then to study the role of the vectors in the epidemiology and the possibilities of the spreading from carnivores to humans to achieve an effective control (see later the preliminary results of this work in a free presentation).

The baseline data of this work would be useful for veterinarians working in the small animal praxis to help them in the recognition of the clinical picture of cutaneous dirofilariosis. Results concerning this zoonotic disease would be useful for the human doctors, as well, and can help in a more effective control. Furthermore, the results of the planned study could be important not only in Hungary, but also may contribute to the European knowledge on this mosquitoes transmitted disease.

Dirofilariosis in humans

The first human filarioid infection in Hungary was detected by Babes in 1879 (cited by Kotlán, 1951). However, an actual evaluation of the early human cases in Hungary was published in the middle of the last century by Professor Academic Kotlán (Kotlán, 1951). The author referred to Desportes (1939-1940), who in his paper presented a historical review of the cases of human filarioidosis know from literature, in which the parasites playing part in the pathological process could be identified with, or was found to be closely related to "*Filaria conjunctivae*" Addario, 1885 (cit. Kotlán, 1951). Having had the opportunity to examine a new case, Kotlán attempted to review all available data at that time, either verbal or published in Hungarian literature. It was stated in his publication that at least 9 cases recorded from 1880 to 1950 in Hungary was probably identical with *D. repens*, however the infection with other filarioid species was not completely excluded. It was not possible to clarify the epidemiological background

of these cases namely the sources of infection. It was supposed that some of these cases probably were autochthonous because the patients never had left the country.

The case published ten years later (Németh and Kugler, 1968), was the first ocular involvement in our country, and it was supposed that the removed worm pieces from a nodule on the sclera of the 21 years old man were belonging to a *D. repens* specimen. More than 30 years later, the next Hungarian human case in which the worm was detected in the spermatic cord was published by Pampiglione et al. (1999). It was thought that these cases were acquired abroad, too.

However, in a little while, six new cases were reported by pathologists with the help of a parasitologist (Elek et al., 2000). Two of these cases might have been acquired in Italy during summer travels. Four patients have never been abroad and these cases must be considered as autochthonous infections. According to the authors, the patients liked dogs and cats, and they were often living in sites along Danube and Tisza rivers, where mosquitoes are abundant. The thickness of the multilayered cuticle of the worm, diameter of the body and the size, form and number of the longitudinal ridges on its surface were used in the histological diagnosis of *D. repens* worms. In fact, the definitive diagnosis of human dirofilariosis is a pathological and parasitological task. In this report the authors called the attention of Hungarian human clinician to ask the patients whether they have been (and when and where) abroad, whether they have animals (dog or cat), and whether they live near water shore where mosquitoes are abundant. The answer may explain the spread of the worm in Hungary.

It seems that in the recent six years more and more new autochthonous human cases become known in our country. However, we can have the information about most of them from oral presentations in conferences or in personal communications, and only partly from the scientific publications. In the new cases the worms were removed from the orbital tissues (Parlagi et al., 2000), subconjuctival space (Szénási et al., 2000; Hári et al., 2002; Kucsera et al., 2002; Aczél et al., 2005; Tornai et al., 2006), upper lid (Salomváry et al., 2005), the fatty tissue of a subcutaneous nodule of the thigh (Lengyel et al., 2005), a cyst situated in the cutaneous and subcutaneous layers of the forearm (Péter et al., 2005) and the subcutaneous tissue of the neck region (Csanády, 2005, personal communication). All these cases, after the detailed anamnesis and identification of the worms or examining of tissue sections were diagnosed as *D. repens* infection.

Nowadays, the medical clinicians who suspect an human filarioid infection in their patients can be supported in the etiological diagnosis by parasitologists sending removed worms for identification or dissected tissue from the patient to specialized laboratories such as the Department of Parasitology of 'Johan Béla' Human Health Institute (see later a free presentation about the human cases by I. Kucsera) or the Department of Parasitology of the Veterinary Faculty of Szent István University, in Budapest.

Conclusion

It seems to the parasitologists, that in Hungary, the occurrence of *D. repens* is much more frequent than it was considered earlier. The tourism with pets and visiting of the different dog or cat exhibitions abroad and in our country, the repeated travelling of Italian hunters with their dogs to Hungary, climate changing, frequent raining and flood may have increased the spreading of dirofilariosis. A close cooperation not only with the parasitologists, but also between human and veterinary clinicians in our country and with scientists from surrounding countries are needed to better understand the epidemiology of dirofilariosis and to control the risk of infection both for the public and animal health in Europe.

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Free Communications

An update on filarial parasites in *Vulpes vulpes* of Tuscany (Central Italy)

M. Magi[1], P. Calderini[2], S. Gabrielli[3], M. Dell'Omodarme[4], F. Macchioni[1], M.C. Prati[4], G. Cancrini[3]

[1]Dip. di Patologia Animale, Profilassi ed Igiene degli Alimenti, Univ. di Pisa, V.le delle Piagge 2, 56124 Pisa, Italy; [2]Ist. Zooprofilattico Sperimentale del Lazio e della Toscana, Dipartimento di Rieti, V. Tancia, 21, 02100 Rieti, Italy; [3]Dip. di Scienze di Sanità Pubblica, Università "La Sapienza", P.le Aldo Moro 2, 00185 Roma, Italy; [4]Scuola Normale Superiore di Pisa, Italy, INFN Sezione di Pisa, Piazza dei Cavalieri 7, 56127 Pisa, Italy

Red foxes (*Vulpes vulpes),* like other wild and domestic carnivores, can be affected by several species of filarial nematodes, namely *Dirofilaria immitis*, *D. repens*, *Acanthocheilonema reconditum*, and *A. dracunculoides*. The first species induces heartworm disease, with severe/extremely severe pathological changes in the animals, whereas the other parasites are etiological agents of subcutaneous/connective tissue filariosis. Furthermore, at least dirofilarial species proved zoonotic and, therefore, where the animal disease is endemic, the human population is at risk for developing ocular lesions or pulmonary/subcutaneous nodules that always give rise to the suspicion of a malignant cause.

Literature data about the role of the fox as reservoir for filarial nematodes are in disagreement. In fact, in spite of adult worms found in this animal seem often immature and microfilaraemia is reported as sporadic (Meneguz et al, Parassitologia 40 suppl: 105), it has been suggested that the fox may play an epizootiologic role in heartworm infection (Mañas et al, 2005, Vet J 169: 118-120). The possible importance of this wild animal as reservoir of infection for people and for companion animals prompted us to include filarioses among the parasitic diseases that have to be checked on the foxes examined during a survey on echinococcosis.

With the aim of contribute to this subject of debate, and to compare the infection rates of filarial worms in wild and domestic canidae of the region, 112 foxes (42 males, and 72 females), killed during the hunting season 2003-2004 in several areas of the Tuscany (Central Italy), were examined. The carcasses of the foxes, coming from the Monte Amiata (Grosseto; n=37), the hills around Cecina (Livorno; n=26), the zones around Cascina and Bientina (Pisa; n=33) and the hills around Siena (n=16), were sent to the laboratory of Pisa. Data on sex, age, weight, and area of origin were collected for each animal, and the carcasses were stored at -20°C until necroscopy. Microfilariae were searched for in pulmonary and splenic blood smears, and species identification was performed using both morphologic and morphometric characteristics, and Barka coloration technique. As far the adults, it was only possible the searching for *D. immitis* in the endocardium and in the pulmonary

artery, because organizational problems hampered the examination of tissues in order to ascertain the presence of adults belonging to the other species. Finally, molecular diagnostics (DNA extraction, PCR using general "filarial" primers S2-S16 and sequencing) were applied to adults and micriofilariae to confirm all identifications. Data were statistically analyzed with the Fisher exact test, and results were considered statistically significant if $P<0.05$.

A total of 20 foxes (17.9%) turned out positive to filarial parasites. In 8 specimens (7.1%) were found mature adults of *D. immitis* (9 males and 14 females), and two of them (1.8%), collected around Cascina (Pisa), had also microfilariae both at pulmonary and splenic level. One of these two foxes was positive also to microfilariae of *D. repens*. Amicriofilaraemic subjects harboured at least an adult couple. A total of 12 foxes (10.7%) were positive to microfilariae of *A. reconditum*, absent only from specimens hunted on the hills around Livorno, where all foxes were found amicrofilaraemic. In the four areas no difference was evidenced among positivity rates to adults of *D. immitis*, whereas there is a significant difference among those to microfilariae ($P = 0.022$). Molecular tools confirmed morphological identifications.

Our findings on *A. reconditum, D. immitis* and/or *D. repens* circulating microfilariae in about 10% and in less than 2%, respectively, of the fox carcasses examined fit in with data regarding Italy. As far Tuscany, after 25 years the prevalence of microfilaraemic specimens is about unchanged (19.4% vs 17.9%) (Gradoni et al., 1980, Ann Ist Sup San 16: 251-256), and it is not so much different from that evidenced on concentrated blood samples of alive dogs (35.4%) (Marconcini & Magi, 1991, Ann Fac Med Vet Pisa, XLIV: 153-156). Our findings, drawn by the examination of very few µl of blood obtained from perished carcasses, support the hypothesis that the fox can be a source of infection for invertebrate hosts and, therefore, wild reservoir for filarial nematodes that can be transmitted to companion animals and people.

Preliminary results of an epidemiological survey on dirofilariosis of dogs and cats in Hungary

Éva Fok[1], Gabriella Kiss[2], Gábor Majoros[1], Olga Jacsó[1], Róbert Farkas[1], Mónika Gyurkovszky[1]

[1]*Department of Parasitology and Zoology, Faculty of Veterinary Science, Szent István University H-1400 Budapest, P.O. Box 2, Hungary;* [2]*Pfizer Animal Health, 1123 Budapest, Alkotás u. 53., Hungary*

Background

In the recent years more and more frequently cases of mosquito-borne filarioid subcutaneous infection with *Dirofilaria repens* Railliet and Henry, 1911 has been reported in animals (mainly dogs) and in humans. However, limited information were available about the frequency of *D. repens* infection in carnivores in Hungary.

For such a reason, epidemiological surveys were carried out to assess the status of this carnivore's filarioid infection and to evaluate the risk for veterinary and medical public health.

Materials and methods

From June 2005 to July 2006, 826 dog and 29 cat blood samples collected from several areas of the country with the help of veterinary practitioners were examined. The presence of microfilariae in the blood samples was assessed by modified Knott's technique. Larvae were identified by morphological criteria.

Results

D. repens microfilariae were found in 116 blood samples from dogs (116/826; 14%) and in 2 samples from cats (2/29; 6.9%). Of the positive dogs, 64 (55.2%) were living along Danube river, 40 (34.5%) along Tisza river, 1 (0.8%) on the shore of lake Balaton and 11 (9.5%) near other wet areas. The positive cats were from the town of Szeged, situated at both sides of Tisza river. Of the positive dogs, 39.4% were aged 3-6 years and 32.1% were 7-10 year-old. The infection prevalence was 53.2% in male dogs and 46.8% in female dogs. Higher prevalence values were found among mongrel (20.7%) and German shepherds (19%). Other breeds such as various hunting breeds (21.6%) and 1 to 2 positives dogs from miscellaneous breeds (38.8%) were also found to be infected.

Skin lesions (such as erythema, papules, alopeca, nodules) were diagnosed in 276 (33.4%) of the examined dogs and from these animals 37 (13.4%) was microfilaria-positive. However, of the 116 positive dogs, 37 (31.9%) showed skin lesions. From the examined cats, 10 (34.5%) showed various skin disorders, but the microfilaria-positive cats were without symptoms.

The prevalence of other parasitic infection was also evaluated according to the other clinical records, animal

maintenance and control against the ecto- and endoparasites.

Conclusion

It has been shown that *D. repens* infection in both dogs and cats is more important than it is currently considered in Hungary. The survey highlights the possible zoonotic risks for humans living in the regions where the positive animals were found. Visiting or living at the coastal areas of rivers seems to be a significant risk factor to acquire the infection. Our further aim is to continue this survey to acquire more information about the occurrence of this worm infection and to study the role of mosquitoes in spreading of the infection in our country.

Review of human dirofilariosis diagnosed at the Department of Parasitology, National Center for Epidemiology, Budapest, Hungary

István Kucsera, Zsuzsanna Szénási, József Danka

National Center for Epidemiology, Department of Parasitology, 1097 Budapest, Gyáli út 2-6, Hungary

In the temperate regions of Europe (mainly Italy, France and Greece, but over the last few years in Hungary as well) a specific filaroid worm, the *Dirofilaria (Nochtiella) repens*, a parasite of dogs, cats and some other carnivores, transmitted by mosquitoes, has occasionally infected people. In the world literature more than 800 cases have been recorded. Usually only one worm can be detected in human tissues. In human cases the microfilariemia and eosinophilia are usually not present. The diagnosis is ordinarily based on morphology of the intact worm and specific histopathological findings. Surgical removal and identification of the worm are both diagnostic and therapeutic.

In the period from 2000 to August 2006 we diagnosed 14 cases of dirofilariosis in 8 female and 6 male patients. Mean age was 57 years for female and 62 for male (average 60 years). This higher prevalence in older-age corresponds to literature data. Of the 14 cases, 7 had ocular localization, 6 subcutaneous and 1 was diagnosed in histopathological section of removed lymph-node in a patient with lymphoid leukemia. Anamnestic eosinophilia was found in 2 cases. For detection of microfilariae we used Knott concentration technique in 7 cases, with negative results.

Based on the available epidemiological data, it can be deduced that most of these cases (8) are autochthon infections. In 2 cases the data were ambiguous and in 4 cases there were no available epidemiological data. The incidence of human dirofilariosis in Hungary is sporadic, and does not show seasonality.

It can be concluded that *D. repens* occurs in Hungary. The possibility of dirofilariosis has to be taken into consideration in cases of predisposed localized nodules. Increased interest of the clinicians and parasitologists resulted in the newly detected cases being completely diagnosed with both morphological and histopathological examinations. The differential diagnosis from the other filaria species which occur in humans is not required in these cases, because the patients' histories contain no data that refer to that possibility. Therefore, we consider that most of our cases are autochthonal. Although the incidence of dirofilariosis is sporadic and the public health importance is not high, the increasing number of cases suggests that direct attention must be paid to this zoonosis.

Dirofilariosis in dogs - the actual situation in the Czech Republic

R. Dobesova, Z. Svobodova, V. Svobodova

Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic

Introduction

In the past, canine dirofilarial infection (*Dirofilaria immitis* and *D. repens*) in the Czech Republic was associated with import and was considered an imported infection. The present study shows at surveying the presence of autochthonous *Dirofilaria* infections in dogs in the area of the Czech Republic where the climatic conditions are favourable for the life cycle of the parasite.

Material and Methods

We have investigated the occurrence of dirofilariosis in dogs in the Czech Republic from Břeclav area known by high abundance of mosquitoes. The study was carried out from November 2005 to November 2006. The group consisted of 104 dogs. The sampled dogs had no history of travelling abroad and were free of clinical symptoms. Microfilariae were detected by using the Knott test and determined with the histochemical method based on the activity of acid phosphatase.

Serum samples were examined with the PetChek kit (IDEXX Laboratories, Portland, USA) for the detection of adult female *D. immitis* circulating antigens. We have also examined 11 out of 104 animals with IFA Heartworm kit (Fuller Laboratories, USA) for the detection of specific antibodies against *D. immitis.* For comparison we inserted also 2 samples positive for *D. immitis* antigen.

Results

Microfilariae were detected in 8 dogs. The result of the acid phosphatase staining for all eight samples agreed with *D. repens* species. No microfilariae of *D. immitis* were detected. Serological testing detected *D. immitis* antigens in 7 dogs. *D. repens* positive dogs were negative on the ELISA for *D. immitis*.

Specific antibodies against *D. immitis* were found in 6 out of 11 dogs including 2 positive for *D. immitis* antigen.

Conclusions

Our results confirmed endemic dirofilariosis in the Czech Republic. Dirofilariosis in the Czech Republic has been slowly spreading as our previous results show.

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Dirofilaria repens infection in dogs in the Czech Republic

Z. Svobodova[1], V. Svobodova[2], C. Genchi[3]

[1]Small Animal Clinic, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic; [2]Department of Parasitology, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic; [3]Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Università degli Studi di Milano, Milano, Italy

Introduction

Dirofilaria repens is the agent of the subcutaneous filariosis in dogs and cats. Its pathogenicity is often underestimated. Global warming, the increasing abundance of mosquitoes, movement of dogs between endemic and non-endemic countries have increased the risk of spreading of filarial infections in formerly *Dirofilaria*-free areas.

Material and Methods

The study was carried out from November 2005 to November 2006 on 80 dogs, 53 males and 27 females, aged 1-12 years. Most were hunting dogs that spend the majority of their time outdoors. The sampled dogs had no history of traveling abroad and were free of clinical symptoms. Samples of whole blood, blood serum and 2 fresh blood smears were obtained from each dog.

Microfilariae

A modified Knott test was used to detect microfilariae (mf) that were then identified on the basis of their morphology. Species identification was confirmed by histochemical staining of acid phosphatase with naphthol AS-TR-phosphate as a substrate, and pararosaniline as a chromogen.

PCR - DNA was extracted from 100 µl of canine blood samples positive for circulating mf using a commercial kit (QIAamp DNA blood; QIAGEN GmbH, Germany). PCR reactions for the amplification of the mitochondrial coxI gene were performed in a final volume of 10 µl using general filarial primers and thermal profile described in Casiraghi et al. (2004). PCR products were gel-purified and sequenced directly using ABI technology. The obtained sequences were compared to those deposited in gene banks.

Results

Seven of the 80 dogs tested were microfilaremic (8.7 %). Five were male aging 6 - 11 years and 2 were female, both aged 2 years, different breeds - German Shorthaired Pointer (3), Jagterier (1), Labrador Retriever (1), Czech Pointer (1) and Tosa Inu (1). Acid phosphatase staining identified *D. repens* species in all the samples. PCR definitely confirmed *D. repens* diagno-

sis in these samples. Numerous adult *D. repens* were found in the subcutaneous tissue during necropsy of one *D. repens* microfilaremic dog euthanized following the owner's request.

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Filariosis in dogs in Serbia

S. Dimitrijević[1], A. Tasić[2], S. Tasić[2], V. Adamović[3], T. Ilić[1], N. Miladinović-Tasić[2]

[1]Department of Parasitic Diseases, Faculty of Veterinary Medicine University of Belgrade, Serbia; [2]Department of Microbiology and Parasitology, School of Medicine, University of Niš, Serbia; [3]Veterinary Clinic VEGE-VET, Požarevac, Serbia

Systematic research on canine filarioses caused by the species *Dirofilaria repens, Dirofilaria immitis,* and *Acanthocheilonema* (*Dipetalonema*) *reconditum* in Serbia has not been reported. Aim of this study was to establish the prevalence of filarial nematodes in dogs and to identify species according to their morphological and morphometric characteristics.

A total of 208 blood samples were drawn from dogs from different areas of Vojvodina and Braničevo region. Modified Knott technique and commercial DIFIL test (EVSCO, BUENA, NJ, USA) were used to detect microfilariae in blood samples. Identification of species of microfilariae was performed according to their morphological and morphometric characteristics.

All morphometric parameters were obtained using automatic image analysis Lucia M (NIKON). Including mixed infections, microfilariae were found in the peripheral blood of 101 examined dogs. *D. repens* infection was found in 97 dogs, *D. immitis* infection in 15 dogs, and *A. reconditum* infection in 4 dogs.

The appearances of dirofilariosis in Serbia - Vojvodina

Sara Savić-Jevćenić[1], Branka Vidić[1], Živoslav Grgić[1], Lolić Zoran[2]

[1]Scientific Veterinary Institute of "Novi Sad", Novi Sad, Serbia; [2]Private practice "Leo", Novi Sad, Serbia

The first information on the existence of dirofilariosis in Serbia was published in 1999 (Sanda Dimitrijević, 1999). The number of infected dogs is getting higher every year. In the region of Vojvodina, as the part of Serbia, the investigation was done and the cases of infection were found (Tasić et al., 2003). In the year of 2004, a group of 100 dogs was observed within the region of Vojvodina (Bačka and Srem). The blood test was performed with a test kit diagnostic device for quick diagnostic of dirofilariosis, named FASTest HW Antigen, produced by MegaCor, Austria and ELISA test was performed in the same blood samples by a DiroCHECK test kit, produced by Synobiotics, USA. After the analysis and the comparison of the results there were 7% of positive samples to dirofilariosis, gained by two different diagnostic methods - fast test and ELISA.

The positive samples were from the dogs of Srem region, and the places were the following: Venas, Bukovac and Ledinci.

During the year of 2006, the blood samples were taken again from the teritories where dirofilariosis was found previusely and analysed for dirofilariosis, again. The test was done on 50 blood samples from Srem region (Ledinci, Paragovo, Krušedol, Selište, Venac, Bukovac and Petrovaradin). The samples were analysed by ELISA method, again with the same test kits - DiroCheck test kits, produced by Synobiotics, USA. The result was that 5 of 50 dogs were positive to dirofilariosis, which makes 10% of the analysed population. The positive samples were from the following places of the Srem region - Ledinci, Krušedol, Venac and Bukovac.

The number of positive dogs to dirofilariosis has grown during the last two years in the region where the disease was primarly found and dirofilariosis is still present at the same places of Srem region with the tendency of spreading during the year 2006. Usually, dirofilariosis is a side finding during the dissection, and a dog is rarely suspected to have dirofilariosis during the life time, even it has clinical symptoms.

Dirofilariosis in dogs and wild carnivores in Romania

Coman Sofia, B. Băcescu, T. Coman, Gh. Pârvu, Cristina Dinu, T. Petruț, N. Bercaru, Adriana Amfim (Padure)

The Veterinary Medicine Faculty, Spiru Haret University, 2 Jandarmeriei, Street, Bucharest, Romania

The increased number of dirofilaria cases and the impact on the human health due to the zoonotical aspect of the helmintosis, it's mainly the reason for the global scientifical research on the matter.

The research schedule was elaborated by the Parasitological laboratory of the Spiru Haret University, Faculty of Veterinary Medicine, Bucharest, Romania. The clinical findings were studied on 52 dogs of different breed and age. The clinical, parasitologic, and pathologic features of this entity are discussed, during the 6 months trial.

From the 52 studied dogs, 12 (23.07%) were *Dirofilaria* positive. Symptoms, commonly chest pain, cough, or hemoptysis, along with cardiac failure and ascites were present in 25% of 3 patients.

We found positive 5 dogs, aged 3-5 years old, 5 dogs aged 6-8, and 2 dogs aged 10-14, from which 8 were females and 4 males.

The hematology examination revealed: microcytar cupripriv hypocrom anemia, neutrophily, lymphocytopeny, eosinophily. Radiologicly was registerd cardiac hypertrophy with left ventricular dilatation and densifications pulmonary parenchim.

Electrocardiographic tests revealed hipertrophic cardiomiopathy.

Histopathological examination revealed pulmonary edema, thromboses within arteryes, and expatiated alveolar capillary. Among the perilobulary septum and the conjunctive tissue, numerous macrophage cells were found, with the purpose of fagocitosing the erythrocytes degradation product, hemosiderin.

Dirofilariosis in dogs and wild carnivores in Bulgaria

Zvezdelina Kirkova, Andrey Ivanov, Dimitrina Georgieva

Department of Parasitology, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria

A study of the prevalence of the dirofilariosis in dogs and wild carnivores in Bulgaria was conducted from 2001 to 2006. Blood samples from 487 dogs out of heartworm prophylaxis originating from different parts of the country were tested. Microfilariemia was found in 42 (8.62%) of the samples by the modified Knott's test. Infected animals were found in all parts of the country.

All microfilaria positive samples were antigen positive by Pet Check HTWMPT test (IDEXX). Three (6.6%) of 45 microfilaria negative samples were antigen positive.

The necropsy of 113 foxes, 56 jackals, 22 wild cats and 21 martens revealed *Dirofilaria immitis* infection in 4 (3%) of the foxes and 5 (8.9%) of the jackals. The intensity of the infection was from 2 to 16 specimens.

Our results show that wild canides are a natural reservoir and source of infection for dogs. The trend of increasing prevalence of dirofilariosis in dogs, when compared to previous studies, suggests that heartworm prophylaxis should be considered in Bulgaria.

Dirofilariosis among dogs in a small animal practice in the Plovdiv region, Bulgaria

Milen Kostadinov

Private Veterinary Practitioner, Veterinary clinic "Novet", Plovdiv, Bulgaria

Dirofilaria immitis is still considered a rare and imported disease for Bulgaria. It is studied by all veterinary students but it is not expected to be found in the practice. Most practicing veterinarians regard the disease as uncommon. As a result routine analyses are not performed, which leads to a lack of up to date information about localization and numbers of dirofilariosis cases.

The data presented here are gathered in a small animal practice for a period of 5 years (from 2001 until 2006), from the city of Plovdiv and its surroundings, southwestern Bulgaria (total population about 500,000 people).

Since 2001 we have registered a total of 87 cases, 56 percent of them in Plovdiv and 44 percent in the surrounding towns and villages.

We analyzed the cases by the following categories: severity of disease at presentation; diagnostic method used; whether or not the dogs were treated; complications after treatment; dogs living outdoors/indoors; use of dogs (hunting dogs, guard dogs, pets); age; gender; location.

Practically no prevention was used on any dog before presentation. The vast majority of clients heard about the disease for a first time when they presented their sick animals.

During the period of investigation the number of the diagnosed cases was increasing every year. We believe this observation doesn't relate to the dynamics of the disease in the region, but is rather due to technical factors.

The increased cases identification is more related to the fact that we changed our approach from sporadic tests (when the clinical condition indicates) to routine analysis of many dogs coming to the practice. Another important factor is the expansion of our practice, thus the physical ability to check more dogs.

The region of Plovdiv is serviced by another 4 practices having the size of ours and about 15 smaller practices. We deduce that our results can be multiplied at least by a factor of 5 to derive the total number of dirofilaria cases presented (but not necessarily diagnosed) in Plovdiv.

Our results show that dirofilarosis is not a rare disease among dogs in Plovdiv region. This imposes the need to educate dog owners about prevention and to incorporate dirofilaria diagnostic methods into the routine checks of all dogs.

Dirofilariasis in Turkey

Bulent Ulutas[1], Tulin Karagenc[2]

Department of Internal Medicine[1] and Parasitology[2], Veterinary Faculty, Adnan Menderes University, Aydın, Turkey

Dirofilariasis is a disease caused by filarial worms of the genus *Dirofilaria* transmitted by mosquitoes. Although dirofilariasis was originally considered a disease of strict veterinary importance, it has been recognized as an emerging zoonosis. Two *Dirofilaria* species, *D. repens* and *D. immitis*, are of special interest to humans because of their harmful effect on our company pets (dogs and cats) and potential zoonotic role. Turkey is one of the world countries where the presence of *D. immitis* and *D. repens* is currently confirmed. The objective of this study was to provide information on dirofilariasis both on animals and humans in Turkey. *D. immitis* was first reported in one dog in Turkey in 1951 and was found in different provinces and canine prevalence ranged from 0.83 to 46.22% at local scale following years. The national prevalence of the disease has not been determined hitherto, yet. Many cases of *D. immitis* have been reported in dogs in Turkey. *D. repens* was first reported in one human in Turkey in 1944. It was only investigated in dogs in the east part of Turkey, in Elazıg province, seropositivity rate was 7.07%. In Turkey, human dirofilariasis were documented in 18 cases. Serologic methods for use in humans are needed for clinical evaluations of patients with pneumonitis living in highly enzootic *D. immitis* regions. These results demonstrate that dirofilariasis is widespread in dogs in Turkey and epidemiological surveys are needed to determine the real extent of this zoonotic infection.

Prevalence of canine heartworm disease in the Gemlik area of Bursa Province, Turkey

T. Civelek[1], A. Yildirim[2], A. Ica[2], O. Duzlu[2]

[1]Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Internal Medicine, Afyon, Turkey; [2]Erciyes University, Faculty of Veterinary Medicine, Department of Parasitology, Kayseri, Turkey

The purpose of this study was to determine the prevalence of heartworm disease (*Dirofilaria immitis*) in dogs in the Gemlik area of Bursa province, Turkey. The study was carried out between June and September 2004. A total of 100 dogs with various ages and sexes were included in the study. Of these, 65 were between 0.5 and 2 years old, 25 were 3-6 years old and 10 were over 7 years old.

Heparinized blood samples obtained from dogs were separately examined with native and Modified Knott techniques in order to detect the presence of circulating microfilariae while sera were examined with commercial ELISA test kit in order to detect the presence of circulating antigens.

No circulating microfilariae were found in peripheral blood of examined dogs by native and Modified Knott techniques, whereas parasite antigens were determined in the sera of two male dogs one of which was aged between 3-6 years and the second was over 7 years. In conclusion, the prevalence of *D. immitis* was determined as 2% among dogs (occult infection) in the Gemlik area of Bursa province, Turkey.

Prevalence and epidemiological aspects of *Dirofilaria immitis* in dogs from Kayseri Province, Turkey

A. Yildirim[1], A. Ica[1], O. Atalay[2], O. Duzlu[1], A. Inci[1]

[1]Erciyes University, Faculty of Veterinary Medicine, Department of Parasitology, Kayseri, Turkey; [2]Erciyes University, Faculty of Veterinary Medicine, Department of Internal Medicine, Kayseri, Turkey

This study was conducted to determine the prevalence of *Dirofilaria immitis* infection and to investigate the risk factors related to heartworm disease in dogs from Kayseri, Turkey. Blood samples were collected from 280 dogs from May 2005 to March 2006 and were examined by membrane filtration-acid phosphatase histochemical staining and antigen Elisa techniques to detect circulating microfilariae and antigens of *D. immitis*, respectively.

Out of the total of 280 dogs, 27 were positive for *D. immitis* with a prevalence value of 9.6%. In addition, 29.6% of positive dogs determined to have occult *D. immitis* infections. *D. immitis* was the only canine filarial parasite present in the study area. The mean number of microfilariae in infected dogs was 4730 ± 5479 per ml of blood. The highest heartworm prevalence were observed in the 7-10 age group (28.6%), followed by the groups 4-6 (17.1%) and 0.5-3 (4.8%) years old. The differences between the 0.5-3 age group and the other age groups were found significant, whereas no statistically significant difference was observed between the 4-6 and the 7-10 age groups. The infection was more prevalent in males, larger breeds and in the dogs not on prophylaxis. No statistically significant difference was observed between stray and owned dogs. Our results suggest that heartworm treatment and prophylaxis should be considered in Kayseri Province.

Wolbachia of *Dirofilaria immitis*: an historical perspective and morphological characteristics

Wieslaw J. Kozek, Jose A. Gonzalez Jr., Lilia Ana Amigo

Department of Microbiology and Medical Zoology, Medical Sciences Campus, University of Puerto Rico, San Juan, Puerto Rico

Collaborative studies between Marshall Hertig and Samuel Wolbach, on the presence and identification of microorganisms in arthropods, resulted in the discovery of *Wolbachia* in *Culex pipientis* in 1924 and description of the genus *Wolbachia* and the species *pipientis* in 1936. Subsequent studies have demonstrated that *Wolbachia* is widespread in arthropods, infecting about 25-70% of species of insects, and is now known as a remarkable genetic manipulator of the infected arthropod hosts. Application of electron microscopy to elucidate the structure of nematodes revealed that many filariae (17 species reported to date, including *Dirofilaria immitis* and most of the human-infecting species) harbor transovarially–transmitted bacterial endosymbionts, determined by Sironi et al. as belonging to the genus *Wolbachia*. We have examined *Wolbachia* of *D. immitis* in all stages of this parasite: in microfilariae, larvae developing in the mosquito and in the vertebrate host, and male and female adult worms by histological techniques and by electron microscopy, comparing it to the *Wolbachia* of other filariae. *Wolbachia* in *D. immitis* is polymorphic and can be demonstrated by standard histological stains such as Giemsa's, Gimenez, Pinkerton's, Machiavello's and Warthin-Starry. Its life cycle is complex and may consist of two reproductive modes: by binary fission and by a *Chlamydia*-like cycle. The latter mechanism offers a potential survival strategy by producing more progeny than multiplication by binary fission, and appears to be more active during growth embryogenesis and development of the larvae. *Wolbachia* of *D. immits* appears to be slightly larger that *Wolbachia* of other filarids, often occurring in great numbers in oocytes.

The *Wolbachia* are apparently mutualistic endosymbionts required for survival of their hosts and embryogenesis of microfilariae, are present in all larval stages during the life cycle of filariae, and contribute to some of the inflammatory responses and pathological manifestations of filarial infections in the vertebrate hosts. Presence of *Wolbachia* endosymbionts in *D. immitis* complicates our understanding of dirofilariasis in dogs, but also offers possible advantages in treatment and control of this infection.

Susceptibility of *Wolbachia* of filariae to certain antibiotics offers an attractive possibility of treatment and control of dirofilariasis. Detection of wolbachial antigens provides the possibility for early detection of *D. immitis* infections in animals and in man.

Recently sequenced genomes of *Wolbachia pipientis* from mosquitoes and *Wolbachia* from *Brugia malayi* have opened a new chapter in the studies on *Wolbachia* and could provide the means to fully characterize the structure, composition and elucidate the nature of these organisms.

Further studies on *Wolbachia* of *D. immitis*, will undoubtedly reveal additional characteristics that can be applied towards treatment and control of this filarial infection.

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Ultrasound - guided surgical removal of adult heartworms via flexible basket forceps in a dog with caval syndrome

Murat Sarierler[1], Bulent Ulutas[2], Huseyin Voyvoda[2], Abidin Atasoy[2]

Departments of Surgery[1], and Internal Medicine[2],Veterinary Faculty, Adnan Menderes University, Aydın, Turkey

In this case study, ultrasonography-guided heartworm removal was carried out using flexible basket forceps in an Anatolian Shepherd dog (Sivas Kangal Dog). The dog was anaesthetized by intramuscularly injection of 2mg/kg/BW Xylazine HCL (Rompun®, Bayer) and 10 mg/kg/BW ketamine HCL (Ketalar®, Eczacibasi) combination following the subcutaneous administration of 0.02 mg/kg/body weight atropine sulphate (Atropin®, Vetas).

The dog was positioned on the left lateral position. The region of right jugular vein was prepared for aseptic surgery. And also the right 5th-7th intercostal spaces were shaved and coupling gel applied for ultrasonographic examination. Following the cut down of right jugular vein, the flexible basket forceps was inserted in to the jugular vein. With the guidance of ultrasonography, heartworm removal was performed. Insertion of the forceps was repeated to remove heartworms as much as possible. A total of 17 adult heartworms could be removed. One day later, the dog died because of poor clinical condition. Immediately, necropsy was performed and 34 heartworms remaining in the right heart and pulmonary artery were collected. The ratio of removed worms via flexible basket forceps was 33.3%.

The low rate of this ratio may be due to the lack of our experience, because this is the first attempt for us and for Turkey.

It was concluded that it is possible to remove adult heartworms from right heart via flexible forceps with the guidance of USG. However, the higher experienced surgeons could give better results.