Dirofilaria immitis Infection and Dirofilariosis

—An animal model of human filariosis—

Mineo　HAYASAKI

犬糸状虫感染と犬糸状虫症

—ヒトのフィラリア症の動物疾患モデルとして—

早　崎　峯　夫

This issue was published by the support program in 2010 of YAMAGUCHI UNIVERSITY FOUNDATION
本書は、(財)山口大学教育研究後援財団の「平成22年度学術研究成果刊行助成事業」の支援のもとに刊行されました。

Dirofilaria immitis infection and Dirofilariosis
An animal model of human filariosis

Mineo HAYASAKI

Professor Emeritus

Yamaguchi National University

Professor Emeritus of Yamaguchi University
Present address: 2-26-8, Wakaba town, Tachikawa City, Tokyo190-0001, Japan

Specialty: Veterinary Internal Medicine, Veterinary Parasitology,
Veterinary clinical Allergy
Special study: *Dirofilaria immitis* (Canine Heartworm) infection
and Dirofilariosis

Printed & Published by
Sakura Printing & Publishing Co., Ltd.
3139-7, Shimo-kosaba, Yamaguchi City,
Yamaguchi 753-0212, Japan
Tel: 083-941-1600
December 30, 2010

COVER: Infectious larvae, as third stage larva of *Dirofilaria immitis*, from the mouth of mosquito.

Acknowledgements

Dedicated to my mentors.

I am especially grateful to the late Professor Emeritus Seiji KUME, who provided me with a faculty position (Assistant professor of Internal Medicine) in his research group at the School of Veterinary Medicine, Tokyo University of Agriculture and Technology, and helped me immeasurably to conduct my studies on *Dirofilaria immitis* infection and Dirofilariosis.

I am also grateful to the late Professor Emeritus Hiroshi OYA of the Department of Parasitology, School of Medicine, Juntendo University, and Professor Emeritus Kentaro YOSHIMURA of the Department of Parasitology, School of Medicine, Akita University, who made many valuable academic suggestions.

I am also grateful to the five committee members of the Board of Examiners for Ph.D. degrees at the University of Tokyo, the late Professor Emeritus Kazuya USUI (Chairman) of the Department of Veterinary Internal Medicine; the late Professor Emeritus Manabu OGATA of the Department of Veterinary Microbiology; the late Professor Emeritus Kousaku FIJIWARA of the Department of Veterinary Pathology; Professor Emeritus Isamu TOMODA of the Department of Veterinary Internal Medicine; and Professor Emeritus Atsuhiko HASEGAWA of the Department of Veterinary Internal Medicine, School of Veterinary Medicine, the University of Tokyo, who accepted my Ph.D. thesis, which was titled "The Immunology of *Dirofilaria immitis* Infection", and gave me a Ph.D. degree from the University of Tokyo (Registered No. 4982).

Contents

1. Introduction

There are three main unsolved problems that affect studies of *Dirofilaria immitis* infection and dirofilariosis; i.e., (1) the development of a complete vaccine against *Dirofilaria immitis* infection; (2) the establishment of the mechanism of vena cava syndrome (hemoglobinuria syndrome); and (3) the verification of the nature of microfilaria periodicity or circadian rhythm.

Regarding the first prpblem, in our preliminary studies we have found a few vaccine candidates that effectively inhibited *D. immitis* infection. However, we have not yet found a complete vaccine material.

Regarding the second problem, we have succeeded in experimentally inducing vena cava syndrome; i.e., hemoglobinuria was experimentally reproduced in three beagle dogs who had been experimentally infected with *D. immitis*. The 3 dogs suddenly developed hemoglobinuria on days 261, 271, and 272 after infection, after being subjected to strong physical stress (hard running once a day for several days). However, the predicted changes in blood coagulation, hematological and blood chemical parameters, and electrocardiograms were not detected.

Regarding the third problem, we were able to reverse the microfilarial periodicity by changing the daily light and dark cycle and the nature of microfilarial periodicity has been actively analyzed by *in vivo* and *in vitro* studies in our laboratory.

The aim of studies of *D. immitis* infection and dirofilariosis is to analyze human filariosis, which is endemic throughout the world, such as bancroftian filariosis and malayan filariosis, in terms of not only the parasitological features of alternative animal models, but also the clinical features of zoonosis and the biological effects of parasitism. Thus, studies of dirofilariosis and *D. immitis* infection are important for the eradication of parasitic infection.

2. Life cycle of *Dirofilaria immitis* (Fig. 1)

D. immitis, the canine heartworm, is a white large nematode with a mean length of 25–26 cm and a mean width of 1.2 mm in females, and a mean length of 15–16 cm and a mean width of 0.8 mm in males. The adult worms parasitize the pulmonary arteries. After mating with a male worm, the female produces a lot of microfilaria, of about 300 μm in length and 7 μm in width, in the host's blood circulation. Mosquitoes, such as those belonging to the genera *Aedes*, *Culex*, and

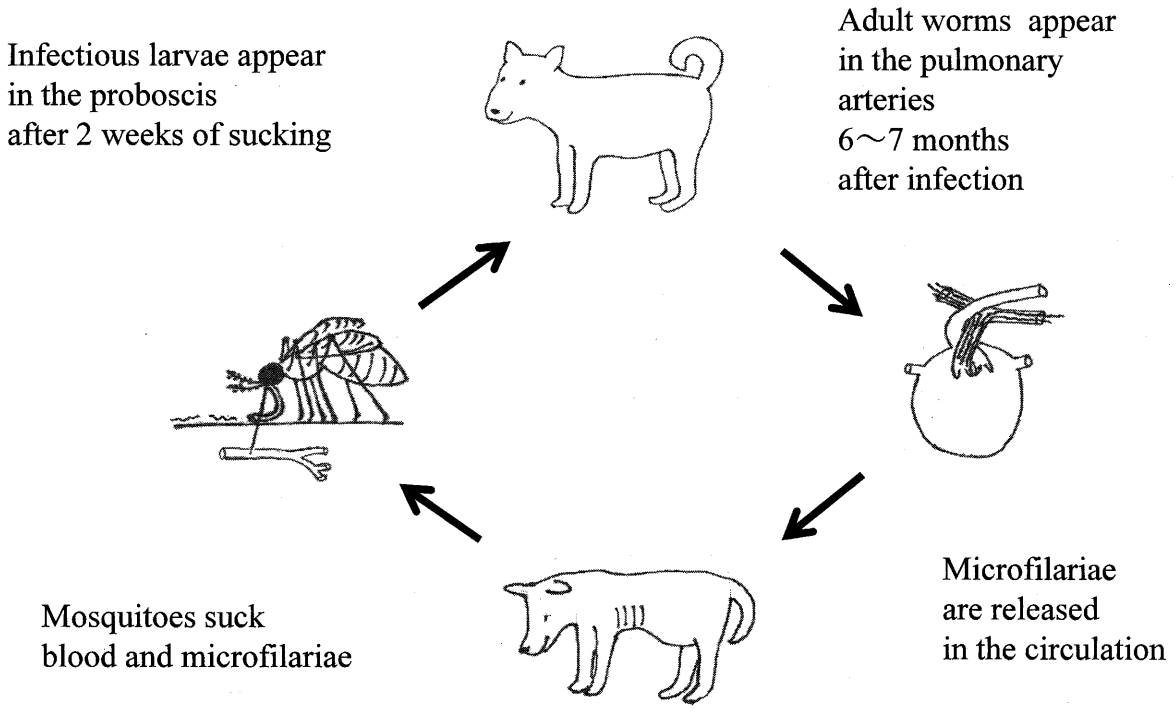

Fig. 1. Life cycle of *Dirofilaria immitis*

Anopheles, etc. act as intermediate hosts and *Aedes togoi* and *Culex pipiens pallens*, and *Aedes aegypti* play major roles as vectors in Japan, and throughout the world. However, fleas and mites never act as intermediate hosts. When a mosquito sucks the blood of its victim, microfilariae are sucked into its body, where they grow into infectious larvae of 1 mm in length within 2 weeks at temperatures of 20–30°C. Infected mosquitoes harboring such infectious larvae are responsible for the wide spread of *D. immitis* infection among dogs. The infectious larvae usually lurk in the thin gap between the needle and the sheath of the mosquito. The sheath opens along longitudinal slits, and the thin space is usually filled with the mosquito's somatic fluid. When the infected mosquito pierces the dermis of its victim with its needle prior to bloodsucking, the sheath stops at the skin surface and flexibly bends upward, and the longitudinal gaps in the sheath open, and the infectious larvae flow out onto the skin. The infectious larvae then undergo positive taxis into the blood plasma, which spills out from the hole in the skin left after the removal of the needle. The infectious larvae then seek out the hole and use it to enter the host.

The infectious larvae inside the host then molts their outer cuticles within a

few days, and grow into the fourth larval stage of development, before migrating into the subcutaneous tissue, adipose tissue, or a subfascial location, where they lurk for about 2 months. After growing to 3–6 cm in length after 60–70 days of infection, the larvae molt again, and then grow into the fifth stage (the final stage of development), although they are still immature adult worms. The immature worm again starts to migrate in order to penetrate a small vein, and then the blood circulation takes it to the pulmonary arteries through the right ventricle. After 3 months, the worm reaches maturity, and after mating with a male adult worm, a fertile female adult worm continuously reproduces a lot of microfilariae in the host's blood circulation, and the host becomes microfilaremic. This is the life cycle of *D. immitis*. This means that *D. immitis* has a long prepatent period of 6–7 months after infection. The life span of *D. immitis* in dogs is about 6 years for adult worms and about 2 years for microfilaria. In Japan, if no adulticidal or preventive larvicidal drugs are administered, about 10 adult worms may infect a dog each year because the infection period of *D. immitis* runs from June or July to October or November, depending on the region.

3. Dirofilariosis

D. immtis parasitizes the pulmonary arteries of its definitive hosts, dogs and the canidae family, and causes pulmonary hypertension in the host animal. The pulmonary hypertension causes chronic col pulmonale, right ventricle hypertrophy, and congestive cirrhosis, which lead to ascites or renal failure and eventually death.

Formerly, it was believed that the adult worms lived in both the right ventricle and the pulmonary arteries because they were mainly detected in the right ventricle when necropsy examinations were performed. Similarly, it was believed that the worms overflowed into the main pulmonary arteries when many adult worms had parasitized the host. However, it was recently confirmed by echo-imaging that the worms only live in the stem of the pulmonary arteries and always swim upstream the blood flow. Therefore, the worm penetrates into the right ventricle when the host dies, and its heart beat and blood pressure are gradually reduced. For this reason, it was thought for longtime that *D. immitis* lives, not only in the pulmonary arteries but also in the right ventricle. In fact, many old veterinary parasitology textbooks state that « *D. immitis* lives in the right ventricle and pulmonary arteries ».

4. Zoonosis

In general, canine dirofilariosis (canine heartworm disease in dogs) progresses mildly and chronically and leads to severe heart failure ; liver failure ; renal failure; and trophonosis, including congestive cirrhosis, congestive ascites, glomerular nephritis, and hemoglobinuria syndrome, although its severity varies from individual to individual.

In feline dirofilariosis (canine heartworm disease in cats), the host's inflammatory immune attack against immature *D. immitis* worms is often very violent compared to that of dogs, although many asymptomatic feline cases have been reported. Large numbers of immuno-activated leukocytes and platelets accumlate against the invading immature worms, and cause violent focal inflammations in large areas of the pulmonary arteries and connecting lung tissues, particularly in the caudal lung lobes, where the immature worms prefer to live. These inflammmatory reactions produce many thromboembolisms in both smaller and large pulmonary arteries leading to acute cardiac failure caused by pumonary hypertension and pneumonia, which often lead to sudden death; however choosing an appropriate treatment is generally difficult because the cause of the problems is difficult to diagnose. Vena cava syndrome (hemoglobinuria syndrome), a specific symptom of dirofilariosis, has also been detected in infected cats, although it is very rare, and surgery to remove the heartworms from the vena cava and right atrium is an effective cure as in infected dogs; however, the administration of adulticidal drugs should be contraindicated because it induces pulmonary thromboembolisms, leading to sudden host death.

Human dirofilariosis (canine heartworm disease in humans) has been reported in over 200 patients in the past 150 years throughout the world. Human dirofilariosis can be categorized into two pathological types, human pulmonary dirofilariosis and human extra-pulmonary dirofilariosis. Human pulmonary dirofilariosis has a similar pathological mechanism to feline dirofilariosis. *D. immitis* occasionally develop into immature worms and reach the pulmonary arteries after their final migration in the human host. Then, the immature worms parasitizing the pulmonary arteries are exposed to a violent immune attack, and most immature worms usually die within a short period. However, both the inflammatory immune attack against live immature worms and the pathological reaction against dead worms produce pulmonary infarctions. These lesions consecutively induce mild to severe pulmonary

arteritis and focal pneumonitis, and then, coughing, thoracodynia, bloody phlegm, dyspnoea, fever, and hydrothorax, although the condition can be cured with appropriate treatment. However, asymptomatic patients are also known to account for half of these patients. Usually, pulmonary dirofilarial infarctions are surgically treated by thoracic lumpectomy under the suspicion of cancer. However, a detailed differential diagnostic examination will indicate the parasitic infarction. If detected early, its prognosis is not so severe, and symptomatic and conservative treatment may be chosen to avoid the high risk of thoracic surgery.

5. Epidemiology

Dirofilariosis is widely endemic throughout the world, mainly in the tropical zone and the temperate zone, where mosquitoes are highly endemic. In Japan, until 40–50 years ago, dirofilariosis was not endemic on Hokkaido, a large northern island located in the subarctic zone, or in the high altitude towns and villages on Honshu, the central and largest island, which is located in the temperate zone. However, it has recently been become in these areas endemic. Similarly, it has been endemic in Anchorage, Alaska in the USA, a typical arctic region since the 1990s. It is believed that global warming has caused this increase in its distribution.

D. immitis has been detected in many kinds of animals (Table 1), although most of these animals are classified as non-definitive hosts, and most of these cases were thought to represent accidental infections. The infected animals included dogs and canidae, cats and felidae, sea mammals, bears, horses and deer, rabbits, and even some birds (penguines), indicating that *D. immitis* has a wide host range including carnivores and herbivores, primates, and birds.

6. Summary of previous papers

1) Reviews

Immunology of *D. immitis* infection [1]

This review investigated the host's humoral and cell-mediated immune responses, the somatic and excretory-secretory antigens of microfilaria and male and female adult worms, the immuno-pathological mechanism of clinical syndrome, the microfilaricidal mechanism of platelets under diethylcarbamazine administration, immunological diagnosis, the microfilaricidal mechanism of occult infection, and

Table 1. Animal species first detected *Dirofilaria immitis* in the world

Animal	Country	Reporter	Animal	Country	Reporter
Dog	UK	Wright (1845)	Coyote	USA	Gier & Ameel (1959)
Human	Brazil	Magalhães (1887)	Brazil otter	Venezuela	Vogelsang (1959)
Wolf	Japan	Janson (1892)	Panther	Japan	Chiba et al. (1961)
Jaguar	Srinum	Horst (1899)	Mink	Japan	Kume & Ohishi (1961)
Cat	Brazil	Travassos (1921)	Japanese deer	Japan	Nishimura et al. (1964)
Tiger	China	Schwarz (1925)	Ferret	Japan	Ohishi (1965)
Eared seal	USA	Faust (1937)	Horse	Japan	Kiryu et al. (1970)
Fox	Japan	Itagaki & Kume (1938)	Beaver	USA	Foil & Oriel (1975)
Fur seal	Japan	Itagaki & Kume (1938)	American black bear	USA	Johnson (1975)
Jackal	Japan	Hiraiwa (1938)	Wild cat	Panama	Otto (1975)
Racoon dog	Japan	Itagaki & Kume (1938)	Jaguarundi	Panama	Otto (1975)
Musk cat	Japan	Itagaki & Kume (1938)	Wa-mon-seal	Japan	Tago-oka (1975)
Haku-bi-sin	Japan	Itagaki & Kume (1938)	Lion	Japan	Hayasaki (1975)
otter	Venezuela	Vogelsang (1940)	Black badger	USA	Williams & Dade (1976)
Dingo	Australia	Faust (1941)	Steller's sea lion	Japan	Kamiya & Kagoshima (1977)
Racoon	USA	Fox (1941)	Lesser panda	USA	Harwell & Craig (1981)
Go-ma-fu seal	Mexico	Faust 1941)	A-ka-ge Monkey	USA	Beskin & Eberhard (1982)
Zu-ki-n seal	?	Faust 1941)	Rabbit	Japan	Narama et al. (1982)
Musk rat	USA	Goble (1942)	Clouded leopard	Japan	Okuda et al. (1983)
White nose badger	Mexico	Caballero (1944)	Japanese weasel	Japan	Unidentified (1993)
White collar bear	Japan	Itagaki & Taniguchi (1948)	Snow leopard	Japan	Murata et al. (2003)
Orangutan	Malaysia	Sandosham (1951)	Penguin	Japan	Sano et al. (2005)

trial studies on immunological prevention of the infection.

D. immitis infection and dirofilariosis [2]

This review investigated the clinical and epidemiologic characteristics of dirofilariosis, including its progression. It also compared canine dirofilariosis and feline dirofilariosis and examined the historical and present statuses of the infection in Japan and the expansion of host susceptibility into various animal species.

Clinics in feline heartworm disease [3]

This review focuses on the specific clinical features of feline dirofilariosis,

including clinical findings; methods used for diagnosis and the evaluation of disease severity; therapy for symptomatic patients; and surgical (for Vena Cava Syndrome with Hemoglobinuria), adulticidal, and preventive treatments.

Guidelines for the diagnosis, treatment, and prevention of heartworm (*D. immitis*) infection in cats, as the recommendations of the International Feline Heartworm Disease Council [4]

The preamble of this review stated that: Feline heartworm disease is becoming more common. The regional prevalence of heartworm infection in cats, which is currently more of a medical curiosity than an important clinical entity, is gradually changing as heartworm infections in cats are recognized with increasing frequency. The clinical importance of heartworm in cats is increased by the fact that even light infections are capable of producing severe disease with potentially life-threatening consequences. Furthermore, there are significant differences between feline heartworm disease and its canine counterpart, which are generally not appreciated. These include the host responses to the parasite, clinical manifestations, the reliability of diagnostic methods, and therapeutic opinions. Consequently, the management of feline heartworm disease is often based on misconceptions and so is accompanied by considerable uncertainty. This document is intended to promote understanding and provide guidance for the diagnosis, treatment, and prevention of feline heartworm disease.

2) Parasitology of *D. immitis* infection

Ultrastructure of microfilaria [5]

The ultrastructure of *D. immitis* microfilariae has mainly been observed by transmission electron microscopy; however, the details of the morphological features of microfilariae remain poorly understood. Our study presented clear images of a mouth-like cavity, a lip-like process, a triangular hook, and two small pores on a cephalic disk together with a central canal and a cephalic ciliary channel in the anterior section of the microfilaria, in addition to a body wall, an excretory pore, an anal pore, and a tail as outer ultrastructures. We also found by scanning electron microscopy that a large number of nuclear column cells or spherical cells were distributed throughout the body in addition to radially running microstrings connecting these cells to each other.

Mechanism of microfilaria agglutination ("Medusa-head formation") [6]

Microfilariae obtained from a microfilaremic dogs show agglutination or "Medusa-head formation", when cultured *in vitro* in serum taken from another dog with an occult infection. It has been speculated that this is caused by a lethal process working against the microfilariae. However, the mechanism remained poorly understood. Our study revealed that the phenomenon was due to an immune complex precipitating the microfilariae and attaching them to each other. Only live microfilariae were agglutinated, and the agglutinated microfilariae remained alive for as long as one month in *in vitro* culture, indicating that the process was not lethal.

Characteristics of worm antigens [7-9]

The antigenic characteristics of *D. immitis* microfilariae and adult worms were analyzed using somatic components and excretory-secretory products by examining the cross reactivity between them using immunofluorescence, SDS-PAGE and immunoblotting, and we also analyzed these characteristics among the intestinal parasites of dog. Our studies demonstrated that they consisted of many protein components and that their antigenicity is very complex. In addition, the antigenic complexicity of microfilariae was similar to those of adult male and female worm, despite microfilariae having a very simple morphological structure with no digestive tract or genital glands. *D. immitis* antigens were partially cross-reactive with those of *Toxocara canis*, *Ancylostoma caninum*, *Trichuris vulpis*, and *Dipylidium caninum*. Such cross-reactivity among nematodes may have been acquired by natural selection as part of their evolution and adaptation to their host.

3) Biology of *D. immitis* infection

Re-migration capacity of immature worms [10]

Our study first demonstrated the migration capacity of immature adult *D. immitis*. For this purpose, 5th stage juvenile adult worms recovered from the pulmonary arteries of infected dogs 145-147 days after infection were transplanted into the subcutaneous tissue of uninfected dogs. One month later, these transplanted worms were recovered from the pulmonary arteries of the recipient dogs, indicating that the immature adult *D. immitis* retained their re-migration ability until the age of, at least, 145-147 days after infection, despites these worms having already reached the pulmonary arteries.

Can *D. immitis* larvae infect its host by transplancental infection from a mother to her offspring? [11]

Our study assessed whether the transplacental infection of *D. immitis* from a mother to her offspring is possible. One female dog was used in this study and was mated on the 21th day after being experimentally infected with infectious larvae. The dog became pregnant and delivered 6 fetuses after about 2 months. No worms were recovered from these 6 puppies at 59 days after birth; i.e., about 4 months after the experimental infection. These results indicated that the transplacental infection of *D. immitis* is not possible.

4) Susceptibility of unusual hosts to *D. immitis*

Susceptibility of cats [12, 13]

Our study first demonstrated that cats are showing a gradually increasing susceptibility to *D. immitis* infection, although the reason for this remained unknown. Our results revealed that *D. immitis* were able to attain near-normal growth to sexual maturation, and a sufficient number of circulating microfilariae was detected, which showed a nocturnal sub-periodic diurnal rhythm. Therefore, it was considered that such diurnal changes may depend on factor(s) endogenous to microfilariae, including phototaxis-like behavior, rather than on the host immune response. The prevalence of *D. immitis* infection among cats reared in Yamaguchi prefecture, Japan, was investigated by immunoblotting, and 6.0% (19 of 315 cats tested) were found to be positive, indicating that the regional prevalence of *D. immitis* infection in domestic cats is persistently low.

Susceptibility of lesser pandas [14]

This was a report on *D. immitis* infection in lesser pandas reared in Tama zoological park in Tokyo. When the pandas died, a necropsy examination was performed. One mature male worm each was recovered from cases 1 and 2, and 3 immature worms (2 males and 1 female) from case 3, suggesting that *D. immitis* have been the causative agent of death. *D. immitis* infection in lesser pandas may be increasing in frequency.

Susceptibility of raccoon dogs [15]

Wild raccoon dogs are susceptible to *D. immitis* infection. In the central area of Japan, 2 of 63 wild racoon dogs investigated by necropsy examination were

infected with *D. immitis*. One individual had 6 worms (3 males and 3 females), and another had 3 worms (1 male and 2 females), and microfilariae were detected when their circulating blood was examined with the lung stamp smear technique. This may indicate that the susceptibility of raccoon dogs to *D. immitis* infection is similar to that of dogs.

5) Immunological Parasitology of *D. immitis* infection

Immunological studies of canine heartworm disease [16]

The purpose of this present study was to provide baseline data on the clinical application of serological testing to the diagnosis of *D. immitis* infection and also to reveal the kinetics of humoral antibody production and the cell-mediated immune response during worm growth in the host.

Improvement of indirect hemagglutination serological testing [16, 17]

In terms of immunological specificity and sensitivity, the indirect hemagglutination (IHA) test was the most useful for detecting the *D. immitis*-specific antibody in infected dogs among the IHA test, complement fixation test, and agar gel diffusion test.

Four kinds of *D. immitis* antigen, prepared from intrauterine microfilariae (I-Mf), circulating microfilariae (C-Mf), migrating larvae, and adult worms (A-Di), were assessed using the IHA test. Interestingly, it was demonstrated that I-Mf showed the highest specificity and sensitivity among these antigens, when phosphate buffered saline (PBS) extract was used. In contrast, the PBS extract of C-Mf was found to have extremely low antigenicity. The cross reactivity between *D. immitis* and intestinal nematodes, such as *Toxocara canis*, *Ancylostoma caninum*, and *Trichulis vulpis*, were evaluated using the agglutinin absorption assay. No substantial antigenic cross reactivity was present between I-Mf or A-Di and the above three intestinal nematodes.

Difference in antigenicity between microfilariae and adult worms [16]

The agglutinin absorption assay also suggested that the antigenicity of A-Di antigen differes from that of I-Mf, although they shared some common antigens. The present results indicate that I-Mf antigen is useful for IHA testing for *D. immitis* infection in dogs. I-Mf and A-Di antigens were analyzed to assess their antigenic differences in IHA tests performed using Sephadex G-200 gel filtration, DEAE

cellulose column chromatography, and polyacrylamide gel DISC electrophoresis. The IHA positive protein fraction was recovered from the first gel filtration assay peaks of both antigens. However, DEAE cellulose column chromatography of the first peak materials of each antigen showed that their antigenic activities were mainly recovered from different fractions. The DISC electophoretic patterns of these two fractions also showed the different antigenicities of I-Mf and A-Di. It was therefore revealed that the physico-chemical properties of I-Mf antigen differ from those of A-Di.

Sensitivity of the indirect fluorescent antibody test [18]

A study to clarify the antigenic reactivity of I-Mf, C-Mf, and A-Di was undertaken usig the indirect fluorescent antibody (IFA) test and serum samples collected from dogs that had been experimentally infected with *D. immitis* and non-infected controls. In negative IFA test, yellowish autofluorescence was observed in the lumen of the intestines, and slight reddish-brown autofluorescence was also observed in the muscle layer and the cuticle of the I-Mf, but no specific fluorescence was found. In positive IFA test, specific fluorescence was detected in the lateral chord, the muscle layer, the intestine, the uterus, the ovary and the cuticle of the I-Mf. Intact I-Mf and C-Mf were thoroughly washed with PBS and tested for IFA. Specific fluorescence was observed on the surface of I-Mf, but not on that of the C-Mf. Thus, the different antigenicities of I-Mf and C-Mf were confirmed not only by the IHA but also by the IFA tests.

Kinetics of antibody production [19]

The kinetics of reaginic and hemagglutinating antibody production in dogs that had been experimentally infected with *D. immitis* were studied using the passive cutaneous anaphylaxis reaction and IHA test. The production of these two antibodies was demonstrated throughout the prepatent and patent periods of infection. Reaginic antibody was first detected on the 65th day of infection, which coincided with the fourth molt of worm development, and its level was increased when microfilaremia became evident. The reaginic activity of the sera was detected by heating at 56°C for 60 min followed by reduction and alkylation procedures. Sephadex G-200 gel filtration analysis indicated that reaginic activity was recovered in the ascending portion of the second (IgG) peak, suggesting that its molecular weight was slightly higher than that of IgG. The hemagglutinating antibody

responses showed two distinct peaks, which coincided with the fourth larval molt and the occurrence of microfilaremia. These data therefore indicate that new borne-fourth stage larvae and new borne-microfilariae strongly stimulate marked host antibody production.

Kinetics of the passive transfer of anti-*D. immitis* antibody from the mother to its offspring [20]

This study was the first to demonstrate that anti-*D. immitis* antibody is passively transferred from mother to offspring. The antibody was examined using the IHA test and was found to be transferred to the puppies via colostrum and to persist in the puppies for approximately two months. No antibody was detected in the new-born fetus before it had consumed its mother's colostrum. The colostral antibody titer was identical to that of the maternal serum. This result may aid better understanding of the mechanism behind the false positive serological reactions observed in non-infected puppies.

Cell-mediated immunity [21]

The cell-mediated immune responses of dogs infected with *D. immitis* were evaluated using the macrophage migration inhibition (MI) test (indirect method). Thirteen non-infected dogs and 20 naturally infected dogs were found to be negative on the MI test when they were examined with antigens prepared from I-Mf and A-Di. The MI tests were also negative in these dogs throughout the prepatent period of infection. These data suggested that the cell-mediated immune responses of infected dogs are immunologically suppressed by *D. immitis* infection.

6) Clinico-immunology of filarial infection

Specific bands in the immunoblotting test can be used to predict the presence of living *D. immitis* in cats [22]

The immunoblotting test was used to identify specific worm antigen bands that are useful for diagnosing active *D. immitis* infections in cats by examining serial serum samples from experimentally infected cats. Common specific antigen bands with molecular weights of 36, 32, 22, 19, and 14 kDa, were detected throughout the experiment, indicating that they could be used to predict positive adult worm infection.

Does immunization and immunosuppressant drug administration affect *D. immitis* infection? [23, 24]

The role of the canine immune system in the rejection of *D. immitis* infections still remains poorly understooding. About 40% of inoculated infectious *D. immitis* larvae reach the pulmonary arteries of dogs, as demonstrated by experimental infection, although this means that about 60% of inoculated larvae are killed by host protective mechanisms. Accordingly, attempts were made to evaluate the host protective immunity mechanisms employed against experimental *D. immitis* infection by means of immunization with antigenic materials and the administration of immunosuppressants. The results showed that immunization with heterologous worm antigen induced a marked protective effect against the infection. On the contrary, homologous worm-somatic antigen permitted the infection. On the other hand, the immunosuppressive drugs, azathioprine and predonisolone, did not increase the infection rate, although a marked reduction in antibody production was seen. From this, it is likely that the larvae-killing immune attack is controlled by an other mechanism.

The role of anti-idiotype monoclonal antibody specific to *Onchocerca volvulus* in microfilaricidal activity of platelets activated by diethlycarbamazine [25]

Anti-idiotype monoclonal antibody to microfilariae of *Onchocerca volvulus*, which has infected an estimated 18 million people in Africa and Latin America, causing severe eye damage and eventual blindness, was first produced in filarial nematodes.

In this study, I produced hybridoma cells secreting an anti-idiotype monoclonal antibody specific to *O. volvulus* microfilaria, using a radioimmuno assay. Diethylcarbamazine (DEC), which has been the most widely used agent for the treatment of filarial diseases for over 35 years, eliminates microfilariae, but its mode of action remains unknown. Our group recently reported that the microfilaricidal activity of DEC is mediated through an antibody-independent mechanism by blood platelets with additional triggering caused by a filarial excretory antigen. Our study demonstrated that the microfilaricidal action of DEC against *O. vulvulus* is mediated by platelet cytotoxicity. Briefly, platelets are activated by DEC treatment. DEC-activated platelets kill microfilariae, but they also require the additional triggering by a soluble filarial excretory product. The need for the filarial excretory product was demonstrated by their effective killing action after the addition of anti-idiotype antibody specific to *O. volvulus*.

7) Clinical study of dirofilariosis

Determination of the parasitic location of living adult *Dirofilaria immitis* by echocardiography [26]

The parasitic location of living *D. immitis* was examined in the main pulmonary arteries of the infected dogs by 2-dimensional echocardiography (2-DE) and the pulse doppler method (PD). Thirty living adult *D. immitis* worms were surgically implanted into the jugular veins of 2 normal dogs and immediately invaded the pulmonary arteries. When these 2 dogs were anesthetized with pentobarbital, the worms moved from the inner part of the pulmonary arteries toward the right atrium through the pulmonary artery valve and the right ventricle according to the decrease in heart blood output. These observations indicated a developmental mechanism for dirofilariosis-associated vena cava syndrome (hemoglobinuria syndrome), which occurs simultaneously with acute severe cardiac failure and severe hemoglobinuria, as lethal symptoms.

Immunological treatment of coughing in dogs with dirofilariosis [27]

A persistent, spasmic and productive cough known as filarial cough often occurs in dogs with dirofilariosis, and is considered to be due to an allergic response to *D. immitis*. Twenty-one dogs with filarial cough were subcutaneously injected with worm antigen (200 μg of protein concentration) extracted from adult *D. immitis* once a day for 5 days. These injections were effective in 17 (81%) of the dogs, resulting in a complete cure for 7 dogs and marked improvement in 10 dogs.

Paradoxical embolism due to canine filariae [28, 29]

Dogs were diagnosed with paradoxical embolism induced by canine filariae by clinico-pathological examinations, and radiography indicated arterial embolism in the hind quarters. Autopsy revealed that congenital heart malformations; i.e., openings in the ductus arteriosus and foramen ovale were present, indicating that the adult *D. immitis* parasitizing the pulmonary arteries had invaded the left atrium or the aorta through these openings and then had induced an embolism in the peripheral arteries of the hind quarters.

***D. immitis* found in the feces of a dog infected with *D. immitis* [30]**

Two yellowish dead female *D. immitis* containing many microfilariae in uteru were passed in the feces of a dog 15 days after it had been treated with an arsenical

(Trimelarsen). Coughing and bloody vomitus were recorded on this day. It seems likely that the worms had entered the bronchus via a post-treatment hemorrhage, were coughed up, and then swallowed. The dog was sacrificed nine days later (i.e., 24 days post-treatment. Emboli in the pulmonary artery with related hemorrhagic areas were seen in the right middle lobe, and a dead worm was found in that branch of the bronchus. Sixty-seven dead worms were found in the pulmonary arterial system, and 19 living worms were found in the right ventricle. Thus, a total of 70 dead worms and 19 living (79% dead worms) were recovered after treatment with Trimelarsen.

Inhibition and prevention of mosquito bloodsucking and *D. immitis* infection via the administration of a topical insecticide [31]

The inhibition and prevention efficacy of a topical insecticide against *D. immitis* infection were evaluated. The results showed that mosquito bloodsucking was significantly reduced, thereby significantly inhibiting *D. immitis* infection.

8) Development of filaricidal drugs against *D. immitis* larvae

Establishment of dose and schedule of Ivermectin administration for clinical use [32, 33]

The recommended dose and administration schedule of Ivermectin for preventing *D. immitis* infection were established in experimental infection studies. The results suggested that a single oral administration of ivermectin at a dose of 6 μg/kg killed all 30 to 60-day-old larval worms. Therefore, the administration of Ivermectin once a month at a dose of 6 μg/kg throughout the infection period is recommended. Accordingly, in Japan, six administrations from July until December are necessary because the infectious period lasts persisted from June to October.

Prophylactic effects of levamisole hydrochloride against *D. immitis* larvae in infected dogs [34-38]

Levamisole hydrochloride was examined for its prophylactic activity against fourth-stage larvae of *D. immitis*, which is parasitic in the subcutaneous, adipose tissues, and/or the subfascial gap as an intermediate location of the host. Experimental infection studies indicated that both of a dose of 2.5 mg/kg/day administered orally every day for 6 months from June to November and the same dose administered every other day for the same period killed all larvae, meaning

that the development to adult *D. immitis* was completely inhibited. Other studies, such as field and intermittent administration trials, were also performed.

References (*: Corresponding author)

1 Hayasaki, M.(1991): Review: Immunology of *Dirofilaria immitis* infection. Journal of Japanese Veterinary Medical association, 44, 781–789.(in Japanese)
早崎峯夫（1991）：総説「犬糸状虫感染の免疫学」。日本獣医師会雑誌、44, 781–789.

2 Hayasaki, M.(1997): Review: *Dirofilaria immitis* infection and dirofilariosis. Journal of Veterinary Medicine, 50, 463–469.(in Japanese)
早崎峯夫（1997）：総説「犬糸状虫感染と犬糸状虫症」。獣医畜産新報、50, 463–469

3 Hayasaki, M.(1997): Review: Clinics of feline heartworm disease. Journal of Veterinary Medicine, 50, 330–333.(in Japanese)
早崎峯夫（1997）：総説「猫の犬糸状虫症の臨床」。獣医畜産新報、50, 330–333.

4 Atkins, CE, Atwell, RB, Dillon, R, Genchi, C, Hayasaki, M, Holmes, RA, Knight, DH, Lukof, DK, McCall, JW, Slocombe, JOD.(1996): American Heartworm Society Guidelines for the Diagnosis, Treatment, and Prevention of Heartworm (*Dirofilaria immitis*) infection in cats, Recommedations of the International Feline Heartworm Disease Council. *In*: Proceeding of Recent Advances in the heartworm symposium'95, 1–4.

5 Song, K.H., Tanaka, S., Hayasaki, M.* (2009): Scanning electron microscopic observation of ultrastructure of *Dirofilaria immitis* microfilaria. Journal of Veterinary Medical Science, 71, 779–783.

6 Hayasaki, M.* (2001): Immunological analysis of agglutination in *Dirofilaria immitis* microfilariae. Journal of Veterinary Medical Science, 63, 903–907.

7 Hayasaki, M.* Nanamura, F. and Konno, K.(1994): Immunoblotting analysis of somatic components of *Dirofilaria immitis*. Journal of Veterinary Medical Science, 56, 1181–118.

8 Kaneko, H., Hayasaki, M.* and Ohishi, I.(1990): Antigenic identification of excretory–secretory products of adult *Dirofilaria immitis*. Japanese Journal of Veterinary Science, 52, 995–1000.

9 Konno, K. and Hayasaki, M.*(1995): Antigenic cross reactivity among *Dirofilaria immitis* and four intestinal parasites–species in the dog. Japanese Journal of Parasitology, 44, 161–164.

10 Hayasaki, M.*(1996): Re–migration of fifth stage juvenile *Dirofilaria immitis* into pulmonary arteries after subcutaneous transplantation in dogs, cats, rabbits.

Journal of Parasitology, 82(5), 835-837.

11 Hayasaki, M., Oishi, I. and Kume, S. (1973): Is transplacental infection possible in *Dirofilaria immitis* infection in dogs? Journal of Japanese Veterinary Medical Association, 26, 417-418. (in Japanese)
早崎峯夫、大石　勇、小林茂雄、久米清治（1973）：犬糸状虫*Dirofilaria immiti*の胎盤感染について。日本獣医師会雑誌、26, 417-418.

12 Hayasaki, M.*, Okajima, J., Song, K.H. and Shiramizu, K.(2003): Diurnal variation in microfilaremia in a cat experimentally infected with larvae of *Dirofilaria immitis*. Veterinary Parasitology, 111, 267-271.

13 Hayasaki, M.*, Katsuya, A. and Song, KH.(2007): Immunoblot analysis of prevalence of canine heartworm infection in 315 cats in Yamaguchi. Journal of Japanese Veterinary Medical Association, 61, 549-552.(in Japanese with English summary)
早崎峯夫*、勝矢朗代、Song, KH(2007)：免疫ブロット法を用いた山口県における猫の犬糸状虫感染調査。日本獣医師会雑誌，61, 549-552.

14 Narushima E., Hashizaki, F., Kouno, N., Saito, M., Tanabe, K., Hayasaki, M.* and Oishi, I.(1984): *Dirofilaria immitis* infection in lesser pandas (*Ailurus fulgens*) in Japan. Japanese Journal of Parasitology, 33, 475-481. (in Japanese with English summary, tables and figures)
成島悦雄、橋崎文隆、河野典子、斉藤　勝、田辺興記、早崎峯夫*、大石　勇（1984): 日本のレッサーパンダ*Ailurus fulgens*における犬糸状虫*Dirofilaria immitis*の寄生。寄生虫学雑誌、33, 475-481.

15 Hayasaki, M.* and Oishi, I. (1982): Incidence of canine heartworm, *Dirofilaria immitis*, in wild raccoon dogs in the central area of Japan. Japanese Journal of Parasitology, 31, 175-183. (in Japanese with English summary, tables and figures)
早崎峯夫*、大石　勇（1982）：日本の野生タヌキにおける犬糸状虫*Dirofilaria immitis*の流行について。寄生虫学雑誌、31, 177-183.

16 Hayasaki, M. (1984): Immunological studies on canine heartworm disease. (Ph. D. thesis, University of Tokyo, Ph.D. No.4982), In: Bulletin of the Faculty of Agriculture, Tokyo University of Agriculture and Technology, No.25, 1 -26. (in Japanese with English summary, tables and figures)
早崎峯夫（1984）：「犬糸状虫症の免疫学的研究」（農学博士、東京大学No.4982)、東京農工大学農学部学術報告第25号，1 -26.

17 Hayasaki, M.(1981): Indirect hemagglutination test for diagnosis of canine filariasis. Japanese Journal of Veterinary Science, 43, 21-26.

18 Hayasaki, M.(1983): Antigenicity of microfilarial and adult *Dirofilaria immitis* in indirect fluorescent antibody test. Japanese Journal of Veterinary Science, 45, 113–115.

19 Hayasaki, M.(1982): Reaginic and hemagglutinating antibody production in dogs infected with *Dirofilaria immitis*. Japanese Journal of Veterinary Science, 44, 63–70.

20 Hayasaki, M. (1982): Passive transfer of anti-*Dirofilaria immitis* hemagglutinating antibody from the mother dog to its offspring. Japanese Journal of Veterinary Science, 44, 781–786.

21 Hayasaki, M.*, Nakagaki, K., Kobayashi, S. and Oishi, I. (1981): Immunological response of dogs to *Dirofilaria immitis* infection. Japanese Journal of Veterinary Science, 43, 909–914. (in Japanese with English summary, tables and figures)
早崎峯夫*、中垣和英、小林茂雄、大石　勇（1981）：犬糸状虫寄生犬における免疫応答について。日本獣医学雑誌、43, 909–914.

22 Hayasaki, M.*, Mori, N., Hongo, H., Katsuya, A., Song, K. H., Une, S., Ikeda, T., Ooi, H. K. and Uchida, A.(2005): Immunoblot analysis of special antigen bands predictable for *Dirofilaria immitis* infection in cats. Veterinary Parasitology, 131, 325–329.

23 Hayasaki, M.* and Ohishi, I.(1989): Influence of immune treatments against *Dirofilaria immitis* infection in dogs. Japanese Journal of Veterinary Science, 51, 540–546.

24 Hayasaki, M.* and Ohishi, I.(1989): Influence of immuno-suppressants against *Dirofilaria immitis* infection in dogs. Japanese Journal of Veterinary Science, 51, 957–962.

25 Cesbron, J-Y., Hayasaki, M., Joseph, M., Lutsch, C., Grzych, J-M. and Capron, A.(1988): *Onchocerca volvulus*: Monoclonal anti-idiotype antibody as antigen signal for the microfilaricidal cytotoxicity of diethylcarbamazine-treated platelets. Journal of Immunology, 141, 279–285.

26 Ohono, H., Hayasaki, M. and Oishi, I.(1991): Determination of parasitic location of living adult *Dirofilaria immitis* and cardiac function of infected dogs as assessed by echocardiography. Journal of Japanese Veterinary Medical Association, 44, 1115–1120.(in Japanese with English summary)
大野弘子、早崎峯夫、大石　勇（1991）：犬糸状虫成虫の寄生部位と血行動態の超音波画像による検討。日本獣医師会雑誌，44, 1115–1120.

27 Hayasaki, M.*, Harayama, A., Seki, H., Konno, K. and Ohishi, I.(1991):

Immunological treatment of cough occurring in dogs with dirofilariosis. Journal of Veterinary Medical Science, 53, 651–653.

28 Hayasaki, M., Oishi, I., Kobayashi, S., Kume, S., Mogami, Y. and Sejima, T.(1974): Seven cases of paradoxical embolism due to canine filariae. Journal of Japanese Veterinary Medical Association, 61, 549–552.(in Japanese with English summary)
早崎峯夫、大石　勇、小林茂雄、久米清治、最上義典、瀬島　孟（1974）：犬糸状虫による奇異性塞栓症７例について。日本獣医師会雑誌、27, 2－9.

29 Hayasaki, M., Sejima, T., Oishi, I., Kobayashi, S. and Kume, S.(1973): A case report on inveterate paradoxical embolemia caused by canine filariae. Journal of Japanese Veterinary Medical Association, 26, 13–19.(in Japanese with English summary)
早崎峯夫、瀬島　孟、大石　勇、小林茂雄、久米清治（1973）：犬糸状虫による陳旧性奇異性塞栓症の１例。日本獣医師会雑誌、26, 13–19.

30 Hayasaki, M., Oishi, I., Kobayashi, S. and Kume, S.(1972): *Dirofilaria immitis* found from feces of a dog. Journal of Japanese Veterinary Medical Association, 25, 598–599.(in Japanese)
早崎峯夫、大石　勇、小林茂雄、久米清治（1972）：犬の糞便中に発見された犬糸状虫*Dirofilaria immitis*について。日本獣医師会雑誌、25, 598–599.

31 Hayasaki, M.*, Saeki, H.(2009): Inhibition and prevention efficacy against mosquito bloodsucking and *Dirofilaria immitis* infection by administration of topical insecticide. Journal of Veterinary Medical Science, 71, 1049–1052.

32 Ohishi, I., Katae, H., Hayasaki, M., Nakagaki, K. and Tada, Y.(1987): Prophylactic activity of ivermectin against *Dirofilaria immitis* infectionin dogs: Establishment of effective dose and administration schedule. Japanese journal of Veterinary science, 49, 439–445.

33 Ohishi, I., Katae, H., Hayasaki, M. and Tada, Y.(1987): Prophylactic activity of ivermectin against *Dirofilaria immitis* infection in dogs: Larvicidal activity of ivermectin against *D. immitis* larvae 30 days after infection. Japanese Journal of Veterinary Science, 49, 115–120.

34 Suzuki, Y., Kodera, S., Kimura, H., Kobayashi, S., Hayasaki, M., Oishi, I. and Sugimura K.(1985): Field trial on prophylactic effects of levamisole hydrochloride by intermittent medication against *Dirofilaria immitis* infection in dogs. Journal of Japanese Veterinary Medical Association, 38, 216–218.(in Japanese with English summary)
鈴木嘉尚、小寺主司、木村　肇、小林茂雄、早崎峯夫、大石　勇、杉浦邦紀（1985）：

塩酸レバミゾールの間欠投与による野外での犬糸状虫の予防効果。日本獣医師会雑誌、38, 216-218.

35 Hayasaki, M. and Oishi, I.(1985): Prophylactic effects of levamisole hydrochloride by intermittent medication against *Dirofilaria immitis* infection in dogs. Journal of Japanese Veterinary Medical Association, 38, 154-157.(in Japanese with English summary)
早崎峯夫、大石　勇（1985）:塩酸レバミゾールの間欠投与による犬糸状虫の予防試験。日本獣医師会雑誌、38, 154-157.

36 Hayasaki, M. and Oishi, I.(1985): Prophylactic effects of levamisole hydrochloride by long-term medication against *Dirofilaria immitis* infection in dogs. Journal of Japanese Veterinary Medical Association, 38, 8-12.(in Japanese with English summary)
早崎峯夫、大石　勇（1985）:塩酸レバミゾールの長期投与による犬糸状虫予防試験。日本獣医師会雑誌、38, 8-12.

37 Hayasaki, M., Nakagaki, K. and Oishi, I.(1984): Larvicidal effects of the short-term medication of levamisole hydrochloride to developing stages of *Dirofilaria immitis* in infected dogs. Japanese Jouranl of Parasitology, 33, 573-576.(in Japanese with English summary, tables and figures)
早崎峯夫、中垣和英、大石　勇（1984）：犬糸状虫幼虫に対するLevamisole短期投与の殺滅効果。寄生虫学雑誌、33, 573-576.

38 Hayasaki, M., Kobayashi, S. and Oishi, I.(1984): Prophylactic effects of intermittent medication of levamisole hydrochloride against *Dirofilaria immitis* infection in dogs. Japanese Jouranl of Parasitology, 33, 429-433.(in Japanese with English summary, tables and figures)
早崎峯夫、小林茂雄、大石勇（1984): Levamisole塩酸塩の間欠投与による犬における犬糸状虫予防効果。寄生虫学雑誌、33, 429-433.