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

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

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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 “general 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 oviposition, 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
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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), mutate 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 haemocoel 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-

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**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 rhythmic 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 pharyngeal 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 pharyngeal 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).
Vectors of *Dirofilaria* nematodes 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
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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*,...
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*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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