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Aedes albopictus is a natural vector of *Dirofilaria immitis* in Italy

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Abstract

Investigations were carried out in Padova town (Veneto region, NE Italy) to define the actual role of *Aedes albopictus* in the natural transmission of *Dirofilaria* nematodes, and to assess the risk that its presence might represent for veterinary and medical health. During summer 2000–2002 daytime captures of human-attracted mosquitoes were carried out in three areas of the town. The presence of filarial parasites in mosquitoes was evaluated by PCR, and sequencing confirmed species assessment. DNA extraction was performed separately on pools of the insect abdomen and thorax-head, to discriminate between *Dirofilaria* infected/infective specimens. A total of 2721 mosquitoes were caught and *A. albopictus* was the most abundant species (2534). Filarial DNA was found in 27.5% (19/69) of the abdomen pools formed with mosquitoes collected in summer 2000, and in 11.1% (16/144) and 4.9% (6/123) thorax-head pools coming from samplings 2001 and 2002, respectively. Filarial DNA was belonging to *D. immitis* and all studied areas harboured infective specimens. These results prove *A. albopictus* as natural vector of *D. immitis* in Italy. Moreover, they support the hypothesis that the presence of the mosquito could affect the transmission pattern of canine heartworm disease in urban environment and, considering the aggressive anthropophylic behaviour of the species (30–48 bites/h) proven in Padova town, could enhance the circulation of filarial nematodes from animals to humans.

Keywords: *Aedes albopictus*; Natural vector; *Dirofilaria immitis*; Molecular diagnostics; Italy

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1. Introduction

Aedes albopictus, the "tiger mosquito", is an aggressive daytime biting nuisance mosquito species, but receives international attention over all for its possible role as a vector for parasites. In fact, it has been shown as a natural/experimental vector of some arboviruses, etiological agent of infectious diseases like Dengue, Yellow Fever, and several Encephalites (Zhang, 1990; Savage et al., 1994; Mitchell, 1995; Gerhardt et al., 2001; Turell et al., 2001). Concerning nematodes, experimental infections documented the development of three filarial species (*Dirofilaria repens*, *D. immitis* and *Setaria labiatopapillosa* Italian populations) in a laboratory colony of *A. albopictus* originating from specimens collected in central Italy, so indicating that the mosquito may be a competent intermediate host of these parasites (Cancrini et al., 1995). The mosquito proved efficient vector also for heartworm population present in Taiwan (Lai et al., 2001). Few data are available on the actual involvement of the "tiger" in the natural transmission of filariae. Ahid and Lourenco-De-Oliveira (1999) reported the absence of *D. immitis* in specimens captured in an endemic area of Brazil, whereas Lai et al. (2001) found *D. immitis* infected mosquitoes in a Taiwan endemic area.

The spreading of *A. albopictus* in Italy raises questions on its possible role as a vector mainly for indigenous nematodes. In fact, filarial species common among dogs, cats and wild carnivores (belonging to the genus *Dirofilaria*), cattle, equines, pigs and wild ruminants (belonging to the genus *Setaria*), present also in Italy, could be transmitted by this mosquito. Filarial nematodes in this country are mostly in sympatry and in some cases two species are present in the same host. Therefore biting mosquitoes could pick up together, for example, *D. repens* and *D. immitis*, even if their competence/efficiency as a vector could be, of course, different for each species. As a consequence, in absence of different geographical distribution it's difficult to reliably identify the filarial parasite present in the mosquito sampled and to single out mixed infections. Until recently species identification was only possible through enzymatic loci analysis (Cancrini et al., 1989) applied to fresh material (analysed immediately or frozen), and so not very practical, especially for the tiny larvae present in the insect vector. Now, molecular techniques of DNA amplification using polymerase chain reaction (PCR) assays, which are highly sensitive and specific, allow the parasite species present in each single mosquito sample (dehydrated, frozen, or stored in 70% alcohol, in Carnoy's fixative, or in isopropanol) is reliably identified (Favia et al., 1996).

The present study reports on human-attracted *A. albopictus* in Padova town (Veneto region, NE Italy) in summer 2000–2002, and analysed to detect *Dirofilaria* infection. The aims of the survey were to define the actual role of the "tiger mosquito" in the natural transmission of these parasites, and to assess the risk that its presence might represent for veterinary and medical health. In fact, dirofilariae could be occasionally transmitted from animals to humans because of the feeding pattern shown by *A. albopictus*, which is an opportunistic blood feeder, choosing predominantly mammals (less frequently birds), like dogs, cows, rabbits and a variety of wild animals (Hawley, 1988), but also humans.

Pathogen detection was achieved by PCR-based technologies, particularly appropriate in surveys on vector-borne diseases carried out in areas where several filarial species are sympatric, and useful to analyse large numbers of specimens (Favia et al., 1997; Massung

et al., 1998). DNA sequencing confirmed species assessment. Unfortunately, a problem might arise since the different stages of the filarial worm cannot be distinguished by molecular tools, so hampering the discrimination between mosquitoes which may transmit each filarial species and those taking up microfilariae but not involved in the transmission. Some tricks have been applied to overcome this constraint.

2. Study area

The town of Padova was chosen on the basis of both the high mosquito density and the presence of *Dirofilaria* nematodes (Capelli et al., 1996; Pietrobelli and Frangipane di Regalbono, unpublished data). Three areas of the town, pointed out by people complaining mosquito bites, were checked for the presence of *A. albopictus*. The sampling sites were the following: the garden of the Psychiatric Hospital (located in the outskirts), an Urban Park and the Botanical Garden (both located in the centre of the city).

3. Materials and methods

3.1. Mosquito sampling

Mosquito sampling was carried out during summer 2000 (from July to September, a total of 10 sampling days), 2001 (from July to October, 12 sampling days) and 2002 (August, 5 sampling days) by two humans employed as bait to attract mosquitoes. Collections were made from 9.00 to 11.00 a.m. and/or from 5.00 to 7.00 p.m. by aspirating females landed on the baits with a paper cup aspirator (Coluzzi and Petrarca, 1973). The female mosquitoes sampled in 2000 were immediately killed and fixed in 70% ethanol. Those collected in 2001 and 2002 were kept under controlled conditions (25–27 °C, ∼90% r.h.) for 5 days and then killed and fixed in 70% ethanol. This trick was applied to better investigate on the positive abdomens. In fact, supposing a last infective blood meal at least 3 days before the capture, keeping mosquitoes for 5 days should allow that the larval development in Malpighian tubules is completed or, in case of incompetent host, the blocked microfilariae are expelled.

Before fixing, identification of mosquito species was made in accordance with the key proposed by Snow (1987).

3.2. DNA extraction

The presence of filarial parasites in mosquitoes (grouped for species and for sampling area) was evaluated by PCR examination of the specimens in pools (usually 10 specimens each for insects collected in 2000 and 2001, and five each for those caught in 2002). Since the different larval stages of filarial parasites cannot be distinguished by the PCR-based method, DNA extraction was performed separately on the insect abdomen and thorax-head to discriminate at least between *Dirofilaria* infected/infective specimens (Favia et al., 1996).

Genomic DNAs were extracted from pools by grinding the mosquitoes in a buffer (1% SDS, 25 mM NaCl, 25 mM EDTA). After incubation at 65 °C for 30 s, each pool was

treated with CH_3COOK 8 M at 0 °C. Phenol/chloroform/isoamylic alcohol was used for further DNA purification. DNA was ethanol precipitated and pellet was suspended in 50 μl of double distilled water.

3.3. DNA amplification

To detect filarial parasites, pooled samples were analysed with "filarial" specific ribosomal primers named S2–S16 (Xie et al., 1994). Conditions for the detection of filarial DNA were those described for the amplification of the spacer 5S of the ribosomal gene (Favia et al., 2000). The reactions give rise to amplification products of approximately 400 bp for most filarial species. In *D. repens* the amplification pattern yields an additional fragment of about 350 bp. Positive samples were checked with primers specific for *D. repens* and *D. immitis* previously designed (Favia et al., 1996) and, in addition, were analysed by sequencing.

3.4. Fragment purification

The 400 bp fragments produced were excised from the agarose gel and purified by the ConcertTM Rapid Gel Extraction System (Gibco BRL), following manufacturer instructions.

3.5. DNA sequencing and analysis

The sequence analyses of the PCR purified products were performed by MWG-Biotech. Sequence comparison was achieved by CLUSTAL W analysis (Thompson et al., 1997).

3.6. Calculation of the infection rate

Minimum infection rates (MIRs) observed were calculated by the standard formula: (number of positive mosquito pools)/(total number of mosquitoes tested) × 100. According to a binomial distribution of the parasites, expected infection rates (P) were also evaluated (Cinco et al., 1998), and calculated as follows: $P = 1 - \sqrt[k]{n/N}$, where n is the number of negative pools, N the number of tested pools and k the average number of specimens in each pool.

4. Results

A total of 2721 specimens were caught in the whole sampling period (summer 2000–2002). As expected, human-attracted mosquitoes were almost all (97.1%) *A. albopictus* (2534), followed by *A. geniculatus* (162), *A. caspius* (11), *Culex pipiens* (9), *Cx modestus* (2), *A. vexans* (1), *Anopheles maculipennis* s.l. (1) and *Culiseta annulata* (1).

Results concerning *A. albopictus* sampling are reported in Table 1.

Considering the exposition time to bites, in the first sampling summer (year 2000) *A. albopictus* population was more abundant in the garden of the Psychiatric Hospital and at

Table 1
A. albopictus females collected in summer 2000–2002 in three sampling sites of Padova town, and exposition time to bites

Year	Garden of the Psychiatric Hospital		Urban Park		Botanical Garden		Total	
	Mosquitoes (no.)	Exposition to bites (h)	Mosquitoes (no.)	Exposition to bites (h)	Mosquitoes (no.)	Exposition to bites (h)	Mosquitoes (no.)	Exposition to bites (h)
2000	125	4	283	16	305	10	713	30
2001	60	4	8	2	1148	24	1216	30
2002	–	–	–	–	605	14	605	14
Total	185	8	291	18	2058	48	2534	74

the Botanical Garden (about 31 and 30 bites/h, respectively) than in the Urban Park (about 18 bites/h). Next samplings were carried out mainly in the Botanical Garden, where an increase of *A. albopictus* population was registered (about 48 and 43 bites/h in summer 2001 and 2002, respectively).

Results of PCR analyses on pools of all collected *A. albopictus*, MIRs observed and *P*-values are shown in Table 2.

As regards "tiger mosquito" collected in summer 2000, filarial DNA was found in 19 out of 69 pools (27.5%). Filarial DNA was not evidenced in mosquito thorax-head but only in the abdomen, and identified as *D. immitis*. As for mosquitoes collected in summer 2001, 24 pools had filarial DNA only in abdomen, eight pools either in abdomen or in thorax-head, whereas eight pools were positive only at level of thorax-head. Concerning summer 2002, 16 pools showed filarial DNA only in abdomen, two pools both in abdomen and in thorax-head and four pools were positive only at level of thorax-head. Therefore, the percentage of positive pools was 27.8% (40/144) and 17.9% (22/123), respectively, for mosquitoes collected in summer 2001 and 2002. Assuming the presence of only one positive mosquito/positive pool, we may calculate MIRs of 2.67% (19/713) in summer 2000, 3.29% (40/1216) in summer 2001 and 3.64% (22/605) in summer 2002. Allowing for the estimated infection rates, the probability to single out positive specimens was 3.07, 3.78 and 3.93%, respectively.

Specific primers and sequencing identified all filarial DNA as belonging to *D. immitis*, so *A. albopictus* proved natural vector of *D. immitis*.

A picture of *D. immitis* infections detected in *A. albopictus* and related to the sampling sites is reported in Table 3. All studied areas harboured infected mosquitoes.

Table 2
Minimum infection rates (MIRs) evidenced by PCR and expected infection rates (*P*)

Year	Specimens (no.)	Pool size (minimum–maximum)	Positive/tested pools	MIRs	*P* (95% C.I.)
2000	713	8–12	19/69	2.67	3.07 (1.99–4.71)
2001	1216	1–11	40/144	3.29	3.78 (2.81–5.06)
2002	605	4–5	22/123	3.64	3.93 (2.61–5.93)

Table 3
D. immitis infection (positive/tested pools) by sampling sites

Year	Garden of the Psychiatric Hospital		Urban Park		Botanical Garden	
	Thorax-head	Abdomen	Thorax-head	Abdomen	Thorax-head	Abdomen
2000	0/12	6/12	0/29	2/29	0/28	11/28
2001	2/6	5/6	0/2	0/2	14/136	27/136
2002	–	–	–	–	6/123	18/123
Total	2/18	11/18	0/31	2/31	20/287	56/287

5. Discussion

A total of 2721 mosquito females were collected in Padova town during 74 h of daytime captures on human baits, and *A. albopictus* was the most abundant species (2534 specimens). The examination by PCR-based technologies of 713 specimens sampled in summer 2000 and immediately killed, allowed the detection of 19/69 (27.5%) pools of about 10 specimens each infected by *D. immitis*.

Lacking of positive thorax-heads did not allow defining the actual value of this mosquito as a natural vector for *D. immitis*, what can be certainly drawn by the results of the analyses designed for specimens collected in 2001 and 2002. In both years the mosquitoes were kept for 5 days to allow that the development in Malpighian tubules is overcome or, in case of incompetent host, the blocked microfilariae are expelled, yielding a negative PCR. In fact, it has been shown that the insect defensive mechanisms against dirofilariae are efficient only on microfilariae recently ingested or penetrated in primary cells of the Malpighian tubules, being haemocytes, at the contrary, totally unable to block migrating L3 (Vegni-Talluri and Cancrini, 1994). Therefore, all pools proved positive have to be considered as due to larvae developed in a suitable and competent host, and *A. albopictus* acts as a natural vector of Italian *D. immitis*.

MIRs evidenced by molecular tools fit in with estimated values, and indicate an increasing probability to single out positive specimens in the three studied years. These results prove the risk for heartworm disease in the town of Padova and support what hypothesised in 1995, e.g. that the stable presence of *A. albopictus* in Italy should have increased the probability of transmission of endemic animal filariae, mainly in urban areas where *Cx pipiens* has been so far the main vector, and that this should be particularly relevant for *Dirofilaria* transmission (Cancrini et al., 1995). In fact, experimental infections had shown that 86.1% of the *A. albopictus* Italian strain fed on dog (microfilaremia = 6000 mf/ml) developed *D. repens* infective L3 (larvae mean number = 2.8), and 70.3% of the specimens infected with *D. immitis* through membrane feeding (6000 mf/ml) became infective (larvae mean number = 4.8), with a vector efficiency index of 0.18 and 0.86, respectively (Cancrini et al., 1995). These data have been later confirmed on the Taiwan strains of *D. immitis* and *A. albopictus*, and the "tiger" proved more efficient for heartworm transmission if compared to *Cx quinquefasciatus* (Lai et al., 2001). Previous laboratory data, joined together the high number of natural infections now evidenced, support the hypothesis that the presence of *A. albopictus* could affect the transmission pattern of canine and human dirofilariosis in urban

environment. The circulation of filarial nematodes between animals might be improved and enhanced and, considering the aggressive anthropophylic behaviour of the species (30–48 bites/h) proven in Padova town, from animals to humans. These results fit in with heartworm prevalence observed in this area and, in general, in Italy (mainly northern Italy populations), with cases of human dirofilariosis reported in Italy (Pampiglione et al., 2001), and with seroprevalences of *D. repens*/*D. immitis* antibodies evidenced in human population present in Italy (Prieto et al., 2000).

By the technical point of view, the use of S2–S16 primers could allow the detection of any filarial DNA, and it is of great concern because a lot of species possibly mosquito-transmitted (like *S. labiatopapillosa*, *S. equi*, *S. tundra*, *Foleyella furcata*, *D. repens*, *D. immitis*, *Cardiofilaria*, *Micipsella*, *Filaria*) have been reported in animals living in Italy (Pietrobelli et al., 1995; Mancianti et al., 2001; Cancrini, unpublished data.). Sequencing confirmed the unambiguous identification of *D. immitis* as the only filarial species transmitted by *A. albopictus* circulating in Padova town. The result is not surprising, being the capture sites all urban areas, far away from farms and habitat having different animal species as possible source of microfilaremic bloods. Concerning canine *D. repens*, our results fit in with previous data on the absence of the subcutaneous species in the town, and therefore the role of "the tiger" as natural vector of *D. repens* needs further evaluation.

A. albopictus templates will be tested for the presence of viral infections. In fact, high prevalence of antibodies against Tahyna and West Nile has been evidenced among people living in northeastern and in northern and central Italy, respectively, and to western equine Encephalitis virus in domestic animals. This suggested the possibility that an Alphavirus and a Flavivirus related to West Nile might circulate (Verani et al., 1967, 1995). The hypothesis on Flavivirus has been recently documented by an epidemic of West Nile disease in 14 horses from Tuscany (central Italy) (Cantile et al., 2001), and should be interesting to evaluate the actual role of *A. albopictus* as natural vector in Italy also for indigenous viruses.

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