Detection of *Dirofilaria immitis* (Nematoda: Filarioidea) by Polymerase Chain Reaction in *Aedes albopictus*, *Anopheles punctipennis*, and *Anopheles crucians* (Diptera: Culicidae) From Georgia, USA

BETH LICITRA,[1] ERIC W. CHAMBERS,[1] ROSMARIE KELLY,[2] AND THOMAS R. BURKOT[1,3]

J. Med. Entomol. 47(4): 634–638 (2010); DOI: 10.1603/ME09277

ABSTRACT Potential mosquito vectors of *Dirofilaria immitis* (Leidy) (Nematoda: Filarioidea), the causative agent of dog heartworm in the southeastern region of the United States, were collected with CDC light traps and gravid traps in seven counties in the state of Georgia, USA. The presence of *D. immitis* in these mosquitoes was detected by polymerase chain reaction using species-specific primers for the *D. immitis* surface or cuticular antigen. Overall, 1,574 mosquitoes of 13 species in seven genera were collected; 92% of the specimens were *Aedes albopictus* (Skuse), *Aedes vexans* (Meigen), or *Anopheles punctipennis* (Say). *Ae. albopictus*, *An. punctipennis*, and *Anopheles crucians* Wiedemann were positive for *D. immitis* DNA. *Ae. albopictus* had the highest maximum likelihood rate of infection (2.30%; 95% confidence interval [CI] = 1.15–4.00%) followed by *An. crucians* (1.38%: 95% CI = 0.04–6.93%), and *An. punctipennis* (0.85%: 95% CI 0.03–4.29%). The detection of *D. immitis* DNA in the heads and thoraxes of *Ae. albopictus* (0.40%; 95% CI = 0.12–2.02%) indicates that these mosquitoes can support the development of *D. immitis* to the infective stage 3 larvae.

KEY WORDS *Dirofilaria immitis*, *Aedes albopictus*, Georgia

Dog heartworm is caused by infection with *Dirofilaria immitis* (Leidy) (Nematoda: Filarioidea), a mosquito-transmitted parasite of canines found throughout the world (Acha and Szyfres 2003). Sixty-three species of mosquitoes are thought to be potential vectors of *D. immitis*, with 28 of these being found in the United States (Ludlam et al. 1970); 12 of these species [*Aedes albopictus* (Skuse), *Aedes sollicitans* (Walker), *Aedes sticticus* (Meigen), *Aedes taeniorhynus* (Christophers), *Aedes trivitattus* (Coquillett), *Aedes vexans* (Meigen), *Anopheles bradleyi* King, *Anopheles crucians* Wiedemann, *Anopheles punctipennis* (Say), *Culex nigripalpis* Theobald, *Culex quinquefasciatus* Say, and *Culex salinarius* (Coquillett)] are found in the southeastern United States and have been implicated as presumptive vectors of *D. immitis* (Lok et al. 2000). Although it was once believed that transmission occurred year-round in the warmer southern United States, recent studies have shown that *Dirofilaria* transmission may be seasonal in some warm climate states (Watts et al. 2001).

The *D. immitis* infections in canines have been historically common in the southeastern United States. In 1968 in Atlanta, *D. immitis* infection rates of 5.4 and 12.5% were found in privately owned dogs and dogs in shelters, respectively (Thrasher et al. 1968). In North Carolina and Virginia, a 19% *D. immitis* infection rate in dogs was found (Falls and Platt 1982). The *D. immitis* infections in dogs residing in animal shelters in northwestern South Carolina ranged from 8.7% (1991–1992) to 12.7% (1999–2000) (Yabsley et al. 2004).

In canines, dog heartworm is a chronic infection by adult *D. immitis* worms within the right ventricle and pulmonary arteries. In humans, infection with *D. immitis* can result in pulmonary dirofilariasis, a benign condition in which the parasites migrate to the heart and die and are then carried to the lungs. In the lung the parasites aggregate to form a pulmonary nodule (1–4 cm) known as a "coin" lesion (Acha and Szyfres 2003). These coin lesions can be mistaken for pulmonary malignancies and are often removed unnecessarily (Rodrigues-Silva et al. 1995). Although infections in humans are rare, four cases of human dirofilariasis were reported from Savannah, GA, between 1992 and 1999 (Echeverri et al. 1999).

Traditionally, field studies to incriminate vectors or to determine seasonality and intensity of transmission involved the dissection of large numbers of field-collected mosquitoes to determine whether a mosquito species was naturally infected with the infectious stage 3 larvae (L3) of *D. immitis*, which is transmitted from the vector to a vertebrate during blood feeding.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

[1] Division of Parasitic Diseases, Centers for Disease Control and Prevention, 4770 Buford Hgwy., Mailstop F-42, Chamblee, GA 30341.
[2] Georgia Division of Public Health, Atlanta, GA 30303-3109.
[3] Corresponding author, e-mail: tburkot@cdc.gov.

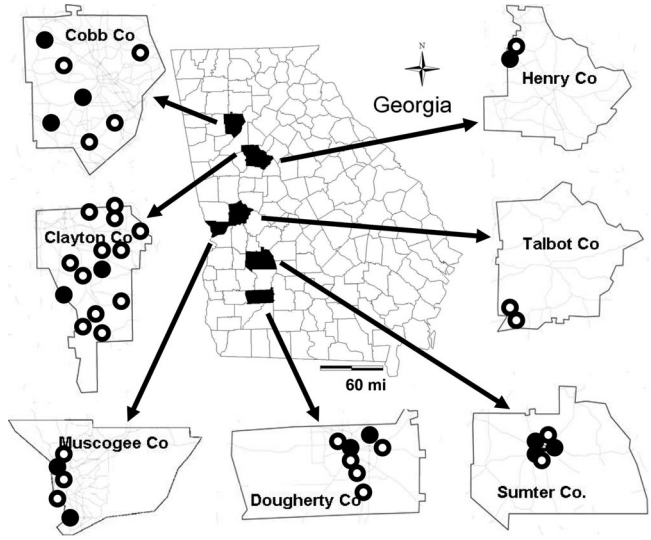

Fig. 1. Map of the state of Georgia with locations of the seven counties where mosquitoes were analyzed for *D. immitis* DNA. Approximate locations where all mosquitoes were negative for *D. immitis* DNA are shown as empty circles. Approximate locations with at least one mosquito positive for *D. immitis* DNA are shown as solid circles (map created 2009; source Georgia Division of Public Health: Centers for Disease Control and Prevention; Transverse Mercatur projection; UTM 1983, zone 16). Distance scale is for the map of Georgia.

It is logistically challenging to perform an adequate number of dissections for these studies and technically difficult to identify the L3s of different *Dirofilaria* species by morphology (Sauerman and Nayar 1983). However, sensitive and specific polymerase chain reaction (PCR)-based assays to circumvent these obstacles have been developed to identify *D. immitis* DNA in pools of mosquitoes (Scoles and Kambhampati 1995, Watts et al. 2001).

The current study identified potential mosquito vectors of *D. immitis* in Georgia, USA, by estimating the parasite infection rate by PCR analysis using specific primers to detect *D. immitis* DNA and emphasized the identification of *D. immitis* in non-*Culex* species (which constitute 10 of the 12 presumptive vectors of *D. immitis* in the southeastern United States; Lok et al. 2000) that were available from collections performed as part of a West Nile virus (family *Flaviviridae*, genus *Flavivirus*, WNV) study. PCR analysis of only the mosquito head and thorax was also conducted to identify mosquitoes that may be potentially infective (because only *D. immitis* DNA from L3s should be present in a vector mosquito's head and thorax).

Materials and Methods

Mosquito Collections. Mosquitoes were collected using both CDC light traps and gravid traps at 49 sites in seven counties in Georgia as part of the state WNV surveillance program (Fig. 1). Mosquitoes were collected between 29 September 2006 and 13 October 2006 and again between 11 May 2007 and 25 July 2007. *Culex* spp. [other than *Culex erraticus* (Dyar & Knab)] were not available for screening for *D. immitis* because they had been processed previously by the Georgia Division of Public Health for WNV detection and then discarded. Mosquitoes were pooled by species, trap location, and date of collection and were stored at −80°C until analyzed for *D. immitis.*

Extraction of DNA From Mosquitoes. DNA from mosquito pools was extracted using the DNeasy kit (QIAGEN, Valencia, CA) protocol, with the only modification being that the column was washed twice with buffer AW1 and once with buffer AW2 before DNA was eluted from the column with 125 μl of AE elution buffer. The purified DNA then was used for the PCR assay.

Extraction Controls. A negative DNA extraction control from a pool of noninfected laboratory-reared *Ae. albopictus* mosquitoes was run with each set of PCR reactions. In addition, a positive DNA extraction control and positive PCR control were included with each set of PCR reactions. The positive DNA extraction control consisted of a pool of laboratory-reared *Ae. albopictus* mosquitoes spiked with three to six *D. immitis* L3s (larvae provided by the National Institutes of Health/National Institute of Allergy and Infectious Diseases Filariasis Research Reagent Repository Center). The positive PCR control consisted of three to six *D. immitis* L3s.

PCR Amplification With *D. immitis* Primers. PCR assays were modified from the reaction conditions first published by Scoles and Kambhampati (1995) as described by Chambers et al. (2009) using primers based on the tandemly repeated *D. immitis* surface or cuticular antigen present at 20–50 copies per haploid genome. The repeat sequence (GenBank No. M82811) of this surface protein is 133 amino acids long with a molecular weight of 15 kDa (Culpepper et al. 1992, Poole et al. 1992). Each 50-μl PCR reaction

Table 1. Filarial *D. immitis* DNA-positive rates in mosquitoes collected in Georgia, USA

Species	Tested (no.)	Pools (no.)	Avg. no. mosquitoes per pool	*D. immitis* DNA-positive rate[a]	95% CI
Ae. albopictus	604	98	6.2	2.30	1.15–4.00
Ae. vexans	551	54	10.2	0	
An. punctipennis	121	20	6.1	0.85	0.03–4.29
An. crucians	77	10	7.7	1.38	0.04–6.93
Anopheles quadrimaculatus Say	23	7	3.3	0	
Cx. erraticus	10	5	2	0	
Aedes canadensis (Theobald)	4	2	2	0	
Psorophora columbiae (Dyar & Knab)	3	3	1	0	
Orthopodomyia signifera (Coquillett)	3	3	1	0	
Psorophora ciliate (F.)	2	1	1	0	
Aedes atlanticus Dyar & Knab	1	1	1	0	
Coquilletidia perturbans (Walker)	1	1	1	0	
Uranotaenia sapphirina (Osten Sacken)	1	1	1	0	
Total	1,401	206	6.8	1.11	0.58–1.87

[a] Parasite DNA rates are max likelihood estimates as calculated by PoolScreen2 software (Katholi et al. 1995).

contained 1× *Taq* buffer (QIAGEN); 3 mM $MgCl_2$; 0.20 mM each dATP, dCTP, dGTP, and dTTP; 10 pmol of *D. immitis* surface antigen forward and reverse primers (5′-ACG TAT CTG AGC TGG CTC AC-3′ and 5′-ATG ATC ATT CCG CTT ACG CC-3′, respectively; Scoles and Kambhampati 1995), 1.25 U of HotStarTaq DNA polymerase, and 1 μl of genomic DNA. PCR reactions were run on an I-Cycler (Bio-Rad Laboratories, Hercules, CA). The PCR conditions consisted of a single step of 95°C for 15 min and 50°C for 5 min (needed to activate the HotStar *Taq*DNA polymerase; S. Williams, personal communication), followed by 35 cycles of 72°C for 1 min, 90°C for 1 min, and 50°C for 1 min. The final step was a 5-min extension at 72°C. PCR products were size fractionated on 2% agarose gels stained with GelRed (Biotium, Hayward, CA). Agarose gels were run at 70 V for 1 h and visualized under UV light. Samples positive for the *D. immitis* surface antigen repeat yielded a 378-bp fragment after amplification. Positive samples were verified by repeating the PCR reaction in duplicate. A pool was considered positive if at least one of the two confirmatory repeat samples were also positive. Analysis of potential infections with *D. immitis* L3s was conducted by PCR using DNA extracted from the head and thorax of all *Ae. albopictus* captured in light and gravid traps during July 2007.

Estimation of Mosquito Infection Rates. Mosquito infection rates by PCR were calculated as maximum likelihood estimates, with 95% confidence intervals (CI) based upon the likelihood ratio method using PoolScreen2 software (Katholi et al. 1995).

Results and Discussion

In the current study, 13 species in seven genera of mosquitoes (total of 1,401 mosquitoes in 206 pools) were analyzed from 49 trap locations in seven counties in Georgia (Fig. 1). Ninety-two percent of mosquitoes analyzed in this study were *Ae. albopictus* ($n = 796$), *Ae. vexans* ($n = 551$), and *An. punctipennis* ($n = 122$) (Table 1).

Three mosquito species were positive for *D. immitis* DNA: *Ae. albopictus*, *An. punctipennis*, and *An. crucians* (Tables 1 and 2). This is the first study that reports evidence for the natural infection of mosquitoes with *D. immitis* in the state of Georgia. Many previous studies in other states reported mosquito species infected with filariid nematodes that were presumed (because of the difficulty of identifying *D. immitis* by morphology) to be *D. immitis* (reviewed by Lok et al. 2000), including the three species confirmed with *D. immitis* DNA in our study. Infected *Ae. albopictus* mosquitoes were identified from six of the seven counties in which mosquitoes were collected with 31% of the 49 collection sites ($n = 15$), yielding mosquitoes positive for *D. immitis* DNA, confirming the widespread distribution of this parasite (Fig. 1). Infected *Ae albopictus* were not found in Talbot Co. ($n = 7$). The estimated prevalence of *D. immitis* infection in *Ae. albopictus* for any larval stage was 2.30% (95% CI = 1.15–4.00%) (Table 1), ranging from 0.92% (95% CI = 0.11–3.2%) in Clayton Co. to a high of 17.1% (95% CI = 3.44–44.9%) in Sumter Co. among the six counties where evidence of infection was found. Overall, the prevalence of *D. immitis* L3s based on

Table 2. Numbers of wild-caught mosquitoes tested and number of PCR-pools positive for *D. immitis* (in parentheses) by year and month of collection

Species	2006, Sept.	2006, Oct.	2007, May	2007, June	2007, July	Total
Ae. albopictus	59 (2)	217 (5)	25 (3)	41 (2)	262 (1)	604 (13)
Ae. vexans	0	296 (0)	196 (0)	59 (0)	0	551 (0)
An. punctipennis	0	7	113 (1)	1 (0)	0	121 (1)
An. crucians	0	0	77 (1)	0	0	77 (1)
Others	0	2 (0)	30 (0)	15 (0)	1 (0)	48 (0)
Totals	59 (2)	515 (5)	441 (5)	116 (2)	263 (1)	1401 (15)

detecting *D. immitis* DNA in the heads and thoraces of *Ae. albopictus* was 0.40% (95% CI = 0.12–2.02%).

An. punctipennis was collected from Dougherty, Muscogee, Sumter, and Talbot counties. A single *D. immitis*-positive pool was detected in the town of Americus in Sumter Co. The overall maximum likelihood of infection in *An. punctipennis* was 0.85% (95% CI = 0.03–4.29%) (Table 1). A single infected pool of *An. crucians* was found from Sumter Co.; (the only county in which this mosquito was collected). The maximum likelihood of infection was 1.38% (95% CI = 0.04–6.93%). *D. immitis* DNA was not detected in any of the other 10 mosquito species tested (Table 1), including *Ae vexans* ($n = 551$), which was reported as a presumed vector in studies in 10 states, including the neighboring state of Alabama (Lok et al. 2000).

Ae. albopictus has been implicated previously as both an experimental and natural vector of *D. immitis* in Louisiana, Florida, and Italy (Comiskey and Wesson 1995, Nayar and Knight 1999, Cancrini et al. 2003). Results from our study confirm that not only is *Ae. albopictus* capable of becoming infected with *D. immitis* in Georgia but also *Ae. albopictus* can support the development of *D. immitis* to the L3, based on the presence of *D. immitis* DNA in the head and thorax of this mosquito. Infection rates were higher in *Ae. albopictus* than in either of the other two *D. immitis* DNA-positive species, *An. crucians* and *An. punctipennis*. *Ae. albopictus* positive for *D. immitis* DNA were found in six of the seven counties that were sampled, but *An. crucians*- and *An. punctipennis*-positive mosquitoes were only detected in Sumter Co., although *An punctipennis* was collected in four counties albeit in limited numbers ($n < 5$) except for Sumter Co. where 113 *An. punctipennis* were collected and which yielded the *D. immitis* DNA-positive pool). Although the data presented cannot confirm whether transmission of dog heartworm occurs throughout the year in Georgia, infected mosquitoes were detected in each month in which samples were collected as early as 11 May and as late as 13 October, the latest and earliest dates when mosquitoes were collected, indicating a minimal 6-mo transmission season (Table 2).

Ae. albopictus was first detected in the continental United States in 1985 in Harris Co., TX (Sprenger and Wuithiranyagool 1986), and by 1997 it had spread to 678 counties in 25 states, including Georgia (Moore and Mitchell 1997). *Ae. albopictus* readily feeds on a variety of mammals, including dogs and humans (Savage et al. 1993), and *Ae. albopictus* is now known to support the development of *D. immitis* to the L3. In addition to the daytime feeding *Ae. albopictus*, *An. punctipennis*, and *An. crucians*, two dusk- and nighttime-feeding mosquitoes, were identified as infected with *D. immitis* by DNA analysis. The sampling biases associated with the mosquito collection methods (e.g., light and gravid traps may under-sample some species, including *Aedes triseriatus* Say, a presumptive *D. immitis* vector in the southeastern United States; Lok et al. 2000), as well as the unavailability of *Ae. triseriatus*, *Cx. nigripalpis*, *Cx. salinarius*, and *Cx. quinquefasciatus* for analysis, and the small numbers of mosquitoes from nine other species analyzed ($n < 24$ for each of these nine species), suggests that our study probably underestimated the number of species of dog heartworm vectors in Georgia. However, finding *D. immitis* DNA in mosquitoes that seek bloodmeals during the day, at dusk, and at nighttime suggests that domestic pets in Georgia are potentially at high risk of being exposed to the bites of dog heartworm-infected mosquitoes at all times when outdoors. Further work is needed to confirm the vector status of *An. crucians* and *An. punctipennis* by detecting the presence of *D. immitis* L3s in these suspected vectors.

Acknowledgments

We thank the Filariasis Research Reagent Repository Center (University of Georgia, Athens, GA) for providing L3 stage *D. immitis* and Steve Williams (Smith College) for advice on PCR conditions.

References Cited

Acha, P. N., and B. Szyfres. 2003. Zoonoses and communicable diseases common to man and animals. Pan American Health Organization, Washington, DC.

Cancrini, G., A. Frangipane di Regalbono, I. Ricci, C. Tessarin, S. Gabrielli, and M. Pietrobelli. 2003. *Aedes albopictus* is a natural vector of *Dirofilaria immitis* in Italy. Vet. Parasitol. 118: 195–202.

Comiskey, N., and D. M. Wesson. 1995. Dirofilaria (Filarioidea, Onchocerciadae) infection in *Aedes albopictus* (Diptera, Culicidae) collected in Louisiana. J. Med. Entomol. 32: 734–737.

Culpepper, J., R. B. Grieve, L. Friedman, M. Mika-Grieve, G. R. Frank, and B. Dale. 1992. Molecular characterization of a *Dirofilaria immitis* cDNA encoding a highly immunoreactive antigen. Mol. Biochem. Parasitol 54: 51–62.

Echeverri, A., R. F. Long, W. Check, and C. M. Burnett. 1999. Pulmonary dirofilariasis. Ann. Thorac. Surg. 67: 201–202.

Falls, R. K., and T. R. Platt. 1982. Survey for heartworm, *Dirofilaria immitis*, and *Dipetalonema reconditum* (Nematoda, Filarioidea) in dogs from Virginia and North Carolina. Am. J. Vet. Res. 43: 738–739.

Katholi, C. R., L. Toè, A. Merriweather, and T. R. Unnasch. 1995. Determining the prevalence of *Onchocerca volvulus* infection in vector populations by polymerase chain reaction screening of pools of black flies. J. Infect. Dis. 172: 1414–1417.

Lok, J. B., E. D. Walker, and G. A. Scoles. 2000. Filariasis, pp. 299–375. *In* B. F. Eldridge and J. D. Edman (eds.), Medical Entomology. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Ludlam, K. W., L. A. Jachowski, Jr., and G. F. Otto. 1970. Potential vectors of *Dirofilaria immitis*. J. Am. Vet. Med. Assoc. 157: 1354–1359.

Moore, C. G., and C. J. Mitchell. 1997. *Aedes albopictus* in the United States: ten-year presence and public health implications. Emerg. Infect. Dis. 3: 329–334.

Nayar, J. K., and J. W. Knight. 1999. *Aedes albopictus* (Diptera : Culicidae) : an experimental and natural host of *Dirofilaria immitis* (Filarioidea : Onchocercidae) in Florida, USA. J. Med. Entomol. 36: 441–448.

Poole, C. B., A. G. Gandea, 3rd, C. V. Maine, R. E. Jenkins, M. E. Selkrik, and L. A. McReynolds. 1992. Cloning of a

cuticular antigen that contains multiple tandem repeats from the filarial parasite *Dirofilaria immitis.* Proc. Natl. Acad. Sci. USA 89: 5986–5990.

Rodrigues-Silva, R., H. Moura, G. Dreyer, and L. Rey. 1995. Human pulmonary dirofilariasis: a review. Rev. Inst. Med. Trop. Sao Paulo 37: 523–530.

Sauerman, D. M., and J. K. Nayar. 1983. a survey for natural potential vectors of *Dirofilaria immitis* in Vero-Beach, Florida. Mosq. News 43: 222–225.

Savage, H. M., M. L. Niebylski, G. C. Smith, C. J. Mitchell, and G. B. Craig, Jr. 1993. Host-feeding patterns of *Aedes albopictus* (Diptera: Culicidae) at a temperate North American site. J. Med. Entomol. 30: 27–34.

Scoles, G. A., and S. Kambhampati. 1995. Polymerase chain reaction-based method for the detection of canine heartworm (Filarioidea: Onchocercidae) in mosquitoes (Diptera: Culicidae) and vertebrate hosts. J. Med. Entomol. 32: 864–869.

Sprenger, D., and T. Wuithiranyagool. 1986. The discovery and distribution of *Aedes albopictus* in Harris County, Texas. J. Am. Mosq. Control Assoc. 2: 217–219.

Thrasher, J. P., K. G. Gould, M. J. Lynch, and C. C. Harris. 1968. Filarial infections of dogs in Atlanta, Georgia. J. Am. Vet. Med. Assoc. 153: 1059–1063.

Watts, K. J., G. R. Reddy, R. A. Holmes, J. B. Lok, D. H. Knight, G. Smith, and C. H. Courtney. 2001. Seasonal prevalence of third-stage larvae of *Dirofilaria immitis* in mosquitoes from Florida and Louisiana. J. Parasitol. 87: 322–329.

Yabsley, M. J., C. Dresden-Osborne, E. A. Pirkle, J. M. Kirven, and G. P. Noblet. 2004. Filarial worm infections in shelter dogs and cats from northwestern South Carolina, USA. Comp. Parasitol. 71: 154–157.

Received 18 November 2009; accepted 25 February 2010.