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Mosquito-based survey for the detection of flaviviruses and filarial nematodes in *Aedes albopictus* and other anthropophilic mosquitoes collected in northern Italy

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SUMMARY

In order to optimize resources, an assay allowing the detection of several etiological agents would be useful. This paper describes a polymerase chain reaction (PCR)-based assay for the parallel detection of different pathogens in mosquitoes (Diptera: Culicidae). The assay combines the analysis of both RNA and DNA, hence allowing the detection of both flaviviruses (Flaviviridae: *Flavivirus*) and filarial nematodes (Spirurida: Onchocercidae). The sensitivity of diagnostic assay for both flavivirus and nematode infections was tested by means of spiked samples and resulted adequate for the purpose of screening mosquito samples. The assay was implemented to check pathogen infections in 637 mosquitoes field-collected in Romagna, northern Italy. The integrity of RNA extracted from mosquito pools was checked by control amplifications targeting the 18 S rRNA of mosquitoes. Control amplifications were successful in 118 out of 119 pools. Flavivirus RNA was not found in any of these 118 pools, whereas DNA of *Dirofilaria immitis* (Leidy) was detected in one pool of *Aedes albopictus* (Skuse).

KEY WORDS: Mosquito-based surveillance of pathogens, Mosquito-borne diseases, *Flavivirus, Dirofilaria, Aedes albopictus*, RT-PCR

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INTRODUCTION

Mosquitoes (Diptera: Culicidae) are the most important vectors of diseases affecting humans and animals all over the world. Exotic mosquito species are frequently introduced into new geographic areas thus adding new potential vectors to the native culicid fauna. This is the case of the Asian tiger mosquito, *Aedes albopictus* (Skuse), which has been introduced in many countries, including Italy (Knudsen *et al.*, 1996; Romi, 2001). Its diffusion in Southern Europe (Aranda *et al.*, 2006) raised serious concerns due to its possible role in the transmission of viruses and parasitic nematodes (Gratz, 2004).

The actual incidence of flaviviruses (Flaviviridae: *Flavivirus*) in Europe may be underestimated because most infections cause non symptomatic or mild febrile illness, and, even in cases of hospitalization of febrile patients, ordinary diagnostic procedures do not include any test for arthropod-borne disease (Kallio-Kokko *et al.*, 2005). Nevertheless, an increasing incidence of flavivirus

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infections has recently been documented in Europe (Kallio-Kokko *et al.*, 2005).
Dirofilaria immitis (Leidy) and *D. repens* (Raillet and Henry) (Spirurida: Onchocercidae) are the etiological agents of canine heartworm and dog subcutaneous granulomas, respectively. Both species can cause parasitic zoonosis: accidental infections in humans may result in subcutaneous, conjunctival, and pulmonary nodules (Pampiglione *et al.*, 1995; Pampiglione *et al.*, 2001). Both parasites are widespread in Italy and many indigenous mosquito species as well as *Ae. albopictus* can potentially serve as intermediate hosts (Cancrini *et al.*, 2003a; Cancrini *et al.*, 2003b; Pampiglione and Rivasi, 2001).
Pathogen detection in field-caught mosquitoes is one of the mainstays of surveillance programs for many mosquito-borne diseases (CDC, 2003). If an adequate number of samples is processed, this method may provide the earliest evidence of transmission in an area. Moreover, mosquito-based surveillance provides crucial information on relevance as vectors, distribution, and abundance of mosquito species (CDC, 2003).
Over the past 15 years, several PCR-based molecular methods have been developed for the detection of pathogens in mosquito samples. Molecular assays for the detection of flaviviruses have recently been reviewed by Lanciotti (2003), and several DNA amplification tests targeting genome of parasitic nematodes have been proposed (Favia *et al.*, 2000; Mar *et al.*, 2002; Rishniw *et al.*, 2006; Scoles and Kambhampati, 1995; Watts *et al.*, 1999). In general, PCR-based methods offer higher sensitivity and much lower processing time than any other alternative techniques, so that large number of mosquitoes, either pooled or individually sorted, can be rapidly screened.
In this study, a PCR-based assay combining both RNA and DNA analysis, and thus allowing the detection of both flaviviruses and parasitic nematodes, is proposed. The assay was implemented to survey pathogen infections in mosquitoes field-collected in northern Italy.

MATERIALS AND METHODS

Specimen collections

Individuals of *Ae. albopictus* used for the development and the optimization of the diagnostic assays were taken from a laboratory colony (Centro Agricoltura Ambiente "Giorgio Nicoli", Bologna, Italy).
Field samplings were carried out in different locations of Romagna, northern Italy. This region has a tourist vocation and some important commercial harbours. Moreover, residual wetlands provide habitat for high-density populations of migratory and resident birds. For these reasons, Romagna can be considered at potential risk of transmission of mosquito-borne diseases.
Female mosquitoes were collected in the field from early September to late October 2005. Two sampling methods were used: lethal traps (Mosquitraps, Biogents, Regensburg, Germany) checked weekly for the presence of mosquitoes, and direct aspiration on human bait performed at daylight for a fixed time of 30 min. The mosquitoes were sorted by species, date, site, and method of collection thus forming 119 pools each including 1-50 individuals. The pools were air-dried in sterile Petri plates and stored at room temperature from 7 to 15 weeks before molecular analyses.
Inactivated viral stocks of West Nile virus (WNV) and of the four dengue (DEN) serotypes were kindly provided as suckling mouse brain suspensions by E. A. Gould (Centre for Ecology and Hydrology, Oxford, UK). The yellow fever (YF) vaccine Stamaril (batch Z5429-5, Aventis-Pasteur, Maidenhead, UK) containing attenuated viral strain 17D was also used.
Filarial nematodes were obtained from the whole blood of three filaremic dogs. Nucleotide sequencing of microfilariae 5 S rRNA region confirmed preliminary morphological identification of the parasites as *D. immitis*.

Nucleic acid extractions

Mosquitoes were triturated with autoclavable pestles (Starlab, Ahrensburg, Germany) in 1.5 ml tubes containing 350-600 µl of RTL lysis buffer (Qiagen, Hilden, Germany). The lysates were homogenized by passing at least 5 times through a 20-gauge needle. Supernatants were clarified from solid debris by high-speed centrifugation, and applied to All Prep DNA/RNA mini Kit columns (Qiagen) for the parallel extraction of both DNA and RNA. To prevent DNA contamination, DNase digestions (RNase-Free DNase Set, Qiagen) were performed on the extracted RNA.

cDNA synthesis

The SuperScript III First-Strand Synthesis for RT-PCR (Invitrogen, Carlsbad, CA) was used for cDNA synthesis. RT reactions were performed in the final volume of 20 µl containing 2 µl of 10X RT buffer, 5 mM of $MgCl_2$, 10 nM of dithiothreitol (DTT), 500 µM of dNTP mix, 40 U of RnaseOUT, 200 U of Superscript III RT, 100 ng of random hexamers, and 7 µl of DNase treated RNA extracts. The mixtures were incubated at 25°C for 10 min, 50°C for 50 min, and 85°C for 5 min. RNA templates were eliminated by digestion with 2 U of RNaseH (Invitrogen) at 37°C for 20 min.

18 S rRNA control PCR amplification

Since samples were conserved at room temperature, PCR amplification targeting mosquito 18 S rRNA was implemented to verify the integrity of RNA in each pool. Primers 18S417 (5'-ACGGGGAGGTAGTGACGAGAAATA-3') and 18S920c (5'- TAATACTAATGCCCCCAACTACTT-3'), which anneal to conserved regions of 18 S rRNA in several mosquito species (Hoffmann *et al.*, 2004), were used.

PCR amplifications were performed in a final volume of 10 µl including 1 µl of cDNA, 1 µl of 10X PCR Gold Buffer, 2 mM of $MgCl_2$, 200 µM of each dNTP, 400 nM of each primer, 0.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA**)**. Cycling conditions were: 10 min at 95°C, 35 cycles of 30 s at 95°C, 1 min at 60°C, and 1 min 20 s at 72°C, followed by a final extension step at 72°C for 7 min.

PCR amplification diagnostic for flaviviruses

Consensus primers DV1 (5'-GGRACKTCAGGW TCTCC-3') and DV3 (5'-AARTGIGCYTCRTCCAT-3'), which target a ≈470 bp region of viral genomes encoding for part of NS3 protein (Chow *et al.*, 1993), were used for flavivirus diagnostic amplifications. Each PCR mixture included 2 µl of cDNA, 2 µl of 10X PCR Gold Buffer, 2 mM of $MgCl_2$, 200 µM of each dNTP, 200 nM of each primer, 0.25 U of AmpliTaq Gold (Applied Biosystems), and bidistilled water to the final volume of 20 µl. The thermocycling program was as follows: a Taq polymerase activation step at 95°C for 10 min followed by 35 cycles of 30 s at 95°C, 1 min at 50°C, and 1 min 20 s at 72°C, and a final extension step at 72°C for 7 min. The assay was tested for specificity attempting PCR amplifications on cDNA obtained from six different flaviviruses (the four DEN serotypes, WN, and YF 17D). To test the sensitivity of the flavivirus-targeting amplifications, non-infected mosquito specimens were spiked with 10-fold serial dilutions of yellow fever vaccine. The number of plaque forming units (PFU) in these dilutions ranged form $\approx 2 \cdot 10^3$ to ≈2. Total RNA isolation and reverse transcription were performed from each spiked sample as previously described. PCR amplification were carried out in triplicate for each cDNA sample.

PCR amplification diagnostic for filarial nematodes

PCR amplifications targeting *Dirofilaria* genome were performed on DNA extracted from mosquito pools using primers S2 (5'-GTTAAG-CAACGTTGGGCCTGG-3') and S16 (5'-TTGACA-GATCGGACGAGATG-3') (Xie *et al.*, 1994). These primers anneal to 5 S rRNA genes and give rise to amplification products that include the intergenic region between repeats of 5 S RNA coding sequences.

Amplification reactions were performed in the final volume of 20 µl including 2 µl of DNA, 2 µl of 10X PCR Gold Buffer, 2 mM of $MgCl_2$, 200 µM of each dNTP, 500 nM of each primer, and 0.25 U of AmpliTaq Gold (Applied Biosystems). Thermocycling profile was as follows: 10 min at 95°C, 35 cycles of 30 s at 95°C, 1 min at 60°C, and 1 min 20 s at 72°C, and a final extension step at 72°C for 7 min.

Modified Knott concentration method (Jackson and Otto, 1975) was performed on 1 ml of dog whole blood to count microfilariae. One hundred microliters of blood, containing 42±18 microfilariae, were added to an equal volume of digestion buffer (50 mM Tris pH 8.5, 1 mM EDTA, 0.5% Tween 20) (Wright and Manos, 1990) including 0.1 mg of proteinase K, and incubated for 1 h at 56°C and 10 min at 95°C. Dilutions of the digested blood (containing the amount of DNA expected to be present in ≈10, 5, 1 and 0.1 microfilariae, respectively) were added to a reared mosquito specimen.

To assess the lower detection limits of diagnostic PCR, DNA extracted from each spiked sample was amplified in triplicate with the conditions described above.

Analysis of field-collected mosquitoes

For routine screening, the parallel extraction of both nucleic acids was carried out for each mosquito pool. After RT reaction, the RNA integrity of each pool was verified by the 18 S rRNA amplification. If the control amplification was successful, the cDNA of the pool was amplified for flavivirus detection, whereas DNA was used for nematode diagnostic PCR. Every batch of pools was accompanied by necessary negative (no DNA) and positive (either cDNA obtained from YF 17D or DNA extracted from blood of filaremic dog) control samples**.** Standard precautions to avoid product contamination were taken throughout the work. Both reverse transcriptions and amplification reactions were performed on Advanced One (Euroclone, Milan, Italy) thermocyclers. Ten microliters of PCR products were resolved on 1.5% agarose gel stained with 1 µg/ml of ethidium bromide and visualized by UV illumination.

Identification of pathogens by sequencing analysis

Given that the use of broadly reactive consensus primers ensures the detection of a wide array of pathogens, their specific identification was achieved by nucleotide sequencing of the resulting bands. The procedure was performed only on the amplicon resulting from the filarial nematode positive pool. The amplicon was ligated into a pCR 2.1 TOPO Vector and used to transform *E. coli* One Shot TOP10 competent cells (TOPO TA Cloning Kit; Invitrogen) according to manufacturer's instructions. Cells were plated onto LB-ampicillin plates with X-GAL (5-bromo-4-chloro-3-indolyl-B-D-galactopyranose) and incubated overnight at 37°C. Four white colonies were picked-up, amplified using M13 primers, and sequenced on an ABI PRISM 3730 automatic sequencer using the Big Dye Terminator Cycle Sequencing Kit v 1.1 (Applied Biosystems) according to the manufacturer's instructions. Parasite identification was done using BLAST and sequences were deposited in GenBank (AN: EU360964-5).

RESULTS

Diagnostic assay set-up

The RT-PCR assay targeting NS3 gene was performed on RNA extracted from the four DEN serotypes, the WNV, and the YF 17D. All the viruses produced DNA fragments of the expected size that were reliably recognized by gel electrophoresis (Figure 1A). When the assay was performed on YF 17D viral RNA mixed to mosquito samples as little as approximately 20 PFU could be detected (Figure 1B).

Favia and co-workers (2000) exhaustively demonstrated that the PCR here proposed can be used to detect both *D. immitis* and *D. repens*. Therefore, the specificity of this PCR was not tested in the present research.

Figure 2 displays the results of PCR preformed on mosquito samples spiked with DNA of *D. immitis* to evaluate the sensitivity of the assay. The amplified products of *D. immitis* were clearly visible for each blood dilution, including the lanes corresponding to the DNA of 0.1 microfilariae.

No bands due to unspecific priming of mosquito RNA sequences were detected in amplification targeting viral genome. On the contrary, filarial diagnostic PCR produced a number of unexpected

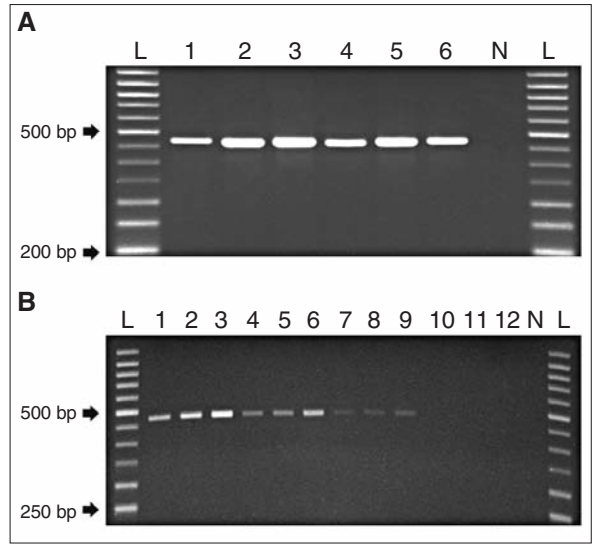

FIGURE 1 - *(A) Ethidium bromide-stained 1.5% agarose gel showing the results of RT-PCR amplifications of ≈470 bp of NS3 gene from different flaviviruses. 1: DEN 1. 2: DEN 2. 3: DEN 3. 4: DEN 4. 5: WNV. 6: YF 17D (Stamaril vaccine, batch Z5429-5, Aventis-Pasteur, Maidenhead, UK). N: Negative control (bidistilled water). L: 50 bp DNA ladder (Celbio, Milan, Italy). (B) RT-PCR amplifications targeting ≈470 bp of NS3 gene performed in triplicate on mosquito samples spiked with different quantities of YF 17D virus. 1-3: ≈2·10^3 PFU. 4-6: ≈2·10^2 PFU. 7-9: ≈20 PFU. 10-12: ≈2 PFU. N: Negative control (bidistilled water). L: 50 bp DNA ladder (Celbio).*

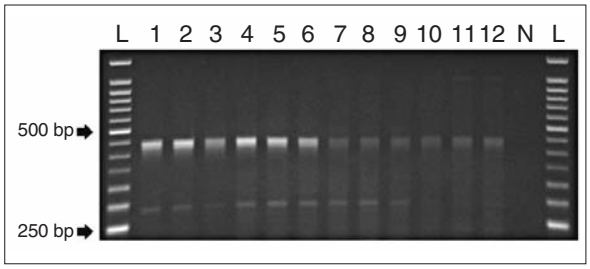

FIGURE 2 - *Ethidium bromide-stained 1.5% agarose gel showing the results of PCR amplifications targeting ≈430 bp of filarial 5 S rRNA region performed in triplicate on mosquito samples spiked with serial dilution of digested filaremic blood. 1-3: 50 µl of undiluted blood corresponding to ≈10 microfilaria DNA. 4-6: 50 µl of 1:2 blood dilution (≈5 microfilaria DNA). 7-9: 50 µl of 1:10 blood dilution (≈1 microfilaria DNA). 10-12: 50 µl of 1:100 blood dilution (≈0.1 microfilaria DNA). N: Negative control (bidistilled water). L: 50 bp DNA ladder (Celbio).*

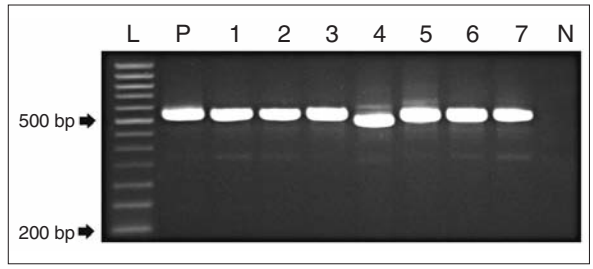

FIGURE 3 - *Representative 1.5% agarose gel of RT- PCR amplifications targeting ≈500 bp of mosquito 18 S rRNA gene performed on samples stored for up to 15 weeks at room temperature. 1-3: Ae. albopictus. 4: Cx. pipiens. 5: Ae. detritus. 6-7: Ae. caspius. P: Positive control (Ae. albopictus stored at -80°C) N: Negative control (bidistilled water). L: 50 bp DNA ladder (Celbio).*

bands in some mosquito samples (Figure 2, Figure 4B). Those bands had molecular weights considerably different from the diagnostic ones and their presence did not negatively bias the assay.

Analysis of field-collected mosquitoes

In total, 637 field-collected female mosquitoes were analyzed (Table 1). Three hundred eighty-seven *Ae. albopictus*, 77 *Aedes caspius* Pallas and 3 *Ae. detritus* Haliday were collected by direct aspiration; whereas lethal traps trapped 141 *Ae. albopictus*, 28 *Culex pipiens* L. and 1 *Ae. caspius*.

The results of surveillance program carried out on field-collected mosquitoes are summarized in Table 1. The control amplifications targeting 18 S rRNA (Figure 3) were successful for 118 out of 119 pools. None of these 118 pools tested positive for flaviviruses (Figure 4A). On the contrary, one pool, including 34 *Ae. albopictus* specimens collected by direct aspiration, resulted positive for filarial DNA (Figure 4B). This parasite was identified as *D. immitis* performing Blast search on sequences. Three out of four clones had the same sequence (AN: EU360965) whereas the remainder (AN: EU360964) showed two nucleotide substitutions and a deletion of two TTA repetitions in the microsatellite region. The uncorrected pairwise distance between the sequences was 0.07.

The *Dirofilaria* minimum infection rate (MIR) calculated for *Ae. albopictus* was 1/528 (0.19%).

TABLE 1 - *Results of assays for RNA integrity and for pathogen detection in field-collected mosquitoes.*

Mosquito species	*No. of specimens*	*No. of pools*	*No. of positive pools*		
			Control amplification	*Flavivirus RNA*	*Nematode DNA*
Aedes albopictus	528	98	97	0	1
Aedes caspius	78	6	6	0	0
Aedes detritus	3	2	2	0	0
Culex pipiens	28	13	13	0	0
Total	637	119	118	0	1

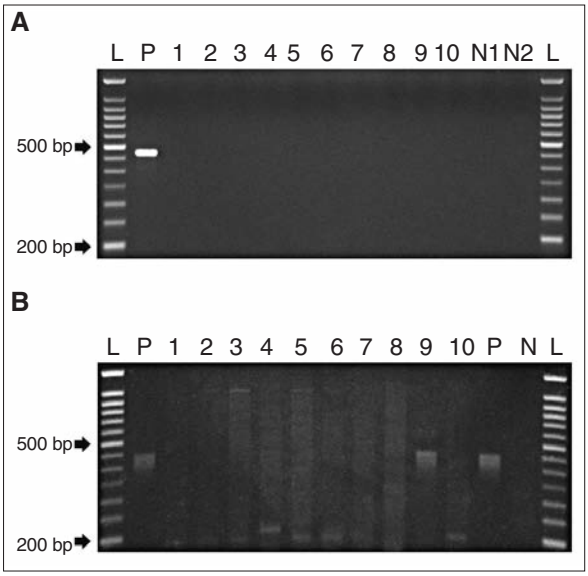

FIGURE 4 - *Representative 1.5% agarose gels showing the results of diagnostic assays for pathogen detection in mosquito pools. (A) RT-PCR assay for flavivirus detection. 1-7: Ae. albopictus pools. 8: Cx. pipiens pool 9: Ae. detritus pool. 10: Ae. caspius pool. P: Positive control (YF 17D virus). N1: Negative control (RT reaction performed on bidistilled water). N2: Negative control (bidistilled water). L: 50 bp DNA ladder (Celbio). (B) PCR assay for filarial nematodes detection. 1-8: Ae. albopictus pools negative for filarial nematode DNA. 9: Ae. albopictus pool positive for Dirofilaria immitis DNA. 10: Cx. pipiens pool negative for filarial nematode DNA. P: Positive controls (filaremic blood). N: Negative control (bidistilled water). L: 50 bp DNA ladder (Celbio).*

DISCUSSION

To detect flaviviruses within mosquitoes, different universal primer pairs (Chow *et al.*, 1993; Gaunt and Gould, 2005; Sanchez-Seco *et al.*, 2005; Scaramozzino *et al.*, 2001) were preliminarily tested (data not shown). Primers DV1-DV3 proposed by Chow *et al.* (1993) were eventually selected for the relatively small size of resulting amplicons (≈470 bp) and for their low affinity with mosquito genome. Using primers DV1-DV3 Scaramozzino and co-workers (2001) failed to amplify YF 17D virus. On the contrary, this virus was amplified reliably by RT-PCR performed in this study (Figure 1A, lane 6), thus confirming results reported in the original paper (Chow *et al.*, 1993). The discrepancy between these findings could be due to a combination of several factors including viral quantities tested, RNA extraction methods, RT and PCR reagents, and slight differences in thermocycling conditions. The possibility to amplify consistently YF virus, besides widening the range of the assay, also allows using the attenuated strain 17D as positive controls for the amplifications targeting flavivirus sequences (Figure 4A). This is useful in routine screening of mosquitoes since the vaccine can be easily obtained and stored and can be manipulated without risks in biosafety level 1 facilities.

The sensitivity of RT-PCR performed on mosquito samples spiked with YF 17D viral RNA was about 20 PFU (Figure 1B). Because of sequence divergence among flaviviruses, it is expected that sensitivity of the assay may vary among viruses (Lanciotti, 2003). Nevertheless, given that vector mosquitoes contain a number of PFU usually comprised between 10^4 and 10^5 (Hadfield *et al.*, 2001; Vanlandingham *et al.*, 2004), the sensitivity of the assay appears to be adequate for the recognition of infective individuals. Some published assays achieved a higher sensitivity in flavivirus detection adopting multi-step nested PCR or real-time PCR (Lanciotti, 2003). The implementation of multi-step amplifications, besides increasing the sensitivity of the assay, increases also the risk of cross contamination. Real-time technology is still not available in most laboratories, and it is considerably more costly than conventional RT-PCR.

Primers S2-S16 anneal to sequences that appear highly conserved among nematodes, and they have been previously used to amplify 5S rRNA and 5S spacer in many different species (Favia *et al.*, 2000; Fischer *et al.*, 2002; Liu *et al.*, 1996; Xie *et al.*, 1994). Although not specifically tested, the proposed assay should allow the detection of several mosquito-borne nematode of medical and veterinary importance such as *Setaria*, *Onchocerca*, *Wuchereria* and *Brugia*.

Very few studies focusing on flavivirus circulation are reported for Italy (Verani *et al.*, 1979) and little is known about actual flavivirus diffusion. The isolation of WNV performed on mosquitoes collected in an area of equine outbreak was unsuccessful (Romi *et al.*, 2004). Similarly, all field-collected mosquitoes analysed in this study were negative for flavivirus RNA. This finding was not unexpected given the number of mosquitoes tested, the species composition, and the probable low

rates of flavivirus circulation in Italy. Albeit the long-term storage of the mosquito pools at room temperature, the absence of flavivirus infections in the samples must not be attributed to compromised RNA integrity. The control amplification targeting 18 S rRNA did not reveal any decrease in the quality of RNA extracted from mosquito pools. Moreover, several authors described RT-PCR assays for the detection of viral RNA in samples held without a cold chain (Bangs *et al.*, 2001; Johansen *et al.*, 2002; Kramer *et al.*, 2001; Turell *et al.*, 2002; Wacharapluesadee *et al.*, 2003). In particular, Guzman and co-workers (2005) reported that WN and YF viral RNA could be found in mouse brain samples dried on filter paper and stored at room temperature for up to 90 days, and Bangs *et al.* (2007) were able to detect dengue RNA in dried specimens of *Aedes aegypti* (L.) exposed to tropical ambient conditions for up to 13 weeks.

Infections of *D. immitis* have been reported for natural populations of *Ae. albopictus* in central and northern Italy (Cancrini *et al.*, 2003a; Cancrini *et al.*, 2003b). Although in the present study a considerably lower MIR was detected, our findings confirm the role of *Ae. albopictus* in the circulation of dog heartworm. Harasawa and co-workers (1997) reported a degree of sequence variation for 5 S rRNA region between individuals of the same strain of *D. immitis* similar to that found in this study. For these reasons it is likely that the sequence variation among the clones analyzed here can be related to different nematode individuals infecting the same mosquito. However the possibility that two or more specimens of *Ae albopictus* in the pool were infected with different filarial strains cannot be ruled out.

In this paper, we describe a rapid protocol for the parallel detection of different mosquito-borne pathogens in insect samples. The assays rely on simultaneous extraction of both RNA and DNA from the same sample. RNA is analyzed by means of RT-PCR to detect flaviviruses; DNA is processed by conventional PCR to reveal filaroid nematode infections. Since primer sets and cycling conditions allow the amplification of several different flaviviruses and parasitic nematodes, nucleotide sequencing is used for specific identification of pathogens in positive samples. This assay could readily be modified to screen any mosquito-borne pathogens of medical and veterinary importance. In countries where very little is known on actual infection rates of mosquito-borne pathogens, a wide-range assay allowing the detection of several etiological agents would be suitable to rapidly assess the level of circulation of different pathogens. Moreover, the vector status of different mosquito species could be accurately evaluated.

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