SHORT COMMUNICATION

Multiplex Polymerase Chain Reaction for Detection of *Dirofilaria immitis* (Filariidea: Onchocercidae) and *Wuchereria bancrofti* (Filarioidea: Dipetalonematidae) in Their Common Vector *Aedes polynesiensis* (Diptera: Culicidae)

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J. Med. Entomol. 34(6): 741–744 (1997)

ABSTRACT In French Polynesia, *Aedes polynesiensis* (Marks) is the vector of the human filarial parasite *Wuchereria bancrofti* (Cobbold) and dog heartworm, *Dirofilaria immitis* (Leidy). A multiplex polymerase chain reaction (PCR) assay was designed to screen pools of field-collected *Ae. polynesiensis* for the presence of both parasites simultaneously using primers specific for each parasite. The sensitivity of detection on purified DNA was 1 and 10 pg, equivalent to 0.1 and 1 L3 larva per pool for *W. bancrofti* and *D. immitis*, respectively. Codetection was performed at an hybridization temperature of 58°C to avoid competition between heterologous DNA and primers that was observed at 55°C. In addition, *D. immitis* was detected by PCR in the blood of infected dogs.

KEY WORDS *Wuchereria bancrofti, Dirofilaria immitis, Aedes polynesiensis,* bancroftian filariasis, canine heartworm, polymerase chain reaction

IN FRENCH POLYNESIA, *Aedes polynesiensis* (Marks) is the vector of 2 filarial parasites, *Wuchereria bancrofti* (Cobbold), the etiological agent of human bancroftian filariasis, and *Dirofilaria immitis* (Leidy), the canine heartworm. Bancroftian filariasis was controlled in Polynesia between 1950 and 1982 with diethylcarbamazine (DEC), leading to a great reduction in prevalence. However, in 1993 a control program based on the semiannual distribution of DEC at 3 mg/kg was reimplemented because of the reappearance of infection. *D. immitis* is widespread throughout the Polynesian islands because of poor veterinary care and the difficulty of vector control.

Surveillance of infection rates in mosquito populations is one of the tools available to determine the success of a control program. Our objective therefore was to develop a more convenient and sensitive technique than the dissection of mosquitoes for determining infection rates in *Ae. polynesiensis* populations. A polymerase chain reaction (PCR) assay was developed for detecting *W. bancrofti* larvae in pools of *Ae. polynesiensis* (Chanteau et al. 1994, Nicolas et al. 1996), based on the primers NV1 and NV2 (Zhong et al. 1996), which amplify a 188-bp repeat DNA sequence specific for *Wuchereria*. This assay detects all geographical strains of *W. bancrofti* tested thus far. However, it is not stage specific because the target DNA was identified from a genomic DNA library. The assay is sensitive because it can detect a single *W. bancrofti* L3 larva in a pool of 100 *Ae. polynesiensis* or the presence of a single mosquito harboring as few as 1–2 microfilariae among 20–50 uninfected mosquitoes (Nicolas et al. 1996). A PCR assay also has been described for detecting *D. immitis* in mosquitoes (Scoles and Kambhampati 1995).

Screening of *Ae. polynesiensis* populations for the presence of both filarial parasites is of interest in epidemiological studies. In this article, we have extended our previous work on *W. bancrofti* detection in *Ae. polynesiensis* to show that both parasites can be searched either separately or simultaneously in the same mosquito extracts by a multiplex PCR. In addition, the *D. immitis* primers can be used to monitor heartworm infection in Polynesian dogs.

Materials and Methods

DNA Preparation. Microfilariae of *W. bancrofti* variety *pacifica* were obtained from 20 ml blood collected in ethylenediaminetetraacetic acid (EDTA) during the day by veinous blood puncture (Desowitz et al. 1973) from a microfilaremic Polynesian patient and purified on Percoll gradient. Similarly, microfilariae of *D. immitis* were purified from 20–30 ml veinous blood collected from the anterior leg of an infected dog. Genomic DNA was

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0022-2585/97/0741-0744$02.00/0

isolated from microfilariae by proteinase K digestion (McReynolds et al. 1986).

Mosquito Extracts. Day-biting *Ae. polynesiensis* were collected by amicrofilaremic persons acting as bait and collectors (Bonnet et al. 1956) on an island from the Society Archipelago, French Polynesia, where bancroftian filariasis and canine heartworm are endemic. Collections were done in the morning from 0630 to 1030 hours and from 1500 to 1800 hours to include the 2 peaks of *Ae. polynesiensis* biting activity (Lardeux et al. 1995). DNA was extracted from each of 50 pools of 5 field-captured mosquitoes and resuspended in 100 μl of TE buffer per pool (10 mM Tris-HCl pH8, 1 mM EDTA) as described in Nicolas et al. (1996). Extracts from noninfected *Ae. polynesiensis* from a laboratory rearing were used as negative controls.

Polymerase Chain Reaction Assay. The sequences of the *W. bancrofti* primers were forward NV1 (21 mer) 5′-CGTGATGGCATCAAAGTAGCG-3′ and reverse NV2 (22 mer) 5′-CCCTCACTTACCATAAGACAAC-3′ (Zhong et al. 1996). The target sequence (*Ssp*I DNA repeat) is present at ≈500 copies per haploid genome. Primers used for detection of *D. immitis* were based on the DNA sequence of a tandemly repeat *D. immitis* surface antigen present at 20–50 copies per haploid genome (Culpepper et al. 1992, Poole et al. 1992). These sequences were forward Di1 (20 mer) 5′-ACGTATCTGAGCTGGCTCAC-3′; reverse Di2 (20 mer) 5′-ATGATCATTCCGCTTACGCC-3pr (Scoles and Kambhampati 1995), and they yielded a major 378-bp fragment and a minor 780-bp fragment by PCR. Polymerase chain reactions were performed in a final volume of 50 μl, as described in Nicolas et al. (1996). Ten microliters of the PCR product were loaded onto a 1.5% agarose gel and visualized by ethidium bromide staining. A negative control for the PCR assay using water instead of template in the reaction mix was included in all runs.

Sensitivity and Specificity of *D. immitis* and *W. bancrofti* Primers on Purified Parasite DNA. The sensitivity and specificity of *D. immitis* or *W. bancrofti* primers used separately were determined on a series of combinations of *D. immitis* and *W. bancrofti* DNA. Crossed combinations of 10-fold concentrations (0, 1, 10, or 100 pg) of DNA from each parasite were added to a same reaction and amplification was carried out with a single pair of primers. Coamplification assays then were carried out on crossed combinations of DNA from the 2 filarial species with the 2 pairs of primers (*D. immitis* and *W. bancrofti*) in the same reaction mix.

Detection of *D. immitis* and *W. bancrofti* in Pools of *Ae. polynesiensis*. PCR was carried out on 5 μl of DNA extracts prepared from 50 pools of 5 field-captured *Ae. polynesiensis*, with *D. immitis* primers and *W. bancrofti* primers, either in 2 separate assays or in a coamplification assay.

Detection of Filarial Parasites in Dog Blood. Venous blood samples (5 ml each) were taken during the day from the anterior leg of 15 dogs. Detection of *D. immitis* microfilariae was done by microscopic examination of 20 μl of blood (blood smear). Blood was frozen at −20°C in 0.1 M EDTA until PCR assay. DNA was extracted from 100 μl whole blood as described in Williams et al. (1996). PCR was carried out on 1 or 5 μl of blood extracts using either *D. immitis* or *W. bancrofti* primers.

Table 1. Detection limits by PCR of *D. immitis* (Di) or *W. bancrofti* (Wb) repeat sequences from mixtures of genomic DNA from both parasites

Parasite DNA in the reaction	Sensitivity of *D. immitis*, pg		Sensitivity of *W. bancrofti*, pg	
	Di	Di + Wb	Wb	Di + Wb
Primers				
Di	10/10[a]	10/10	—	—
Wb	—	—	1/1	1/1
Di + Wb	100/10	100/10	1/1	10/1

[a] Hybridization temperatures = 55/58°C.

Results and Discussion

Screening pools of vectors for the presence of filarial parasites by PCR assays can be a cost-effective strategy and yield more rapid diagnostic information than dissection of individual mosquitoes. We previously have shown that PCR on pools is more sensitive than dissection for detection of *W. bancrofti* in *Ae. polynesiensis* in an endemic situation (Nicolas et al. 1996). Another advantage of PCR is that vector extracts can be screened for the presence of several pathogens including RNA viruses (Chungue et al. 1993) or for differentiating strains that are morphologically similar such as *Onchocerca volvulus* (Leuckart) in Africa (Katholi et al. 1995).

The PCR protocol that we have designed for detecting *W. bancrofti*, either in mosquitoes (Nicolas et al. 1996) or in blood (Williams et al. 1996), did not require hot-start PCR, a technique that avoids false-priming but is more expensive than conventional PCR. Our objective was to use the same protocols for detecting *D. immitis*, and if necessary to screen mosquitoes for the presence of both parasites in a multiplex PCR assay.

In preliminary experiments using purified DNA from a single species and a single pair of primers at the hydridization temperature of 55°C (Nicolas et al. 1996), the sensitivity of the PCR assay was 1 pg for *W. bancrofti* and 10 pg for *D. immitis* (Table 1). PCR on *W. bancrofti* DNA yielded a unique 188-bp fragment, whereas PCR on *D. immitis* yielded a major 378-bp fragment and a minor 780-bp fragment (Fig. 1). Sensitivities remained unchanged when 1–10 μl of *Ae. polynesiensis* extract were added to the reactions. No PCR product was amplified from 100 pg DNA from a filarial species with primers of the other species.

In field *Ae. polynesiensis* populations, it is unlikely that a single mosquito is infected with both *D. immitis* and *W. bancrofti* larvae. However, screening pools enhanced the likelihood of having DNA from

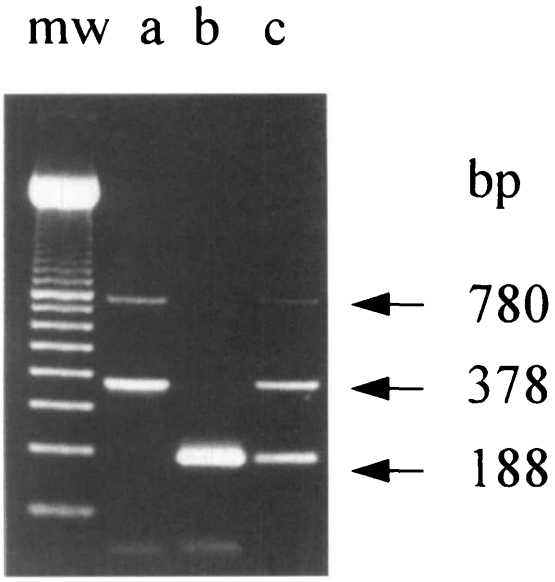

Fig. 1. Detection of D. immitis and *W. bancrofti* DNA by multiplex PCR. DNA templates in the reactions were as follows: lane a, 10 pg of *D. immitis* DNA; lane b, 10 pg of *W. bancrofti* DNA; lane c, 10 pg of *D. immitis* DNA and 10 pg of *W. bancrofti* DNA. mw, Molecular weight marker (100-bp ladder, Pharmacia). Numbers and arrows on the right side indicate the size (bp) of PCR products.

both parasites in the same extract. Therefore, we assayed each pair of primers in the presence of DNA from both parasites. Interestingly, the sensitivity of either *W. bancrofti* or *D. immitis* primers was not altered by the presence of DNA from the other parasite in the reaction (Table 1). Then, we determined the sensitivity of the primers in coamplification assays to screen extracts for the presence of both parasites in a single PCR assay. When the 2 pairs of primers were introduced in the same reaction at a hybridization temperature of 55°C, the amplification of *W. bancrofti* DNA was not inhibited when *D. immitis* DNA was absent, but it was inhibited when it was present (Table 1). In contrast, the sensitivity of *D. immitis* DNA detection was lowered (100 pg instead of 10 pg), even in absence of *W. bancrofti* DNA. Therefore, we repeated the experiments with purified DNA at a more stringent hybridization temperature and observed that the sensitivity of the multiplex PCR assay was restored by a hybridization temperature of 58°C (Table 1). At this temperature, coamplification assays carried out by adding both pairs of primers (*D. immitis* and *W. bancrofti*) in a single reaction mix allowed amplification of the PCR products from both filarial species (Fig. 1).

In agreement with the results of coamplification with parasite DNA, hybridization of DNA extracts from mosquito pools was carried out at 58°C. Out of 50 mosquito pools, 7 were PCR positive with *W. bancrofti* primers. The same extracts were assessed for the presence of *D. immitis*. Preliminary assays with mosquito extracts and *D. immitis* primers indicated a better sensitivity when the PCR was performed on 5 μl of extract instead of 1 μl (data not shown). Using 5 μl extract as a template, 6 out of the 50 mosquito pools were PCR positive with *D. immitis* primers. One pool was PCR positive for both parasites. The results were similar when the 2 pairs of primers were added to the reaction mix in multiplex PCR. Using Poolscreen computer program (Katholi et al. 1995), to predict prevalence of infection in populations from results of pool screening, the predicted infection rates of the *Ae. polynesiensis* population were 2.97% (95% CI, 1.19–6.03%) for *W. bancrofti* and 2.52% (95% CI, 0.92–5.42%) for *D. immitis*.

The *D. immitis* primers also detected *D. immitis* in dog blood using the protocol described in Williams et al. (1996). Among the 15 blood samples examined, 5 were microfilaria-positive for *D. immitis* by blood smear examination and 8 by PCR assay, including all the samples positive by blood smear. None were PCR positive with *W. bancrofti* primers.

In conclusion the *D. immitis* strain endemic in Polynesian islands can be detected using the primers designed by Scoles and Kambhampati (1995) from the major surface antigen of *D. immitis* (the ladder protein) (Poole et al. 1992) in mosquitoes or in dog blood. The *D. immitis* primers were ≈10 times less sensitive than *W. bancrofti* primers, which may be the result of the difference in copy number of the target sequence (25–50 copies for *D. immitis* compared with 500 copies for *W. bancrofti*). Nevertheless, the sensitivity of *D. immitis* detection (10 pg) was sufficient for practical application because it corresponded to 1 *D. immitis* L3 per pool of mosquitoes (if 5 μl extract is used as a template in the PCR reaction). Sensitivity of detection of *W. bancrofti* corresponded to 0.1 L3 per pool of mosquitoes. Therefore, mosquito DNA extracts can be screened for the 2 filarial parasites, either in separate PCR assays or in multiplex PCR. This should be greatly facilitate surveillance programs. The protocol for mosquito DNA extraction that we developed is sensitive enough to detect a single *W. bancrofti* parasite in pools of 50–100 *Ae. polynesiensis* (Nicolas et al. 1996) or *Culex quinquefasciatus* (Say) with NV1-NV2 *W. bancrofti* primers (Furtado et al. 1997). Batches of 1,000–2,000 mosquitoes can be analyzed per day by a technician working 8 h a day, with a single thermocycler. This has to be compared with the ability of a well-trained technician to dissect and examine ≈100–200 mosquitoes a day. Further research should be now carried out to identify more sensitive *D. immitis* primers to attain the field-applicable results obtained with *W. bancrofti*.

Acknowledgments

We thank P. Luquiaud and C. Plichart for skillfull technical assistance and L. Moillon and J. P. Moulia-Pelat for help in collecting dog blood samples. We thank also Frédéric Lardeux for helpful discussion.

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Received for publication 12 February 1997; accepted 13 June 1997.