TRANSACTIONS OF THE ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE (1996) 90, 136–139 N°2

A polymerase chain reaction assay to determine infection of *Aedes polynesiensis* by *Wuchereria bancrofti*

Luc Nicolas[1], Patrick Luquiaud[1], Frédéric Lardeux[2] and David R. Mercer[1] *[1]Institut Territorial de Recherches Médicales Louis Malardé, B.P. 30, Papeete, Tahiti, French Polynesia; [2]ORSTOM (Institut Français de Recherche Scientifique pour le Développement en Coopération), B.P. 529, Papeete, Tahiti, French Polynesia*

Abstract

The sensitivity of a previously described polymerase chain reaction (PCR) assay was improved to detect a single mosquito, infected by as few as 1–2 microfilariae of *Wuchereria bancrofti*, among 20–50 uninfected mosquitoes. Wild-caught *Aedes polynesiensis* were used to compare assessment of infection by dissection of individuals with the PCR assay of pools of mosquitoes. The PCR assay was at least as sensitivite as dissection for detection of mosquitoes infected with *W. bancrofti*.

Keywords: filariasis, *Wuchereria bancrofti*, *Aedes polynesiensis*, polymerase chain reaction

Introdction

At least 120 million people are thought to be infected by *Wuchereria bancrofti* and, to a lesser extent, by *Brugia malayi* or *B. timori* (see OTTESEN & RAMACHANDRAN, 1995). Evaluation of chemotherapy campaigns is based not only on detection of the parasite or specific parasite antigens in patients but also on monitoring the infection of mosquito populations. Traditionally, this has involved the dissection and microscopical examination of hundreds to thousands of mosquitoes, which is very time consuming. Another drawback of the dissection method is the risk of confusing the several filarial species which may co-infect a single mosquito.

The development of deoxyribonucleic acid (DNA) probes and polymerase chain reaction (PCR) assays for detection of parasites has been recently reviewed (WEISS, 1995). Development of molecular techniques to detect filarial parasites in pools of vectors is an objective of the World Health Organization (WHO, 1993). A preliminary PCR assay based on the amplification of a DNA repeat sequence from *W. bancrofti*, the '*SspI* DNA repeat', and using specific primers has been described by CHANTEAU *et al.* (1994). In this assay, a 188 base pair (bp) DNA fragment was amplified specifically from *W. bancrofti* but not from DNA of other filariae, humans or mosquitoes; this fragment is present in all the geographical isolates of *W. bancrofti* so far tested. By contrast, it is not stage-specific.

A PCR method reported by CHANTEAU *et al* (1994) detected a single third-stage larva (L3) of *W. bancrofti* added to a pool of 50 heads of *Aedes polynesiensis*, the main vector of bancroftian filariasis in French Polynesia. However, for convenience, the PCR needs to be performed on pools of whole mosquitoes instead of heads. A PCR assay must also be able to detect any infected mosquito, even by a single parasite, in pools of mosquitoes. With these conditions fulfilled, it should be possible to examine large numbers of mosquitoes in a much shorter time than by dissection. Although the DNA probe considered here is not stage-specific, evaluation of infection rates in mosquito populations, instead of infectivity rates, would be of great help in monitoring chemotherapy campaigns.

We have improved the PCR assay described by CHANTEAU *et al.* (1994) so that it will now detect a single L3 in a pool of 50 to 100 whole *A. polynesiensis* or a single mosquito, infected by about 2 microfilariae (mf) in 20 to 50 whole mosquitoes. Furthermore, we have shown that the PCR assay performed on pools is at least as sensitive as dissection in determining infection rates of wild populations of *A. polynesiensis*.

Materials and Methods

Polymerase chain reaction assay

The extraction of DNA from heads or whole mosquitoes was modified from the method of CHANTEAU *et al.* (1994) as follows. Mosquitoes were dried for 3 h at 90°C, then separated heads or whole insects were carefully crushed with a sterile pestle tissue grinder in a 1·5 mL plastic tube. The crushed material was washed twice with 1 mL of 0·1 M NaCl, 30 mM Tris–HCl (pH 7·8), 30 mM ethylenediaminetetraacetic acid (EDTA), 10 mM 2-β-mercaptoethanol, and 0·5% Nonidet P40®. The supernatant was discarded and DNA was released by lysing the pellet with 100 μL of 0·1 M NaOH and 0·2% sodium dodecyl sulphate for 1 h at 37°C (or 200 μL if the number of mosquitoes was above 10), neutralized with 5 (or 10) μL of 2N Hcl, and then mixed thoroughly with 1 mL of 4·5M guanidine hydrothiocyanate, 50 mM Tris–HCl (pH 6·4), 1·2% Triton X100®, and 20 mM EDTA. DNA was precipitated by addition of 40 μL of silica particle suspension (Sigma) and incubation for 10 min at room temperature. Following centrifugation for 10 s at 12 000 *g* in a Microfuge®, the silica particles were washed twice with 4·5M guanidine hydrothiocyanate and 50 mM Tris–HCl (pH 6·4). The silica pellet was then dried at 56°C for 10 min with the tube cap removed, and the beads were resuspended in 100 μL of TE buffer (10 mM Tris–HCl [pH8], 1 mM EDTA) and incubated at 56°C for 10 min to elute the DNA. After 10 s centrifugation, the supernatant was kept for PCR.

The PCR reaction was performed on 1 to 10 μL of mosquito supernatant in a final volume of 50 μL, and included 2 units of *Taq* polymerase (Promega), 400 pM of both primers, NV1 and NV2 (CHANTEAU *et al.*, 1994), and 200 μM of each dideoxynucleotide in 50 mM KCl, 10 mM Tris–HCl (pH9), 0·1% Triton X100®, and 1·5 mM $MgCl_2$. The temperature programme for the PCR was 5 min at 92°C, then 30 cycles of 15 sec at 92°C, 1 min at 55°C, 1 min at 72°C, and a final 10 min at 72°C.

Ten μL of the PCR product were loaded on to a 1·5% agarose gel and a unique band of 199 bp was visualized by ethidium bromide staining. A negative control for the PCR assay, using water instead of DNA extract in the reaction mix, was included with all runs. A positive control was also included, consisting of 1 pg (or 0·1 pg) of *W. bancrofti* mf genomic DNA, which corresponds to *c.* 1% of an L3 larva per μL.

Because *A. polynesiensis* is also the vector of *Dirofilaria immitis*, the dog heartworm, in French Polynesia, we checked the specificity of the PCR assay for *W. bancrofti*. The sensitivity limit for the assay was 0·1 pg of *W. bancrofti* DNA, but no comparable DNA fragment was amplified from up to 800 pg of DNA from a local strain of *D. immitis*.

Sensitivity and reproducibility of the assay to detect W. bancrofti *L3*

W. bancrofti L3 were obtained from laboratory-bred *A. polynesiensis* fed on a microfilaraemic Polynesian volunteer. Fourteen days after blood-feeding, the mosquito heads were cut off and immersed in RPMI-1640 medium to allow passive release of L3, which were then further

Address for correspondence: Dr L. Nicolas, Institut Territorial de Recherches Médicales Louis Malardé, B.P. 30, Papeete, Tahiti, French Polynesia; fax +689 431 590.

purified by filtration through fine mesh gauze. DNA extraction and the PCR were carried out on pools of 50 or 100 heads of uninfected *A. polynesiensis* or on whole mosquitoes 'spiked' with one L3.

Our ability to detect L3 within whole mosquitoes by the PCR assay was tested with a batch of *A. polynesiensis*, 90% of which were infected with a mean of 6 L3 (range 1–10). Ten pools of 50 mosquitoes containing either 1, 2 or 3 putatively infected individuals were examined.

Negative controls for the extraction procedure were included in all experiments to avoid false positive PCR results. Every tenth extraction of mosquito heads or whole mosquitoes was done using uninfected laboratory-bred mosquitoes. The extracts from these negative controls were then included in the PCR assay.

Sensitivity of the assay for detecting microfilariae in laboratory-bred mosquitoes

The potential of the PCR assay to detect *A. polynesiensis* infected with mf was determined as follows. A batch of *A. polynesiensis* infected with a mean of about 2 mf each was obtained by artificial blood feeding for 1 h on 3 mL aliquots of human blood containing *c.* 1000 mf/mL (FAILLOUX *et al.*, 1991). After the blood meal, fed mosquitoes were separated and frozen at –20°C.

The number of mosquitoes containing mf, as determined by dissection of 30 individuals, was compared with the detection rate by PCR using pools of mosquitoes. The sensitivity of PCR was also investigated using 30 pools of 5, 20 and 50 uninfected mosquitoes 'spiked' with a single putatively mf-infected mosquito to determine the effect of pool size on the sensitivity of the assay.

Comparison of PCR and dissection of wild-caught mosquitoes

A. polynesiensis were collected in 2 distinct geographical areas of Tahaa Island (Society Archipelago, French Polynesia), where the human populations had been previously treated with either ivermectin at 400 μg/kg (IVR area) or a combination of ivermectin at 400 μg/kg plus diethylcarbamazine at 6 mg/kg (IVR+DEC area) (MOULIA-PELAT *et al.*, 1995). Approximately 2000 day-biting *A. polynesiensis* were collected 2, 4 and 6 months after the treatment in the IVR area and 2 and 6 months after in the IVR+DEC area, by uninfected persons acting as both bait and collectors. The mosquitoes were then divided into 2 groups for each drug and time combination. Mosquitoes (>1300) from one group were individually dissected for identification of filaria larvae and developmental stages as described by LARDEUX *et al.* (1995). The other group was killed by freezing within 2–4 h after capture and stored at –20°C until used in PCR assays. In this field study, we examined 50 pools of 5 mosquitoes from each mosquito group to determine the proportion of pools which was positive by PCR.

The PCR results from pools were compared with the results of dissection of individual mosquitoes as follows. Using the real numbers of *A. polynesiensis* dissected and infected as input (e.g., 1369 and 6, Table 2), we randomly sampled by computer 50 groups of 5 among the dissected mosquitoes. A group containing at least one infected mosquito was considered as positive. This allowed us to determine the number of positive groups among 50 in one sampling. This simulation was repeated 1000 times to determine the number N_i and the probability P_i (= N_i/1000) of finding 0, 1, 2, ... i positive pools. The simulated number of positive pools detected by dissection ('dissection positive pools') was the product of $P_i \times i$ and was compared with the number of pools positive by PCR ('PCR positive pools').

Statistical analysis

The χ^2 test was used to test the significance of differences ($P<0{\cdot}05$) in proportions of positive individuals or pools in the detection of mosquitoes infected with mf and in the assay with wild-caught mosquitoes. In this latter experiment, a *t* test for paired observations ($P<0{\cdot}05$) was also used to compare the results of the 2 methods.

Results

Sensitivity and reproducibility of the PCR assay to detect W. bancrofti *L3*

All 10 pools of either 50 or 100 heads 'spiked' with one L3 were positive by PCR. Using whole mosquitoes instead of heads, all 10 extracts from pools of 50 mosquitoes were also positive by PCR, while only 5 of 10 pools of 100 mosquitoes were positive.

The PCR assay detected 8 of 10 pools of 50 mosquitoes containing a single putatively infected mosquito (from a batch of mosquitoes 90% infected with a mean of 6 L3 per infected mosquito). When the number of putatively

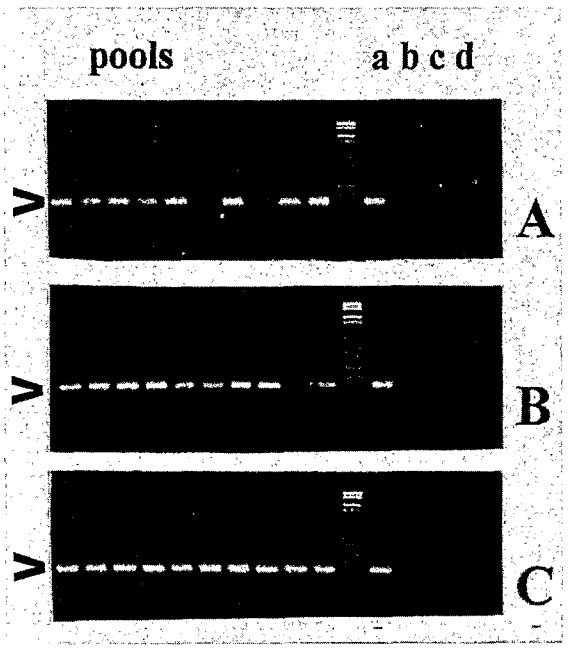

Figure. Detection of *W. bancrofti* in *A. polynesiensis* by PCR assay of 10 pools of 50 whole mosquitoes. A: 49 uninfected mosquitoes + 1 putatively infected; B: 48 uninfected mosquitoes + 2 putatively infected; C: 47 uninfected mosquitoes + 3 putatively infected. Controls used as templates in PCR assays: a, 1 pg *W. bancrofti* DNA; b, 0·1 pg *W. bancrofti* DNA; c, water; d, DNA from 50 uninfected *A. polynesiensis*. Arrow heads indicate the 188 base pair product.

Table 1. Comparison of dissection and PCR for the detection of *A. polynesiensis* experimentally infected with *W. bancrofti* microfilariae

No. of mosquitoes per sample	No. of infected mosquitoes/pool
Dissection	
30 individual blood-fed[a]	
Examination no. 1	24/30[b]
Examination no. 2	25/30
PCR	
30 individual blood-fed	27/30
30 pools of 1 blood-fed+4 uninfected	28/30
30 pools of 1 blood-fed+19 uninfected	25/30
30 pools of 1 blood-fed+49 uninfected	16/30[b]

[a]The 30 individually dissected mosquitoes were examined twice and contained a mean of 1·9 microfilariae per infected mosquito (SE=0·32, range 1–7) at the first examination and a mean of 2·4 microfilariae per infected mosquito at the second examination (SE=0·37, range 1–8).

[b]Significantly different (χ^2, $P<0{\cdot}05$).

Table 2. Comparison of dissection and PCR as a method to estimate *W. bancrofti* infection rates in wild-caught *A. polynesiensis*

	Drug treatment[a]				
	IVR	IVR	IVR	IVR+DEC	IVR+DEC
Months after treatment	2	4	6	2	6
No. of mosquitoes					
Dissected	1369	1815	1774	1635	1944
Infected	6 (0·44%)	15 (0·83%)	24 (1·35%)	11 (0·67%)	6 (0·31%)
Proportion of positive mosquito pools					
Dissection[b]	1·1/50	1·9/50	3·1/50	1·6/50	0·7/50
PCR	5/50	7/50	7/50	4/50	6/50
χ^2 (d.f.=1)	1·47	2·07	0·93	0·37	2·78
P	0·22	0·15	0·34	0·54	0·09

[a]Drug used to treat population in the area where mosquitoes were caught 2, 4 or 6 months later; IVR=ivermectin (400 μg/kg), DEC=diethylcarbamazine (6 mg/kg).

[b]Predicted by simulation analysis from results of individual dissections.

infected mosquitoes was increased to 2 or 3, the PCR detected respectively 9 and 10 of 10 pools (Fig. 1).

Sensitivity of the assay to detect mf in laboratory mosquitoes

Dissection and 2 microscopical examinations of 30 mosquitoes artifically fec on blood containing *W. bancrofti* mf indicated that 78% of these mosquitoes were infected by an average number of 2·1 mf/mosquito. As shown in Table 1, infection rates determined by dissection and PCR of individual mosquitoes were not significantly different (χ^2=1·17, df=1, *P*=0·23).

Moreover, the sensitivity of the PCR assay to detect a single infected mosquito was not affected if this mosquito was mixed with pools of up to 20 uninfected mosquitoes. A significant loss of sensitivity was observed in pools of 50 mosquitoes (Table 1, χ^2=4·80, df=1, *P*<0·02).

Comparison of PCR and dissection of wild-caught mosquitoes

Infection rates of wild-caught mosquitoes ranged from 0·3 to 1·35%. The numbers of 'dissection positive pools', calculated by simulation analysis from the results of individual dissections, were always lower than the numbers of pools which were positive by the PCR assay ('PCR positive pools') (Table 2). The difference between 'dissection positive pools' and 'PCR positive pools' was significant (t_4=7·92, *P*<0·001). However, the differences between proportions of 'dissection positive pools and 'PCR positive pools', for each time and drug combination, were not significantly different (χ^2 test) (Table 2).

Discussion

In this study we improved a PCR assay based on the amplification of a DNA repeat sequence from *W. bancrofti*, the '*SspI* DNA repeat'. We can now detect a single infected whole mosquiot among a pool of as many as 20 to 50 uninfected mosquitoes.

Applied to wild-caught mosquitoes, the assay was shown to be at least as sensitive as dissection. We found higher rates of infection by PCR than by dissection in the 5 groups of mosquitoes examined. Two points must be considered. First, the dissection of several hundreds of mosquitoes was carried out over a short period of time by several workers on populations of mosquitoes infected with few larvae. Infected mosquitoes harboured a mean number of 3·9 L1, 2·3 L2, 3·1 L3 (F. Lardeux, unpublished observations). Detection of L3 is relatively easy. However, detection of the smaller stages (L1) is more difficult as it requires accurate dissection of mosquito thorax muscles to release the parasites and render them visible. It is therefore likely that some mosquitoes infected by these smaller stages might have been missed. By contrast, extraction of DNA from mosquitoes is very efficient because we have shown that a mosquito infected by only 1–2 mf is detectable by PCR. In the field, infected host-seeking females will not usually contain mf, only the later stage larvae which, being larger, are even easier to detect than mf. Therefore, the method should be more sensitive in practice than it proved to be in this experimental study. Secondly, we should also investigate the possible persistence of DNA from *W. bancrofti* parasites which do not complete development within the mosquito but which might still be amplified by PCR, leading to false positive results.

We are currently working to improve the PCR assay to measure accurately the amount of DNA amplified from infected mosquitoes. Although the DNA fragment amplified in the *SspI* PCR assay is not stage specific, it has the great advantage of being specific for *W. bancrofti* and ubiquitous in all the *W. bancrofti* isolates so far tested. At present, this assay estimates the number of pools of mosquitoes which contain at least one infected mosquito. This is of practical interest in areas where infection rates are very low. With the present state of technology, a technician working 8 h per day with a single thermocycler can test about 30–40 pools of mosquitoes (i.e. 1500 to 2000) per day. This is to be compared with the ability of a well trained technician to dissect and examine about 100–200 mosquitoes a day. The PCR assay also allows the screening of mosquito extracts for the presence of other infectious agents, using different molecular probes.

Acknowledgements

We gratefully acknowledge the financial support of the United National Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases, and the French Ministry of Overseas Territories. We thank Dr Paul Martin and Dr Philippe Glaziou for helpful discussion, C. Plichart, A. Ung and M. Chebret for technical assistance, and H. Faipo, N. Tauira, S. Maruae, O. Teroroiria and T. Tupou for help in capturing the mosquitoes.

References

Chanteau, S., Luquiaud, P. Failloux, A.-B. & Williams, S. A. (1994). Detection of *Wuchereria bancrofti* larvae in pools of mosquitoes by the polymerase chain reaction. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **88**, 665–666.

Failloux, A. B., Chanteau, S., Chungue, E., Loncke, S. & Séchan, Y. (1991). Oral infection of *Aedes polynesiensis* by *Wuchereria bancrofti* using Parafilm membrane feeding. *Journal of the American Mosquito Control Association*, **7**, 660–662.

Lardeux, F., Nguyen, L. N., & Cartel, J. L. (1995). *Wuchereria bancrofti* (Filariidae:Dipetalonematidae) and its vector *Aedes polynesiensis* (Diptera:Culicidae) in a French Polynesian village. *Journal of Medical Entomology*, **32**, 346–352.

Moulia-Pelat, J. P., Nguyen, L. N., Hascoët, H., Luquiaud, P. & Nicolas, L. (1995). Advantages of an annual single dose of ivermectin 400 μg/kg plus diethylcarbamazine for community treatment of bancroftian filariasis. *Transactions of the*

Royal Society of Tropical Medicine and Hygiene, **89**, 682–685.
Ottesen, E. A. & Ramachandran, C. P. (1995). Lymphatic filariasis infection and disease: control strategies. *Parasitology Today*, **11**, 129–131.
Weiss, J. B. (1995). DNA probes and PCR for diagnosis of parasitic infections. *Clinical Microbiological Reviews*, **8**, 113–130.
WHO (1993). *Workshop on DNA probes and PCR for detection of filarial parasites in vectors, 11–12 January 1993, Beverly, MA, USA*. Geneva: World Health Organization, mimeographed document TDR/WKSP/FIL/93.1.

Received 1 September 1995; revised 30 October 1995; accepted for publication 31 October 1995

Note added in proof

A computer program (Poolscreen™) which predicts infection rates of populations based upon screening pools was published while this paper was in press (KATHOLI, C. R., TOÉ, L., MERRIWHEATHER, A. & UNNASCH, T. R. (1995). Determining the prevalence of *Onchocerca volvulus* infection in vector populations by polymerase chain reaction screening of pools of black flies. *Journal of Infectious Diseases*, **172**, 1414–1417). Using the Poolscreen™ program, the infection rates (and 95% confidence limits) of mosquito populations calculated from our PCR on pools of mosquitoes (Table 2) were 2·09% (0·67–4·80), 2·97% (1·19–6·03), 2·97% (1·19–6·03), 1·65% (0·44–4·18) and 2·52% (0·92–5·41) respectively for groups IVR month 2, IVR month 4, IVR month 6, IVR+DEC month 2 and IVR+DEC month 6. In this paper we calculated the numbers of 'dissection-positive pools' by simulation analysis using data from individual dissections. The equivalent calculations using the Poolscreen™ program adequately fit our data.

Announcements

Eighth International Training Course on
Identification of Helminth Parasites of Economic Importance
St Albans, UK: 8 July–16 August 1996

Further details can be obtained from: Dr L. M. Gibbons, International Institute of Parasitology, 395A Hatfield Road, St Albans, Hertfordshire, AL4 0XU, UK.

Warwick University Short Courses
Professional Updating Courses in Epidemiology
Design of Vaccination Programmes: from Seroepidemiology to Cost-effectiveness
15–19 July 1996
Immunizing against Hepatitis B virus: from Seroepidemiology to Cost-effectiveness
22–26 July 1996

Both courses will be held at the University of Warwick, Coventry, UK.
Further information can be obtained from Dr Stephen Hicks, Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, UK; phone +44 (0)1203 523540, fax +44 (0)1203 523701.

Tenth International Congress of Virology
Jerusalem, Israel: 11–16 August 1996

This conference is sponsored by the Virology Division of the International Union of Microbiological Societies.
Further information can be obtained from The Secretariat, Xth International Congress of Virology, P.O. Box 50006, Tel Aviv 61500, Israel; phone +972 3 514000, fax +972 3 5175674/5140077.